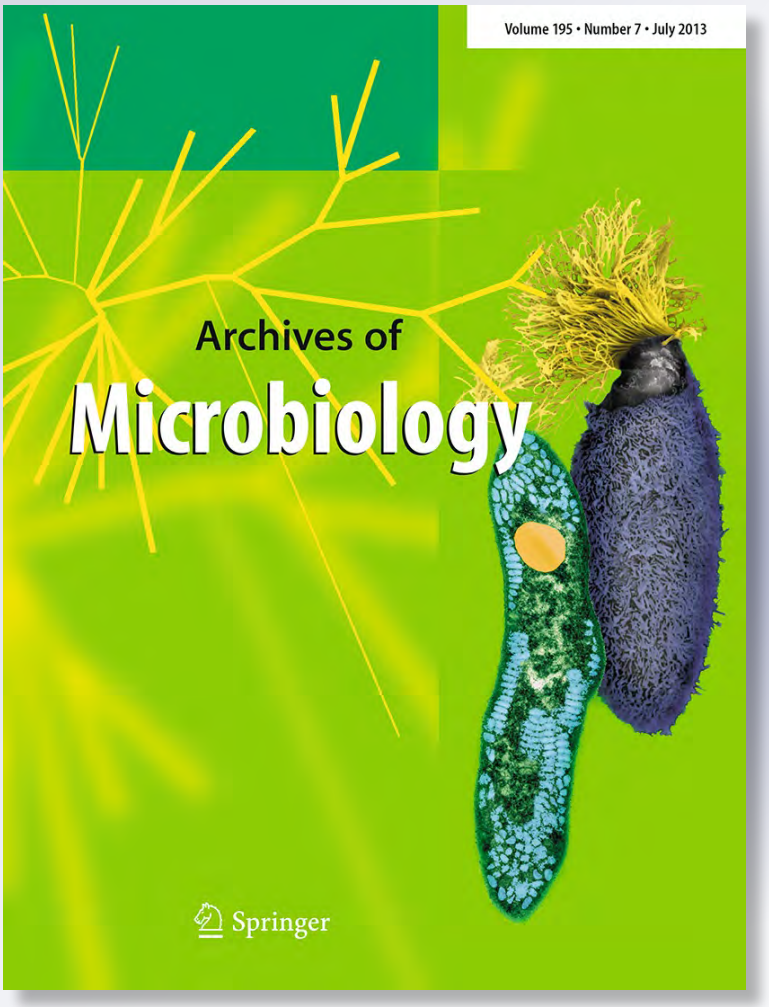
*Colonization of Madagascar periwinkle (*Catharanthus roseus*), by endophytes encoding* gfp *marker*

Adalgisa Ribeiro Torres, Welington Luiz Araújo, Luciana Cursino, Priscilla de Barros Rossetto, Mateus Mondin, Mariangela Hungria, et al.

Archives of Microbiology

ISSN 0302-8933
Volume 195
Number 7

Arch Microbiol (2013) 195:483-489
DOI 10.1007/s00203-013-0897-3



Springer

Arch Microbiol (2013) 195:483–489
DOI 10.1007/s00203-013-0897-3

ORIGINAL PAPER

Colonization of Madagascar periwinkle (*Catharanthus roseus*), by endophytes encoding *gfp* marker

Adalgisa Ribeiro Torres · Welington Luiz Araújo · Luciana Cursino · Priscilla de Barros Rossetto · Mateus Mondin · Mariangela Hungria · João Lúcio Azevedo

Received: 20 December 2012 / Revised: 13 March 2013 / Accepted: 22 April 2013 / Published online: 22 May 2013

Abstract This study reports the introduction of *gfp* marker in two endophytic bacterial strains (*Pantoea agglomerans* C33.1, isolated from cocoa, and *Enterobacter cloacae* PR2/7, isolated from citrus) to monitor the colonization in Madagascar perinwinkle (*Catharanthus roseus*). Stability of the plasmid encoding *gfp* was confirmed in vitro for at least 72 h of bacterial growth and after the colonization of tissues, under non-selective conditions. The colonization was observed using fluorescence microscopy and enumeration of culturable endophytes in inoculated perinwinkle plants that grew for 10 and 20 days. Gfp-expressing strains were re-isolated from the inner tissues of surface-sterilized roots and stems of inoculated plants, and the survival of the *P. agglomerans* C33:1*gfp* in plants 20 days after inoculation, even in the absence of selective pressure, suggests that is good colonizer. These results indicated that both *gfp*-tagged strains, especially *P. agglomerans* C33.1, may be useful tools to deliver enzymes or other proteins in plant.

Keywords Madagascar periwinkle · Endophytes · Green fluorescent protein

Communicated by Erko Stackebrandt.

