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Evaluation of Endophytic Colonization of *Citrus sinensis* and *Catharanthus roseus* Seedlings by Endophytic Bacteria

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Over the last few years, the endophytic bacterial community associated with citrus has been studied as an important component interacting with *Xylella fastidiosa*, the causal agent of citrus variegated chlorosis (CVC). This bacterium may also colonize some model plants, such as *Catharanthus roseus* and *Nicotiana clevelandii*. In the present study, we compared the endophytic colonization of *Citrus sinensis* and *Catharanthus roseus* using the endophytic bacteria *Klebsiella pneumoniae*. We chose an appropriate strain, *K. pneumoniae* 342 (Kp342), labeled with the GFP gene. This strain was inoculated onto seedlings of *C. sinensis* and *C. roseus*. The isolation frequency was determined one week after the inoculation and the endophytic colonization of *K. pneumoniae* was observed using fluorescence microscopy. Although the endophytic bacterium was more frequently isolated from *C. roseus* than from *C. sinensis*, the colonization profiles for both host plants were similar, suggesting that *C. roseus* could be used as a model plant to study the interaction between endophytic bacteria and *X. fastidiosa*.

Keywords: endophytic bacteria, diazotrophic endophyte, green fluorescent protein, *Citrus sinensis*, *Catharanthus roseus*

Endophytic bacteria have been defined as bacteria that can be isolated from the disinfected surfaces of plant tissues or that can be extracted from within the plant, and as bacteria that do not cause visible harm to the host (Hallmann *et al.*, 1997) and external visible structures (Azevedo *et al.*, 2000). They can promote the growth of many field crops by producing plant growth-promoting substances and by fixing nitrogen from the atmosphere (Sturz *et al.*, 2000; Lodewyckx *et al.*, 2002). They also have the potential to act as biocontrol agents against phytopathogens (Sturz *et al.*, 1998) and insects (Azevedo *et al.*, 2000).

The bacterium *Klebsiella* sp. is a common endophyte found in maize (*Zea mays*) (Fisher *et al.*, 1992; McInroy and Klopper, 1995; Chelius and Triplett, 2001), red clover (Sturz *et al.*, 1998), grapevine (Bell *et al.*, 1995), rice (Elbeltagy *et al.*, 2000), sweet potato (Paula *et al.*, 1993; Adachi *et al.*, 2002), alfalfa (Dong *et al.*, 2003a) and soybean (Kuklinsky-Sobral *et al.*, 2004), where it may improve plant growth via nitrogen fixation, as demonstrated by the dinitrogenase reductase protein of *K. pneumoniae* found within the roots of maize (Chelius and Triplett, 2000). Nitrogen (N)-fixing bacteria that inhabit the interior of plants without causing any disease are called diazotrophic endophytes (Iniguez *et al.*, 2004). The *K. pneumoniae* 342 (Kp342) strain is able to produce the NifH protein in maize (Chelius and Triplett, 2000) and wheat (Iniguez *et al.*, 2004).

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The citrus culture in Brazil is an economically important crop, and special attention has recently been given to the study of endophytic bacteria from *C. sinensis* living in xylem vessels, with emphasis on the biological control of citrus disease and the understanding of the interaction between endophytes and phytopathogens that co-habit the same niche (Araújo *et al.*, 2001; Araújo *et al.*, 2002; Andreote *et al.*, 2004; Lacava *et al.*, 2004). This interaction among the endophytic bacteria *Xylella fastidiosa* and their host plants has been studied; however, plant studies are time consuming primarily due to the disease characteristics, which take at least six months to develop into symptoms in citrus plants. Since the bacterium *X. fastidiosa* demonstrates the ability to colonize different hosts while expressing similar symptoms in each host (Lopes *et al.*, 2000, 2003; Monteiro *et al.*, 2001), model plants, such as *C. roseus* and *Nicotiana* spp., have been evaluated to study the interaction between this fastidious bacterium and endophytes. *Catharanthus roseus* has been used as a plant model in the study of important citrus diseases such as citrus variegated chlorosis (CVC) (Monteiro *et al.*, 2001; Andreote *et al.*, 2006). Andreote *et al.* (2006) described *C. roseus* as a model for studying the endophytic bacteria from citrus.

