**Abstract**

Bacteria from the genus *Methylobacterium* interact symbiotically (endophytically and epiphytically) with different plant species. These interactions can promote plant growth or induce systemic resistance, increasing plant fitness. The plant colonization is guided by molecular communication between bacteria-bacteria and bacteria-plants, where the bacteria recognize specific exuded compounds by other bacteria (e.g. homoserine molecules) and/or by the plant roots (e.g. flavonoids, ethanol and methanol), respectively. In this context, the aim of this study was to evaluate the effect of quorum sensing molecules (N-acyl-homoserine lactones) and plant exudates (including ethanol) in the expression of a series of bacterial genes involved in *Methylobacterium*-plant interaction. The selected genes are related to bacterial metabolism (*mxaF*), adaptation to stressful environment (*crtI*, *phoU* and *sss*), to interactions with plant metabolism compounds (*acdS*) and pathogenicity (*patatin* and *phoU*). Under in vitro conditions, our results showed the differential expression of some important genes related to metabolism, stress and pathogenesis, thereby AHL molecules up-regulate all tested genes, except *phoU*, while plant exudates induce only *mxaF* gene expression. In the presence of plant exudates there is a lower bacterial density (due the endophytic and epiphytic colonization), which produce less AHL, leading to down regulation of genes when compared to the control. Therefore, bacterial density, more than plant exudate, influences the expression of genes related to plant-bacteria interaction.

**Key words:** endophyte, *Methylobacterium*, quantitative PCR (qPCR), plant-bacteria Interaction, homoserine.

**Introduction**

Endophytes colonize the plant inner tissues, commonly coming from the soil and entering the plants by intercellular spaces or little root fissure. The genus *Methylobacterium* is constituted by methylotrophic bacteria, which are able to interact symbiotically with different plant species, where they occupy mainly the inner tissues of the host, as endophytes. In agriculture, it has been described that *M. nodulans* is involved with nitrogen fixation and nodulation of plants (Sy et al., 2003), while other species can promote plant growth or induce systemic resistance (Araújo et al., 2002; Lacava et al., 2004; Madahaiyan et al., 2006). In citrus, it has been shown that endophytic
Methylobacterium can interact with Xylella fastidiosa, the causal agent of citrus variegated chlorosis (CVC) (Araújo et al., 2002; Lacava et al., 2004). Gai et al. (2009) manipulated *M. mesophilicum* SR1.6/6 to express the green fluorescence protein (GFP) in *C. roseus*, and observed that the colonization and transmission of *M. mesophilicum* by *Bucephalogonia xanthophis*, the *X. fastidiosa* insect-vector. Their results proved that *M. mesophilicum* is really transmitted by the insect to host plant living in the same niche of *X. fastidiosa* inside of *C. roseus*. Thus, the authors proposed the *M. mesophilicum* as a candidate to symbiotic control of this bacterium can act in plant, influencing the microbial balance in the plant host and participating on the plant development.

The occurrence of plant colonization by pathogenic or non-pathogenic bacteria is dependent of a communication between plants and microorganisms (Rosenbluth and Martinez-Romero, 2006; Hardoim et al., 2008; Wang et al., 2010), which is believed to be made by plant exudation of 17 compounds, such as flavonoids (Hardoim et al., 2008), ethanol (Williams and Yavitt, 2009) and methanol (Sudachat et al., 2009; Sy et al., 2005; Jourand et al., 2005) that attracts microorganisms. Bacterial communication during the plant-interaction can be coordinated physiological and adaptive changes in their population (Cha et al., 1998), favoring its adaptation to specific environments (Bauer and Mathesius, 2004; Joint, 2006; Soto et al., 2006; Sanches-Contreras et al., 2007). Some quorum sense (QS) systems use N-acyl-homoserine lactones (AHLs) as signaling molecules, commonly found in Gram-negative bacteria that living in association with plants (Cha et al., 1998; Loh et al., 2002; Camilli and Bassler, 2006; Sanches-Contreras et al., 2007; Barnard et al., 2007; White and Winans, 2007). QS system allows bacteria to function as multicellular organisms, because the extracellular concentration of auto-inducer increases with bacteria population growth, after reaches a determinate number, this molecules diffuse back into the bacteria and regulate the transcription of different genes that may be related with the secretion of virulence factors, biofilm formation, sporulation, exchange of DNA and others (Zhu and Sun, 2008).