A. R. Torres · L. Cursino · M. Mondin · J. L. Azevedo
Departamento de Genética, Escola Superior de Agricultura 'Luiz de Queiroz', Universidade de São Paulo, PO Box 83, Piracicaba, São Paulo 13400-970, Brazil

A. R. Torres (✉) · M. Hungria
Embrapa Soja, PO Box 231, Londrina, Paraná 86001-970, Brazil
e-mail: adalgisa@cnpso.embrapa.br

Present Address:
W. L. Araújo
Departamento de Microbiologia, Instituto de Ciências Biomédicas, Universidade de São Paulo, Av. Prof. Lineu Prestes, 1374-Ed. Biomédicas II, Cidade Universitária, São Paulo, São Paulo 05508-900, Brazil

Present Address:
L. Cursino
Department of Biology, Hobart and William Smith Colleges, 300 Pulteney Street, Geneva, NY, USA

Present Address:
P. de Barros Rossetto
Instituto de Biologia, Departamento de Genética, Universidade Federal do Rio de Janeiro, Av. Prof. Rodolpho Paulo Rocco s/n Prédio do CCS, 2° Andar, Sala A2-93, Ilha do Fundão, Rio de Janeiro, RJ 21949-900, Brazil

Introduction

Endophytic bacteria are bacteria that reside within living plant tissue without causing apparent harm to the host plant (Quadt-Hallmann and Kloepper 1996), capable of establishing mutualistic associations (Hallmann et al. 1997; Azevedo et al. 2000), promoting plant growth and yield, suppressing pathogens, and helping to remove contaminants, among other benefits (Rosenblueth and Martínez-Romero 2006).

By inhabiting the same sites as vascular pathogens, endophytes may be valuable as biological control (Hallmann et al. 1997) and phytoremediation (Newman and Reynolds 2005) agents. In this context, the release of GMEs represents an important strategy. Indeed, specific endophytes may be highly preferred in comparison with non-specific chemical fertilizers and pesticides, because of cost, time effectiveness, and agricultural sustainability (Compant et al. 2005; Zinniel et al. 2002). The feasibility of this approach has been already pointed out in some studies, for example *Herbaspirillum seropedicae* and *Clavibacter xylii* have been genetically modified to produce and excrete

the δ-endotoxin of *Bacillus thuringiensis*, with insecticide properties (Turner et al. 1991; Downing et al. 2000).

The commercial release of genetically modified endophytes (GMEs) requires both an efficient colonization of the plant tissues and the maintenance of the desirable properties inside the plant.

The first step in colonization studies is the assessment of colonization efficiency, which requires the precise identification of the inoculum. A simple method of tracking bacteria is the introduction of antibiotic resistance markers; however, there are many reports of strains losing their antibiotic-resistant phenotype (Nairn and Chanway 2002). To address this problem, the addition of a second label such as the green fluorescent protein (*gfp*) gene offers an interesting opportunity for ecology studies. This gene does not require any exogenous substrates for its expression and it allows temporal and spatial tracking of the bacteria in the plant tissues (Valdivia et al. 1998; Errampalli et al. 1999; Compant et al. 2005).

The aim of this study was to evaluate the colonization patterns of two *gfp*-tagged strains in Madagascar periwinkle (*Catharanthus roseus*), considered a model plant for microbe interaction studies (Monteiro et al. 2001; Lacava et al. 2007), as well a garden plant.

Materials and methods

Bacterial strains, plasmid, and culture conditions

The bacterial isolates, GMEs, and plasmids are listed in Table 1. The isolates were grown in Luria-Bertani (LB) medium for 18 h. *Escherichia coli* strain DH5α carrying the pPAGFP plasmid was grown in LB medium supplemented with ampicillin (100 μg/ml) at 37 °C.

In a previous study, 68 isolates of endophytic enterobacteria obtained from citrus (*Citrus* spp.) and cocoa (*Theobroma cacao*) plants were evaluated in relation to antibiotic susceptibility and cellulase production (Torres et al. 2008). We chose two ampicillin-sensitive and weak cellulase producer isolates for this study based on their putative endophytic features and on the 16S rDNA sequence characterization, which, according to those authors, indicated that these strains were unrelated to known phytopathogenic bacteria.

Plasmid DNA extraction and transformation of endophytic bacteria

Escherichia coli DH5α was grown overnight in LB medium supplemented with ampicillin (100 μg/ml), and the plasmid was extracted with the "Plasmid Midi kit" (Qiagen).