Furthermore, green fluorescent protein (GFP) is a useful tool for studying the microbial colonization of plants, particularly endophytic bacteria, in time and space without disturbing the bacterium or the host tissue. The use of GFP allows the integrity of the plant structures and the bacteria residing within them to be preserved (Gage *et al.*, 1996; Bloemberg *et al.*, 1997; Tombolini *et al.*, 1999; Chelius and Triplett, 2000). Therefore, the objective of the present study

was to evaluate the colonization of *C. sinensis* and *C. roseus* by *K. pneumoniae* strain Kp342 and to compare the colonization profile in these host plants.

Materials and Methods

Strain and preparation of inoculum

The endophyte *K. pneumoniae* strain Kp342 was labeled with GFP (Chelius and Triplett, 2000) and the wild strain was originally isolated from maize, which was described as a nitrogen-fixing bacterium (Iniguez *et al.*, 2004). The seedling roots were introduced into a PBS bacterial suspension (4×10^9 CFU/ml) at room temperature for 2 h. The seedling roots in the negative control group were introduced with sterile phosphate buffered saline (PBS, NaCl, 8 g/L; KCl, 0.2 g/L; Na_2HPO_4, 1.4 g/L; KH_2PO_4, 0.24 g/L).

Plant growth conditions

C. sinensis Osbeck, var. Natal and *C. roseus* (L.) G. Don (cv. Peppermint Cooler) were used as the experimental hosts. The seeds were planted in the appropriate substrates (sand-vermiculite soil) under greenhouse conditions (photoperiod of 14 h light at 28°C and 10 h dark at 18°C).

Seedlings surface sterilization and isolation of K. pneumoniae 342

The seedlings were washed in running tap water and were superface-disinfected with stepwise washes: 70% ethanol for 5 min, sodium hypochloride solution (2% available per Cl) for 5 min, 70% ethanol for 1 min, and twice with sterile distilled water. To confirm the efficiency of the disinfection process, aliquots of the sterile distilled water used in the final washing were spread over Luria-Bertani (LB, Sambrook *et al.*, 1989) media plates and examined for surface contaminants after 3 days of incubation at 30°C (Andreote *et al.*, 2004). The isolation was performed according to the method described by Araújo *et al.* (2002); fragments from *C. sinensis* and *C. roseus* branches and roots were homogenized in 5 ml of sterile PBS with a blender and serial dilutions were plated onto LB media with 50 μg of benomyl per ml to inhibit fungal growth. The media was supplemented with 25 μg and 50 μg per ml of ampicillin and kanamycin, respectively, to obtain a selection of Kp342 colonies.

Fluorescence microscopy

Seven days after the inoculation, *C. sinensis* and *C. roseus* seedlings (inoculated and non-inoculated) were removed from the pots and washed in running tap water. The roots and branches were cut into small pieces and mounted on a bridged slide with 10% (v/v) glycerol (microscope grade). Fluorescence microscopy was carried out on an Axioskop 2, Zeiss microscope, and the images were taken using the Axivision 2.05 Image Program, Zeiss. GFP-tagged bacterial cells were excited with the 490 nm filter.

Statistical analysis

Analysis of the variances ($P<0.05$) of the dates from isolation were carried out using the SAS software package [Copyright (c) 1989-96]. Bacterial counts were transformed using $\log_{10}$ of X+1 before the analysis of variance.