Although several studies demonstrate the importance of the association between *Methylobacterium*-plants (Pirttila et al., 2000; Sy et al., 2001, Lee et al., 2006; Madhaiyan et al., 2009a; 2009b; Dourado et al., 2012a,b) and that members of the *Methylobacterium* genus produces AHL (Penälver et al., 2006; Pomini et al., 2009; Poonguzhal et al., 2007), little is known about the role of plant exudates and AHL on the expression of bacterial genes that are involved in bacterium plant-interaction. Studies reported that biofilm production seems to be dependent of the production of AHL (Penälver et al., 2006), and that biofilm formation on the plant may be the first step towards endophytic colonization by *Methylobacterium* (Andreote et al., 2006; Rossetto et al., 2011). Despite of the difficulty to understand the complexity of mechanisms involved in plant-microbe interactions, some bacterial genes responsible for metabolism, stress defence and pathogenicity that present an important role on plant bacterial interaction are described. The *mxaF* gene, responsible for the methylotrophic metabolism, encodes a subunit of the dehydrogenase methanol enzyme (MDH) (Zhang and Lindstrom, 2003) its expression confers adaptive advantages in competitive conditions on the plant surface (Williams and Yavitt, 2009), increasing methylotrophic activity during symbioses (Sy et al., 2005).

The genes *phoU* and *sss* are transport genes, where *phoU* gene is responsible mainly for the phosphate homoeostasis regulation, but also controls and interferes on stress, antibiotic production (Li and Zhang, 2007; Gristwood et al., 2009) and virulence gene expression (Cheng et al., 2009) and the *sss* (sodium solute symporter) gene is responsible for the symport transport of solute with the sodium (Scier, 1998). Genes *crI* and *acdS* genes are associated to the stress response (Sandmann, 2009) and to plant metabolism (Hardoim et al., 2008). Phytoene dehydrogenase gene (*crI*) codifies an enzyme that catalyses the denaturation reaction resulting on the lycopene synthesis that protects the cell against oxidative damages, different types of radiations and dissections (Xu et al., 2007) and the *acdS* gene codifies the enzyme carboxylic acid aminocyclopropane deaminase that degrades ACC (1-carboxilate amineciclopropane), forerunner of plant ethylene (Glick et al., 2007; Hardoim et al., 2008).

The *patatin* gene is associated to the pathogenic bacteria and is activated during the pathogeneses process, the *patatin* family genes encode phospholipases enzymes that hydrolyze phospholipids, frequently used by pathogenic bacteria on the effective host colonization hydrolyzing the membrane phospholipids, resulting in a membrane damage and cytotoxicity (Camera et al., 2009), the *phoU* gene is also involved in the bacteria pathogeneses process (Cheng et al., 2009).

Thus, the aim of the present study was to evaluate the expression of several genes previously related to plant-bacteria and bacteria-bacteria interaction (*mxaF, phoU, crI, acdS, sss* and *patatin*) using as model an endophytic bacterium *M. mesophilicum* SR1.6/6, quorum sense molecules (AHL (S)-N-dodecanoyl HSL-extracted from this isolate) and rice and eucalyptus plants as well ethanol as rooting exudate carbon source.

Material and Methods

Microorganism and plant material

The selected bacterial strain SR1.6/6, *M. mesophilicum*, was previously isolated from *Citrus sinensis*
(Araújo et al., 2002). The SR1.6/6 was routinely cultivated at 28 °C in CHOI3 medium (Toyama et al., 1998). The bacteria is stored in dilute liquid CHOI3 supplemented with glycerol at -80 °C. Fresh cultures were started from glycerol stocks for each experiment by plating portions onto CHOI3. The plant hosts used in this study were rice (Oryza sativa) and eucalyptus (Eucalyptus citriodora) seedlings. The axenic seedlings of rice and eucalyptus were obtained from seeds previously disinfected, washing in 70% ethanol for 2 min, sodium hypochlorite solution (2% available Cl- ) for 6 min, and again 70% ethanol for 2 min for rice seeds and washed for 1 min in 70% ethanol, 2 min in sodium hypochlorite solution and 1 min in 70% ethanol for eucalyptus seeds, both seeds followed by two rinses in sterile distilled water. The seeds were germinated on the MS medium. The seeds were washed for 1 min in 70% ethanol, 2 min in sodium hypochlorite solution and again 70% ethanol for 2 min, sodium acetate 50 mM and EDTA 10 mM, containing formaldehyde (0.7%) and ethidium bromide (0.3 g.mL-1). The RNA was quantified by O.D. 260, measured in spectrophotometer NanoDrop ND-1000 (Thermo Scientific, USA). All material used obtain and treat the RNA was sterilized and/or treated with DEPC to eliminated RNase. Total RNA (1-10 µg) was reverse-transcribed into cDNA using random hexamer primers (Invitrogen) and 200 U SuperscriptII RNase H- reverse transcriptase (Invitrogen) according to the procedure supplied with the enzyme. For each RNA sample, a negative RT (no addition of reverse transcriptase) was performed and used as a negative control in subsequent PCRs.