Electro-competent cells of *P. agglomerans* C33.1 and *E. cloacae* PR2/7 were obtained as follows: an overnight culture was diluted in a new fresh medium (1:50), and growth was monitored until the cells reached OD 600 = 0.4–0.7. Cells were then harvested by centrifugation (10,000×*g*, 10 min, 4 °C) and washed three times with ice-cold sterile purified water. The cell suspension was concentrated 50-fold in 10 % sterile glycerol and kept at −80 °C.

Electro-competent cells were transformed by electroporation (2.5 kv, 25 μf, 200 Ω, 4.5 ms) with plasmid pPAGFP. Transformed bacteria were cultured on LB agar medium supplemented with ampicillin (100 μg/ml).

In vitro plasmid stability

Plasmid pPAGFP stability was monitored during 72 h of cultivation of the transformed isolate. During the cultivation, 10 μl of culture was inoculated into new tubes every 24 h. For every inoculation, aliquots were plated on LB medium and 100 random single colonies were transferred using a toothpick to LB agar medium supplemented with 100 μg/ml of ampicillin. Colonies were counted, and the percentage of clones carrying the plasmid was estimated. All experiments were repeated twice.

Detection of plasmid gene expression

Heterologous gene expression was determined after 24 h of growth on plates containing LB solid medium supplemented with ampicillin (100 μg/ml). Bacteria grown in

Table 1 Bacterial strains

Bacterial strains	Characteristics	Source
Pantoea agglomerans C33.1	Endophytic bacteria isolated from cocoa plants	ESALQ/USP
Enterobacter cloacae PR2/7	Endophytic bacteria isolated from citrus plants	(Araújo et al. 2002)
Escherichia coli DH5α	Donor bacteria of the plasmid pPAGFP	Invitrogen
C33.1:*gfp*	C33.1 with pPAGFP	This study
PR2/7:*gfp*	PR2/7 with pPAGFP	This study
Plasmid pPAGFP	Plasmid vector consists of the ligation of the cryptic plasmid pPA3.0 of *Pantoea agglomerans* with the pUC18 vector and the *gfp* gene addition	(Andreote et al. 2008)

* Isolates from endophytic bacteria collection of Laboratory of Microbial Genetics (Department of Genetics, ESALQ/USP, Piracicaba, Brazil)

the presence of antibiotics and expressing the *gfp* gene, detected under UV light at 300–360 nm, were confirmed as carrying both features.

Plant inoculation

Periwinkle plants were propagated using seeds obtained from a commercial variety supplied by local company, without surface sterilization. Prior to inoculation, roots of seedlings (21 days post-germination) were washed in water, soaked in a bacterial suspension (10^9 CFU/ml), and suspended in phosphate-buffered saline [PBS, containing (g/l) Na_2HPO_4 1.44; KH_2PO_4 0.24; KCl 0.20; NaCl 8.00; pH 7.4] for 1 h. Plants were either non-inoculated (control) or inoculated with *P. agglomerans* C33.1, or *E. cloacae* PR2/7, or C33.1:*gfp*, or PR2/7:*gfp* strains, as explained above. Plants were then transplanted into individual pots (one seedling per pot) containing non-sterilized commercial substrate compost (turf, expanded vermiculita and composted material of pine bark) and were maintained in a greenhouse with a 14-h photoperiod and 22/28 °C day/night cycle for collection after 10 and 20 days. The experiment was performed with a complete randomized complete block design with 14 replicates for each treatment.

Enumeration of culturable endophytes in plant tissues

Plants were harvested, placed on a paper towel, and immediately transported to the laboratory, where the substrate was carefully removed from the roots. Stems and roots were separated and weighed. Surface disinfection was achieved by washing the plants with 70 % ethanol for 1 min, with sodium hypochlorite solution (2 % available Cl^-) for 2 min, and with 70 % ethanol for 30 s, followed by two rinses in sterile distilled water. To confirm that the endophytic bacteria reflected only the number of cells inside the plant tissues, a control procedure was always performed to ensure that proper surface sterilization took place, in which aliquots of the sterile distilled water used in the final rinse were also plated on tryptic soy agar (TSA) medium amended with 100 μg/ml of benomyl to inhibit fungal growth. The plates were examined for growth after incubation at 28 °C for 2–15 days. In a further step, fragments of stems and roots were cut into tiny fragments with the aid of a sterilized razor blade in sterile phosphate-buffered saline (1 ml for stems and 2 ml for roots) and serial dilutions were plated on TSA as described above. Samples were also grown on TSA medium supplemented with ampicillin (100 μg/ml), in order to estimate the number of bacteria expressing *gfp*. The plates were incubated at 28 °C for 2–15 days, after which the CFU were determined and the bacterial density was estimated.