Results and Discussion

In the present study, we confirmed that the endophytic colonization only reflects cells within plant tissues by two control methods. In the first method, the root and branch samples were examined by fluorescence microscopy after surface sterilization for GFP-containing cells of the plant surface, but no cells were ever observed using this approach. In the second method, bacterial growth was not observed when the final set of washes used to rinse the tissues was cultured. No disease symptoms were observed in *C. sinensis* and *C. roseus* seedlings inoculated with Kp342 after twenty days of inoculation when compared to the non-inoculated seedlings. Strain Kp342 was significantly ($P<0.05$) and more frequently isolated from *C. roseus* than from *C. sinensis*, while the isolation frequency in the roots was higher than in the branches (Fig. 1). The genus *Klebsiella*, frequently described as endophytic, are known to colonize the rhizosphere, seeds and other parts of plants. The Kp342 strain, in particular, has a very broad host range and is capable of colonizing the interior of many plants (Dong *et al.*, 2003a,b). This enteric bacterium is also described as a more efficient endophytic colonizer of roots than of branches or leaves (Dong *et al.*, 2003b). We found similar results in the present study where the Kp342 strain was more efficient in colonizing in roots than in branches in both plant species studied. Dong *et al.* (2003b) described the ability of the Kp342 strain to colonize different species of host plants; however, the host plants differed in their abilities to be endophytically colonized by the same bacterium and they suggested that the host is an important factor that should be considered in the colonization.

Fluorescence microscopy revealed that the Kp342 strain colonized the xylem vessels of *C. sinensis* roots (Fig. 2) and branches (Fig. 3), and it was able to colonize the xylem vessels of *C. roseus* branches (Fig. 4) and roots. Previous reports have described the ability of the enteric bacteria *K. pneumoniae* to colonize roots and vascular tissue of plants (Dong *et al.*, 2003a). In another study, the authors described the Kp342 strain as being capable of endophytically

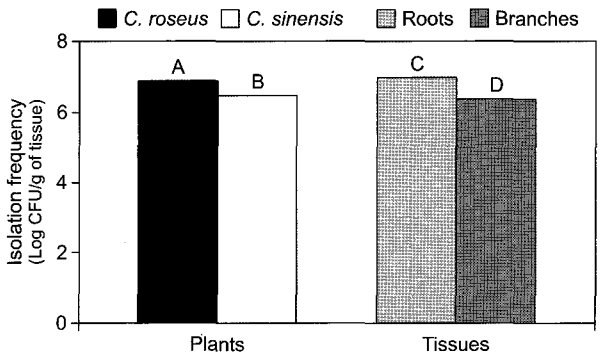

Fig. 1. Isolation frequency of Kp342 in *C. sinensis* and *C. roseus* seedlings from root and branch tissues. Bars with different letters are statistically ($p<0.05$) different by Tukey test (coefficient of variation=2,058052).

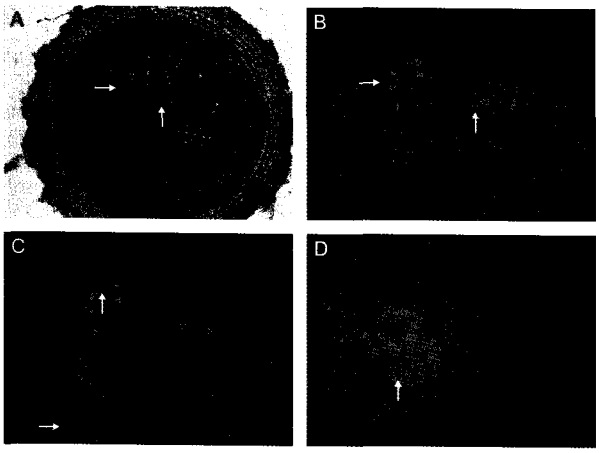

Fig 2. Plant colonization by GFP-labeled *K. pneumoniae* 342 (A, B, C, D). Transversal sections of *C. sinensis* roots showing bacterial colonies inside the xylem vessels (arrows). Bars, 50 μm.