Primers designing and validation

The construction of the primers to amplify the target genes in this study were designed based on the six genomes sequences of Methylbacterium genus (M. extorquens DM4, M. extorquens AM1, M. chloromethanicum CM4, M. populi BJ001, M. nodulans ORS 2060, M. radiotolerans JCM 2831) available at GenBank database (National Center for Biotechnology Information). Sequences from all genes were aligned, and primers were designed, with the software Primer 3 program (v. 0.4.0) (http://frodo.wi.mit.edu/), to anneal in conserved regions of the gene (Table 1). Firstly, the conventional PCR was used to validate the design primers. PCRs were performed in 25 µL reaction containing 1 X enzyme buffer, 3.75 mM of MgCl2, 0.2 mM of each dNTPs, 0.2 µM of each primer and 0.1 U/µL of Taq DNA Polymerase (Invitrogen, Brazil). An initial denaturation was carried out at 94 °C for 5 min, followed by 35 thermal cycles of 30 s at 95 °C, 1 min at 59 °C and 1 min at 72 °C, with a final extension performed at 72 °C for 5 min. All PCR amplificons were checked by electrophoresis on agarose gel (1.5% w/v agarose) and UV visualization of the ethidium bromide stained gels.

The specificity of each primer pair was tested in 17 bacterial strains of Methylbacterium genus, and also in strains affiliated to Rhizobium, Sinorizobium and Bacillus genera (Table 2). All amplification products of the seven genes (mxaF, acdS, crtI, patatin, pshU, sss and recA) were purified, sequenced and compared to the GenBank data by BLASTn (http://blast.ncbi.nlm.nih.gov/) confirming the identity of the amplified fragment.
### Table 1 - Sequencing of designed primers to evaluate the gene expression of the endophytic bacterium *M. mesophilicum* SR1.6/6 in plants.

| Primer          | Target gene | Sequencing (5’-3’) | Fragment length | Reference  |
|-----------------|-------------|--------------------|-----------------|------------|
| MxaFqPCR A/F    | mxaF        | CGTCAACGTCTAGATGCTC/G/T | 250 pb         | This study |
| MxaFqPCR A/R    |             | GATGTCCCTTGGCGAG(A/G)TG |                |            |
| ACC Met1 f      | acdS        | GACCGGGTCGGCCACATC   | 200 pb         | This study |
| ACC Met2 r      |             | AGCCCGCCGTACTTGTGCG  |                |            |
| Patatin F       | Patatin     | CTCAACGGCCAACCTGATG  | 250 pb         | This study |
| Patatin R       |             | CGATCGGGTGATCTTCTT   |                |            |
| PhyF            | crtl        | ATACTTTAAGCCGCTGCTG | 186 pb         | This study |
| PhyR            |             | GACATGCGAGGTACTTGTG  |                |            |
| sssF            | sss         | ATCGACGCGCTGTACAATTAC| 221 pb         | This study |
| sssR            |             | ACCGTCGGTAGTCTGAC    |                |            |
| phoUF           | phoU        | TCGACGGGCTAGTCTAACC  | 189 pb         | This study |
| recoA           | recoA       | CGAAGCTGATGCTGATCTTC | 232 pb         | This study |
| recoR           |             | ATGTGACACTGACCTTGGT  |                |            |
| zwfrF           | zwf         | AGCAGCTGGAACATGTTGTT | 231 pb         | This study |
| zwfrR           |             | CGACGGAGGACGACATTACC |                |            |
| rpoDrF          | rpoD        | ACGACCTGAGGAAACAGTGC | 229 pb         | This study |
| rpoDrR          |             | ACGACCTGAGGAAACAGTGC |                |            |
| proCF           | proC        | CCAAGAGGAGAAGCTAGGC  | 282 pb         | This study |
| proCR           |             | ACAGCTGTCGGTACTGAT   |                |            |
| MMC1            | 16S rRNA    | TACGTGGAGAGATCTACGGTC | 390 pb         | Lacava et al. (2006) |
| MMC2            |             | GTACAAGGCGCCGGACGTC  |                |            |