In planta visualization of *gfp* expressing endophytes using fluorescence microscopy

We selected the strain C33.1:*gfp* for the assessment of the colonization pattern based on its high-level green fluorescence intensity and persistence in periwinkle plants. Seedlings inoculated with C33.1:*gfp* were collected and examined by fluorescence microscopy at various times (24, 48, and 72 h), washed in running tap water, and placed separately on Petri dishes. Hand-cut sections of live leaves, stems, and roots were examined using an Axiophot-2 microscope (Zeiss), and images were captured with a charge-coupled device (CCD) camera, using the software Isis and Ikaros. The filter set 09 (Zeiss) with a 450–490 nm band-pass excitation and 550 nm emission was used for the GFP examination. Phase-contrast microscopy was used to visualize the tissues, and the digital images were merged and processed.

Statistical analysis

Data were analyzed using the SAS software package [Copyright (c) 1989–1996] with a completely randomized analysis of variance ($P < 0.05$). Bacterial counts were transformed using $\log_{10}$ of $x + 1$ before the analysis of variance. Tukey's test was used for comparison of means.

Results

Transformation of endophytic enterobacteria and in vitro plasmid stability

Levels of fluorescence were higher in *P. agglomerans* C33.1:*gfp* than those in *E. cloacae* PR2/7:*gfp* strain. When the plasmid pPAGFP was introduced into the strain *P. agglomerans* C33.1, up to 2×10^4 transformants/μg of plasmid DNA were obtained, indicating a high efficiency of transformation. A moderate efficiency of transformation (up to 4×10^3 transformants/μg of plasmid) was observed with the strain *E. cloacae* PR2/7. The results show that after at least 24 h, the majority of the cells—99.5 % for C33.1:*gfp* and 100 % for PR2/7:*gfp*—were still carrying their respective plasmid. Moreover, after 72 h, the plasmid was detected in 98.5 % of the cells of C33.1:*gfp* and 99.5 % of the PR2/7:*gfp*.

Enumeration of cultivable endophytic populations

The re-isolation of the endophytic bacteria from stems and roots was performed 10 and 20 days after inoculation. Total bacterial population and *gfp*-derived populations were determined for each of the tissues examined. High numbers