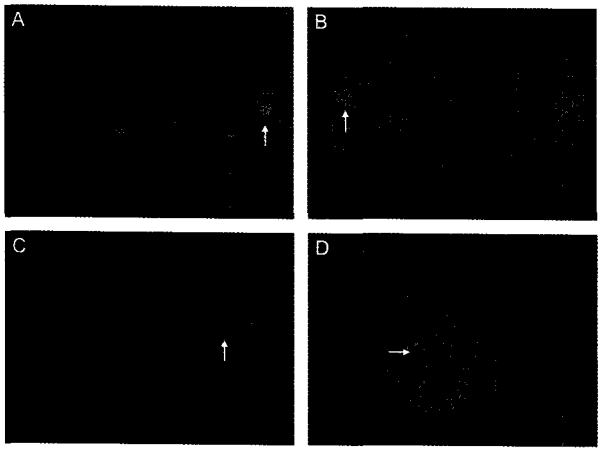

Fig 3. Plant colonization by GFP-labeled *K. pneumoniae* 342 (A, B, C, D). Transversal sections of *C. sinensis* branches showing bacterial colonies inside the xylem vessels (arrows). Bars, 50 μm.

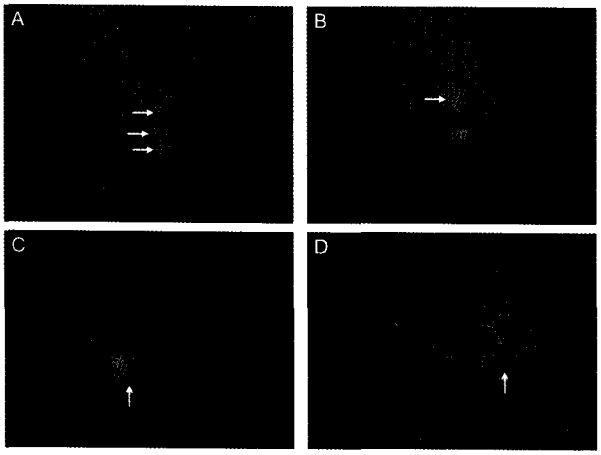

Fig 4. Plant colonization by GFP-labeled *K. pneumoniae* 342 (A, B, C, D). Transversal sections of *C. roseus* branches showing bacterial colonies inside the xylem vessels (arrows). Bars, 50 μm.

colonizing the vascular tissues of *Arabidopsis thaliana* (Dong *et al.*, 2003b). We found that the Kp342 strain was capable of colonizing the vascular tissue of *C. sinensis* and *C. roseus*, which are from the same class as *Arabidopsis*, dicotyledonae, which was in agreement with the finding of Dong *et al.*, (2003b). Bacterial GFP production can provide a simple and powerful tool for monitoring the endophytic colonization of plant tissues.

GFP expression was used to demonstrate the ability of the Kp342 strain to colonize the cortex and intercellular spaces of maize, as well as its ability to colonize and express the *nif* genes in maize roots (Chelius and Triplett, 2000; Dong *et al.*, 2003a, 2003b; Iniguez *et al.*, 2004). In this study, we demonstrated this same ability in *C. sinensis* and *C. roseus* using the same tool, indicating that the results obtained in *C. roseus* may be similar to those observed in *C. sinensis*. This data confirm that *C. roseus* could be used as a model plant in the study of the interaction between endophytic bacteria and citrus plants. This suggests a unique colonization system, making it possible to study the control strategies employed by this model plant because the results can be obtained more quickly than with traditional host cultures, such as citrus, coffee and plums.

To summarize, we used a specific genetic marker (the green fluorescent protein) and isolation technique to evaluate the endophytic colonization profile of *K. pneumoniae* in *C. sinensis* and *C. roseus* plants. This study showed that this colonization is similar, suggesting that the results obtained with *C. roseus* could be used to aid in the understanding of the endophyte-citrus interaction. Since the effect of the endophytic community on *X. fastidiosa* was proposed (Araújo *et al.*, 2002; Lacava *et al.*, 2004), the use of GFP is a good approach to be used in elucidating the role of endophytic bacteria in citrus plants and it will be important to develop a system for investigating the interaction between citrus endophytes and the development of CVC symptoms.

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