### Table 2 - Isolates of *Methylobacterium* spp. and other genera from different plant hosts, identified by partial 16S rRNA gene sequencing, and used to evaluate primers specificity.

| Isolate                  | Host plant | Genes                      |
|--------------------------|------------|----------------------------|
| *Methylobacterium* spp. TC3-6 | Coffee     | mxaF + acdS + Patatin + crtl + phoU + sss + recoA + |
| *Methylobacterium* spp. TC3-7 | Coffee     | mxaF + acdS + Patatin + crtl + phoU + sss + recoA + |
| *M. fujisawaense* F5      | Sugarcane  | mxaF + acdS + Patatin + crtl + phoU + sss + recoA + |
| *Methylobacterium* spp. F7 | Sugarcane  | mxaF + acdS + Patatin + crtl + phoU + sss + recoA + |
| *Methylobacterium* spp. F8 | Sugarcane  | mxaF + acdS + Patatin + crtl + phoU + sss + recoA + |
| *Methylobacterium* spp. F9 | Sugarcane  | mxaF + acdS + Patatin + crtl + phoU + sss + recoA + |
| *M. mesophilicum* SR1.6/6 | Citrus     | mxaF + acdS + Patatin + crtl + phoU + sss + recoA + |
| *Methylobacterium* spp. SR3/27 | Citrus | mxaF + acdS + Patatin + crtl + phoU + sss + recoA + |
| *M. mesophilicum* PR1/3   | Citrus     | mxaF + acdS + Patatin + crtl + phoU + sss + recoA + |
| *Methylobacterium* spp. PR3/11 | Citrus | mxaF + acdS + Patatin + crtl + phoU + sss + recoA + |
| *Methylotrophic bacteria* TP7 | Sweet peper | mxaF + acdS + Patatin + crtl + phoU + sss + recoA + |
| *M. hispanicum* TP8       | Sweet peper | mxaF + acdS + Patatin + crtl + phoU + sss + recoA + |
| *Methylobacterium* spp. R3E | Eucalyptus spp. | mxaF + acdS + Patatin + crtl + phoU + sss + recoA + |
| *Methylobacterium* spp. MA2.9 | Laguncularia racemosa | mxaF + acdS + Patatin + crtl + phoU + sss + recoA + |
| *Methylobacterium* spp. MA3.1 | Aviscena shaueriana | mxaF + acdS + Patatin + crtl + phoU + sss + recoA + |
| *Methylobacterium* spp. MB1.1 | Rhizophora mangle | mxaF + acdS + Patatin + crtl + phoU + sss + recoA + |
| *Methylobacterium* spp. MB1.3 | Rhizophora mangle | mxaF + acdS + Patatin + crtl + phoU + sss + recoA + |
| *Rhizobium* SP             | Laboratory collection | mxaF + acdS + Patatin + crtl + phoU + sss + recoA + |
| *Sinorhizobium* SP         | Laboratory collection | mxaF + acdS + Patatin + crtl + phoU + sss + recoA + |
| *Bacillus* cereus          | Laboratory collection | mxaF + acdS + Patatin + crtl + phoU + sss + recoA + |

Total of 20: 17 8 11 5 15 15 14
RT qPCR analysis

All the amplification reactions by qPCR used the thermocycler iQTM5 (Bio-Rad) programmed to an initial denaturation of 5 min at 94 °C, followed by 40 cycles of 15 seconds at 94 °C and 1 min at 62 °C. The specificity of qPCR primer sets were evaluated by the melting curve with gradient from 60 to 96 °C ranging 1 °C each 30 s. Each amplification reaction it was used 2 μL of cDNA (100 ng), 10 μM of each primer and the Platinum SYBR Green qPCR SuperMix-UDG kit (Invitrogen). The primer-efficiencies were calculated through LinRegPCR software program (Ramakers et al., 2003).