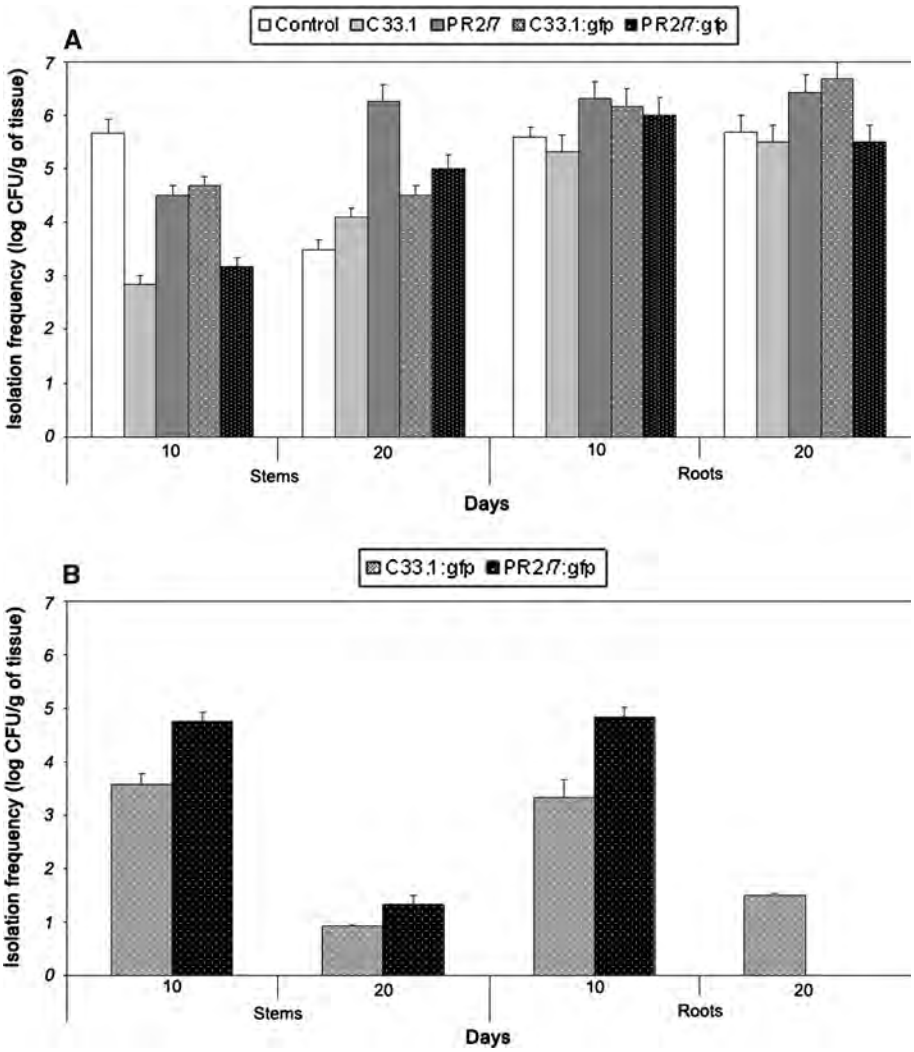


Fig. 1 Isolation frequency of endophytic bacteria recovered from stems and roots of seedlings 10 and 20 days after inoculation. **a** Colonies recovered in tryptic soy agar (TSA) medium without antibiotic. **b** Colonies recovered in TSA medium supplemented with ampicillin, isolated from plant tissues (fresh weight). The *data bars* represent the means, and the *error bars* indicate standard errors of the means of results from four treatments (at least 30 plants were sampled)

of endophytes were recovered in the absence of antibiotics (Fig. 1a). Total population of endophytic bacteria in periwinkle plants was estimated at 10^5 and 10^6 CFU g^{-1} on medium without antibiotic. The density of cultivable endophytic bacteria varied with the plant tissue, being greater in roots than in stems; furthermore, density was higher on the medium without antibiotic (Fig. 1a) than on the medium supplemented with ampicillin (Fig. 1b). The endophytic population expressing *gfp* decreased drastically after 20 days in plants inoculated with both C33:*gfp* and PR2/7:*gfp* under selective conditions (Fig. 1b). In addition, we did not observe endophytes naturally resistant to ampicillin recovered from plants inoculated with C33.1 and PR2/7 strains. Furthermore, few morphological types were observed among the isolated endophytic bacteria in all treatments (data not shown).

In planta visualization of *gfp*-tagged endophytes

GFP-expressing cells were visible at high densities in roots, and root hairs were clearly colonized (Fig. 2a, b). Colonies of *gfp*-tagged cells were also observed in the stem xylem (Fig. 2c, d). Stem cross-sections showing colonization by *gfp*-tagged cells were also observed 48 h after inoculation. Very few cells were observed in the leaf tissues 24 h after inoculation, and although colonization increased 48 h after inoculation, with several cells being detected in the leaves, it decreased drastically 72 h after inoculation (data not shown).

Discussion

The plasmid pPAGFP was stably maintained in the PR2/7 and C33.1 strains for at least 72 h of bacterial cultivation on medium without antibiotics. The abundance of *gfp*-tagged bacteria colonizing the plant tissues did not suggest significant marker loss, highlighting the role of pPAGFP as a valuable tool for studies of bacterial communities grown in the absence of antibiotic selection. *Gfp*-expressing strains were re-isolated from the inner

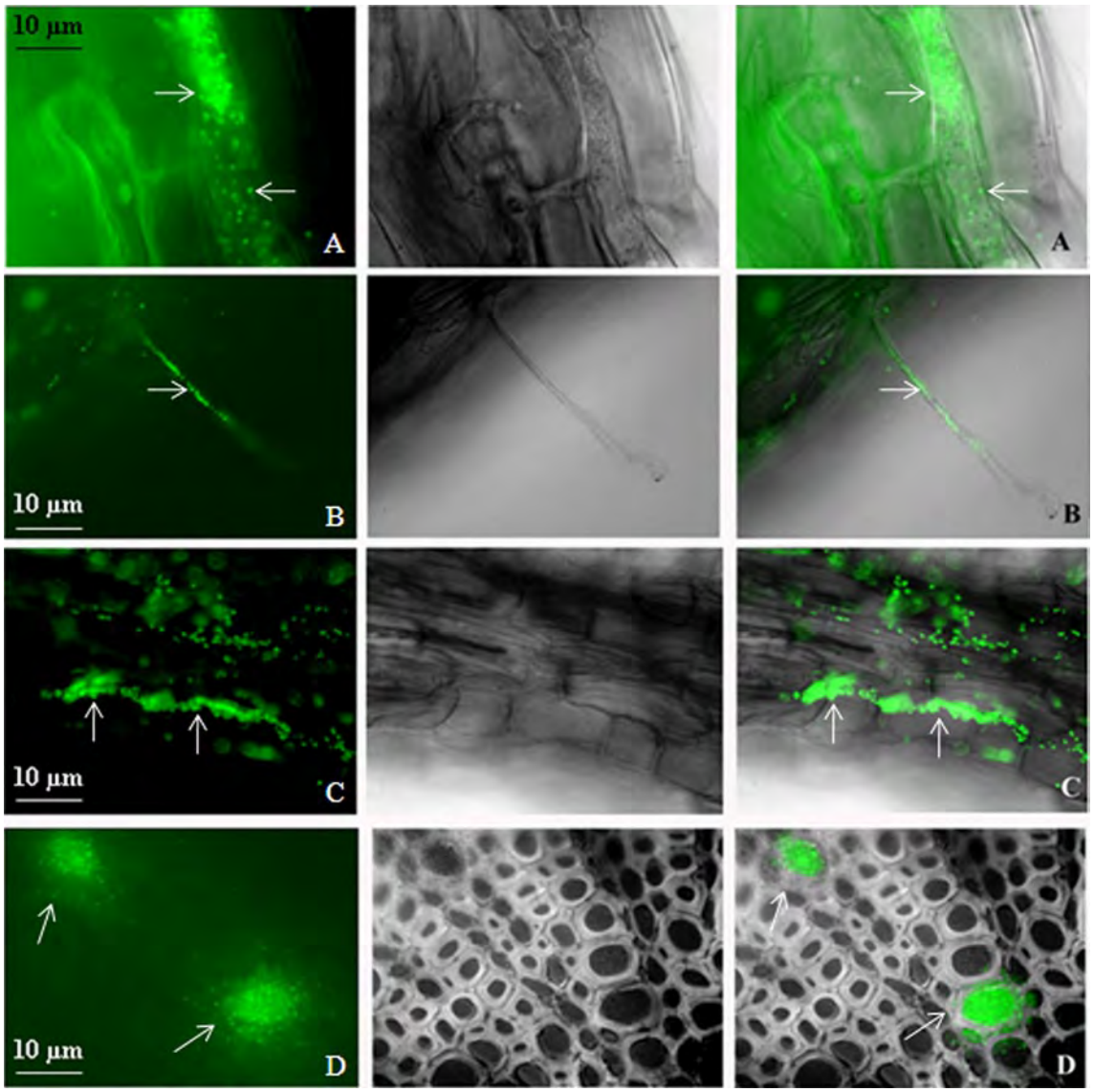


Fig. 2 Colonization of periwinkle seedlings by C33.1:*gfp* (630×). Seedlings were inoculated with C33.1:*gfp* (10^9 CFU/ml) and microscopically analyzed for GFP fluorescence. Sections were visualized 24 **c** or 48 **a**, **b**, **d** h after inoculation. **a** GFP-expressing cells growing in root (*arrows*). Note apparent occupation of intracellular space. **b** GFP-expressing cells colonizing root hairs (*arrows*). **c** GFP-tagged cells inhabiting the intercellular spaces of stem xylem cells (*arrows*). **d** Stem cross-section showing colonization by GFP strain (*arrows*). *Scale bar* = 10 µm

tissues of surface-sterilized roots and stems of inoculated plants, and the survival of the *P. agglomerans* C33:1*gfp* in plants 20 days after inoculation, even in the absence of selective pressure, suggests that is good colonizer. This observation is in agreement with previous study (Verma et al. 2004). The authors have compared endophytic colonization ability of two deep-water rice endophytes, *Pantoea* sp. and *Ochrobactrum* sp., using green fluorescent protein reporter. Although both *Pantoea* sp. and *Ochrobactrum* sp. were able to establish endophytically in the rice root, *Pantoea* sp. was more aggressive invader in the rice tissues.

The use of plasmid pPAGFP, built with a cryptic plasmid of *P. agglomerans*, probably favored the greatest expression of *gfp* gene in that strain. Furthermore, the rapid spread of *gfp*-derived strains in periwinkle plants indicates that the vascular system is the probable route for systemic colonization, which is consistent with previous reports in other plants (Dong et al. 2003; Germaine et al. 2004; Chi et al. 2005). It has also been suggested that *P. agglomerans* can be transported through the colonization of intercellular spaces in root and aerial tissues (Compant et al. 2005). Populations of cultivable endophytes in both roots and stems of periwinkle plants were on average 10^4 CFU g^{-1}

fresh weight, and similar values were previously reported in other plants (e.g. Andreote et al. 2006; Kuklinsky-Sobral et al. 2004; Lacava et al. 2007). In addition, the cultivable indigenous endophytic population was markedly higher in the roots in comparison with the stems, what may result from the higher abundance of nutrients in the rhizosphere to support bacterial growth and metabolism (Glick 1995).

Another important feature is that the results from our study indicate that *Pantoea agglomerans* C33.1:*gfp* strains may be used as vectors to deliver enzymes or other proteins inside the plants, as their monitoring can be easily performed by detecting the expression of the *gfp* gene. In this context, bacteria of the species *P. agglomerans* have been considered one of the most widespread groups in terms of endophytic interaction with plants (Torres et al. 2008). Bacteria of this species have been found in studies using several plant species, such as citrus (Araújo et al. 2001), eucalyptus (Ferreira et al. 2008), and rice (Verma et al. 2004).