The selection of endogenous gene, to be used as a normalizer was made by testing the Ct values for five different genes (16S rRNA, recA, zwf, rpoD e proC) (Stevenson and Weimer, 2005). Thereby, the recA presented the lowest deltaCt and the higher amplification efficiency, resulting in its selection as the normalizer for gene expression analysis.

The Pfaffl method (Pfaffl, 2001) was used to determine a relative quantification of the target genes in comparison to the reference gene. The DNA recombination and repair protein - recA gene was used as a reference gene.

Results and Discussion

The designed primers (Table 1) were specific for 17 Methylobacterium isolates exhibiting PCR products with expected size. However, these fragments were not observed from Sinorhizobium sp., Rhizobium sp. and Bacillus cereus, except for the primer that amplifies acdS gene, which amplified the target gene from Rhizobium sp. (Table 2). Therefore, these primers were used to evaluate the effects of bacteria gene expression under the following conditions: 1. AHL (S)-N-dodecanoyl-HSL; 2. Rice root exudates; 3. Eucalyptus root exudates and 4. Ethanol as carbon source.

No differences were observed between the bacterial growth with and without AHL (S)-N-dodecanoyl-HSL as well in the assay with ethanol as carbon source. However, there were less growth in the presence of the plant, due to bacteria colonization. During experimental period all plants exhibited normal growth and healthy aspects, with no lesions or chlorotic spots. In general, bacterial cell growth actively in culture medium with plant roots and after 48 hours some cells were observed attached on root surface. Taking in mind that one of our objective was to evaluate the differential gene expression in response to AHL, root exudates and carbon source, and that the planktonic cells should vary the gene expression response when attached to the root when plant-associated, in the present study only planktonic cells of M. mesophilicum SR1.6/6 under the mentioned conditions were accessed.

Looking specifically to each analyzed gene, the gene mxaF was over-expressed (more than 10 times) in the presence of AHL, and it was repressed only during the bacterial incubation in medium with ethanol as the carbon source (Figure 1A), the bacteria of eucalyptus treatments presented an increase in gene expression, but no significant differences occurred between the gene expression of M. mesophilicum in control and rice host.

The mxaF gene codifies an enzyme responsible for the transformation of methanol on formaldehyde (McDonald et al., 2005; Zhang and Lindstrom, 2003), and it was over-expressed in the presence of AHL showing that this gene responds to quorum sensing therefore, in the presence of ethanol the expression of this gene was repressed.

On leaves, the production and liberation of methanol is readily metabolized by M. mesophilicum, fact that could confer competitive advantage to the bacterium over other bacteria that do not present such metabolic alternative (Sudtachat et al., 2009; Sy et al., 2005; Jourand et al., 2005), different from rhizosphere, where (considering plant treatments) no significant differences occurred between the gene expression of M. mesophilicum in control and plant hosts, considering the conditions found in this study, where the bacterium was not under competition with other microorganisms, it is possible to generate the hypothesis that the expression of mxaF gene is stimulated mainly by quorum sensing molecules (produced by itself or other bacteria), and in a competing condition, produces mxaF gene as an advantage.

The expression of genes related to plant metabolism and bacterial stress (acdS and crtl genes, respectively) presented the same pattern. It was not affected by ethanol amendment, however it was super expressed in the presence of AHL and repressed in bacterial-rice and Eucalyptus interaction (Figure 1C and E). Considering the genes roles, it can be suggested that on the evaluated conditions, the bacteria did not feel under stressing conditions while in contact with plant roots and its exudates. It is believed that the product of acdS gene is responsible for increasing the plant capacity to support physic and biological stresses. Tittabutr et al. (2008) showed that the introduction of multiples copies of acdS gene increased the enzyme ACC deaminase activity in Rhizobium sp and enhances its symbiotic efficiency on plant host (L. leucocephala). Besides that, it was demonstrated that in some rhizobia strains the acdS gene is regulated by the promoter responsible for the transcription activation of nif genes, which encode for nitrogen fixation (Glick et al., 2007). Therefore, with ACC deaminase enzyme activity there is an increase in plant growth (Cheng et al., 2009).