In a recent work, the strain *P. agglomerans* 33.1:pNK-GFP was able to colonize sugarcane plants, promoting their growth. The growth promotion observed in colonized plants may be related to the ability of *P. agglomerans* 33.1–36 synthesize IAA and solubilize phosphate (Quecine et al. 2012).

Furthermore, genetic engineering is much easier in endophytic bacteria than in plants, and only one bacterium may successfully colonize multiple plants (Newman and Reynolds, 2005).

In summary, the two *gfp*-tagged endophyte strains used in our study proved to be stable for at least 72 generations as well as after colonization of the tissues under non-selective conditions. Moreover, no damages were observed in inoculated plants, making these *gfp*-tagged strains—especially *P. agglomerans* C33.1—useful to deliver enzymes or other proteins *in planta*.

Acknowledgments Funding for this research was provided by FAPESP. A. R. Torres and M. Mondin are CAPES fellows. P. Rossetto and W. L. Araújo are FAPESP fellows. L. Cursino, M. Hungria, and J. L. Azevedo are CNPq fellows. The authors thank M. C. Quecine for help in statistical analysis and Drs. Glaciela Kaschuk and Paulo Lacava for suggestions on the manuscript.

References

Andreote FD, Lacava PT, Gai CS, Araújo WL, Maccheroni W Jr, van Overbeek LS, van Elsas JD, Azevedo JL (2006) Model plants for studying the interaction between *Methylobacterium mesophilicum* and *Xylella fastidiosa*. Can J Microbiol 52:419–426. doi:10.1139/w05-142

Andreote FD, Rossetto PB, Souza LCA, Marcon J, Maccheroni W Jr, Azevedo JL, Araújo WL (2008) Endophytic population of *Pantoea agglomerans* in citrus plants and development of a cloning vector for endophytes. J Basic Microbiol 48:338–346. doi:10.1002/jobm.200700341

Araújo WL, Maccheroni W Jr, Aguilar-Vildoso CI, Barroso PAV, Saridakis HO, Azevedo JL (2001) Variability and interactions between endophytic bacteria and fungi isolated from leaf tissues of citrus rootstocks. Can J Microbiol 47:229–236. doi:10.1139/w00-146

Araújo WL, Marcon J, Maccheroni W Jr, van Elsas JD, Van Vuurde JWL, Azevedo JL (2002) Diversity of endophytic bacterial populations and their interaction with *Xylella fastidiosa* in citrus plants. Appl Environ Microbiol 68:4906–4914. doi:10.1128/AEM.68.10.4906-4914.2002

Azevedo JL, Maccheroni Jr W, Pereira J, Araújo WL (2000) Endophytic microorganisms: a review on insect control and recent advances on tropical plants. Elect J Biotech. www.ejbiotechnology.info/content/vol3/issue1/full/4/index.html. ISSN0717-3458

Chi F, Shen SH, Cheng HP, Jing YX, Yanni YG, Dazzo FB (2005) Ascending migration of endophytic rhizobia, from roots to leaves, inside rice plants and assessment of benefits to rice growth physiology. Appl Environ Microbiol 71:7271–7278. doi:10.1128/AEM.71.11.7271-7278.2005

Compant S, Reiter B, Sessitsch A, Nowak J, Clement C, Barkia EA (2005) Endophytic colonization of *Vitis vinifera* L. by plant growth promoting bacterium *Burkholderia* sp PsJN. Appl Environ Microbiol 71:1685–1693. doi:10.1128/AEM.71.4.1685-1693.2005

Dong Y, Iniguez AL, Triplett EW (2003) Quantitative assessments of the host range and strain specificity of endophytic colonization by *Klebsiella pneumoniae* 342. Plant Soil 257:49–59. doi:10.1128/AEM.69.3.1783-1790.2003

Downing KJ, Leslie G, Thomson JA (2000) Biocontrol of the sugarcane borer *Eldana saccharina* by expression of the *Bacillus thuringiensis cry1Ac7* and *Serratia marcescens chiA* genes in sugarcane-associated bacteria. Appl Environ Microbiol 66:2804–2810. doi:10.1128/AEM.66.7.2804-2810.2000

Errampalli D, Leung K, Cassidy MB, Kostrzynska M, Blears M, Lee H, Trevors JT (1999) Applications of the green fluorescent protein as a molecular marker in environmental microorganisms. J Microbiol Meth 35:187–199. doi:10.1111/j.1574-6941.1999.tb00651.x

Ferreira A, Quecine MC, Lacava PT, Azevedo JL, Araújo WL (2008) Diversity of endophytic bacteria from *Eucalyptus* species seeds and colonization of seedlings by *Pantoea agglomerans*. FEMS Microbiol Lett 287:8–14. doi:10.1111/j.1574-6968.2008.01258.x

Germaine K, Keogh E, Garcia-Cabellos G, Borremans B, van der Lelie D, Barac T, Oeyen L, Vangronsveld J, Moore PF, Moore ERB, Campbell CD, Ryan D, Dowling DN (2004) Colonisation of poplar trees by *gfp* expressing bacterial endophytes. FEMS Microbiol Ecol 48:109–118. doi:10.1016/j.femsec.2003.12.009

Glick BR (1995) The enhancement of plant growth by freeliving bacteria. Can J Microbiol 41:109–117. doi:10.1139/m95-015

Hallmann J, Quadt-Hallmann A, Mahaffee WF, Kloepper JW (1997) Bacterial endophytes in agricultural crops. Can J Microbiol 43:895–914. doi:10.1139/m97-131

Kuklinsky-Sobral J, Araújo WL, Mendes R, Geraldi IO, Pizzirani-Kleiner AA, Azevedo JL (2004) Isolation and characterization of soybean-associated bacteria and their potential for plant growth promotion. Environ Microbiol 6:1244–1251. doi:10.1111/j.1462-2920.2004.00658.x

Lacava PT, Araújo WL, Azevedo JL (2007) Evaluation of endophytic colonization of *Citrus sinensis* and *Catharanthus roseus* seedlings by endophytic bacteria. J Microbiol 45:11–14

Monteiro PB, Renaudin J, Jagoueix-Eveillard S, Ayres AJ, Garnier M, Bové JM (2001) *Catharanthus roseus*, an experimental host plant for the citrus strain of *Xylella fastidiosa*. Plant Dis 85:246–251. doi:10.1094/PDIS.2001.85.3.246

Nairn JD, Chanway C (2002) Temporary loss of antibiotic resistance by marked bacteria in the rhizosphere of spruce seedlings. FEMS

Microbiol Ecol 40:167–170. doi:10.1111/j.1574-6941.2002.tb00949.x

Newman LA, Reynolds MC (2005) Bacteria and phytoremediation: new uses for endophytic bacteria in plants. Trends Biotechnol 23:6–8. doi:10.1128/AEM.69(12),7319-7327.2003

Quadt-Hallmann A, Kloepper JW (1996) Immunological detection and localization of the cotton endophyte *Enterobacter asburiae* JM22 in different plant species. Can J Microbiol 42:1144–1154

Quecine MC, Araújo WL, Rossetto PB, Ferreira A, Tsui S, Lacava PT, Mondin M, Azevedo JL, Pizzirani-Kleiner AA (2012) Sugarcane growth promotion by the endophytic bacterium *Pantoea agglomerans* 33.1. Appl Environ Microbiol 78:7511–7518. doi:10.1128/AEM.00836-12

Rosenblueth M, Martínez-Romero E (2006) Bacterial endophytes and their interactions with hosts. Mol Plant-Microb Interac 19:827–837. doi:10.1094/MPMI-19-0827

Torres AR, Araújo WL, Cursino L, Hungria M, Plotegher F, Mostasso FL, Azevedo JL (2008) Diversity of endophytic enterobacteria associated with different host plants. J Microbiol 46:373–379. doi:10.1007/s12275-007-0165-9

Turner JT, Lampel JS, Stearman RS, Sundin GW, Gunyuzlu P, Anderson JJ (1991) Stability of the δ-endotoxin gene from *Bacillus thuringiensis* subsp. *kurstaki* in a recombinant strain of *Clavibacter xyli* subsp. *cynodontis*. Appl Environ Microbiol 57:3522–3528

Valdivia RH, Cormack BP, Falkow S (1998) The use of green fluorescent protein in prokaryotes. In: Chalfie M, Kain S (eds) Green fluorescent protein: properties, applications and protocols. Wiley, New York, pp 121–138

Verma SC, Singh A, Chowdhury SP, Tripathi AK (2004) Endophytic colonization ability of two deep-water rice endophytes, *Pantoea* sp. and *Ochrobactrum* sp. using green fluorescent protein reporter. Biotechnol Lett 26:425–429

Zinniel DK, Lambrecht P, Harris NB, Feng Z, Kuczmarski D, Higley P, Ishimaru CA, Arunakumari A, Barletta RG, Vidaver AK (2002) Isolation and characterization of endophytic colonizing bacteria from agronomic crops and prairie plants. Appl Environ Microbiol 68:2198–2208