In this context, Madahaiyan et al. (2006) described that Methylobacterium is capable of inducing systemic resistance in rice and sugarcane. However, considering that in the presence of AHL increases acdS expression and in the presence of both plants reduces acdS gene expression, suggesting that this gene is regulated by quorum sense (AHL), and probably the treatment of planktonic bacteria with the plant presented lower bacteria cell density, conse-
quenty less AHL, than planktonic bacterial cell without plant, despite of the same initial inoculums, in the presence of the plant, some bacterial colonizes plants (endophytically and epiphytically) (Andreote et al., 2006; Rossetto et al., 2011).

In this way, the results presented suggests that the expression of phytoene dehydrogenase gene (crtI), responsible for the lycopene synthesis that protects the cell against oxidative stress (Xu et al., 2007), in the evaluated conditions were similar to acdS gene. It is associated to the fact that during the bacterium-plant interaction, the microbial defense system is probably not activated due the fact that the plant does not recognize the endophytic bacterium as a pathogen (Figure 1E).

In studied conditions, the expression of genes related to pathogenicity (patatin and phoU genes) are not differently expressed in the presence of plant exudates or ethanol, and it seems to be not regulated by plant interaction. But in the presence of AHL patatin gene is induced, but does not influence the phoU gene expression (Figure 1B and D).

The gene phoU is a transport gene, responsible for stress control (Gristwood et al., 2009; Li and Zhang et al., 2007) and bacteria pathogeneses process (Cheng et al., 2009). While patatin gene is a phospholypases enzymes that hydrolyze phospholipids, activated during the patho-

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**Figure 1** - Relative effects of ethanol, plant exudates and AHL on SR1.6/6 transcription of plant-interaction genes: mxaF (A), patatin(B), acdS (C), phoU (D), crtl (E) and sss (F). The gene expression ration were determined by the method of Pfaffl. The recA as a reference gene. The results are means +/-standard errors of three replicates. Values of asterisks (*) or **) differ statistically of control treatment (a = 0.05 and 0.01 respectively) according to t test of Student.
geneses process to hydrolyze the membrane phospholipids (Camera et al., 2009). The expression of patatin and phoU genes, both related to bacterial pathogenicity, do not vary in the presence of plant exudates (Figure 1B and D), corroborating with previous studies that state M. mesophilicum as an endophyte able to colonize plants without cause damages to the host (Barnerji et al., 2008). Also, the absence on the expression variation of these two genes in the different treatments shows that although present, the regulation of such genes in this endophytic bacterium does not function as their homologous found in pathogenic bacteria. However, these pathogenicity genes respond differently to AHL. It induces only patatin expression and does not influence phoU gene.

The sss (sodium solute symporter) gene responsible for the symport transport of solute with the sodium, in studied conditions, seems to be not regulated by plant interaction (Figure 1F). However, the sss gene expression was induced in medium containing ethanol and in the presence of AHL (Figure 1F).

This study allowed a better understanding of the endophytic gene expression during plant recognition and planktonic bacterial cell interaction with rice and eucalyptus, the effect of ethanol (root exudates) and the effect of quorum sense molecule (AHL (S)-N-dodecanoyl-HSL). It was observed that homoserine lactone induces all analyzed genes mxaF, acdS, crrU, sss and patatin, expect for phoU gene (it does not influence its expression). It was observed that the mxaF gene was not induced in rice, only in eucalyptus, possibly due to the differences on plant-bacteria interaction and on using different metabolic routes, also suggesting that the plant can induce the expression of this gene and indirectly increase the bacteria fitness during the plant host interaction.

In addition, the plant-related environment was stated as a free-of-stress niche for bacterial colonization, based on the analysis of the genes crrU, acdS and phoU expression. Hence, such symbiotic interaction was confirmed by the absence of induction for gene related to pathogenesis characteristics (patatin and phoU). Future studies might reveal differences in specific genes, modulated by the plant genotype, what will add information about the differential behavior of bacteria, according to the host plant. Concluding, we remark that more than plant exudates, bacterial density influences the expression of genes related to metabolism, stress and pathogenesis.

Acknowledgments

This work was supported by a grant from the FAPESP - Foundation for Research Assistance of São Paulo State, Brazil (Proc. 2003/14143-3; Proc. 2010/07594-5). We thank CNPq for the Fellowship to M.N.D. and A.C.B. and also FAPESP for the fellowship to M.C.Q. (Proc. 2005/53748-6).

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