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Impact of Genetically Modified *Enterobacter cloacae* on Indigenous Endophytic Community of *Citrus sinensis* Seedlings

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***Enterobacter cloacae* (strain PR2/7), a genetically modified endophyte (GME) in citrus plants, carrying different plasmids (pEC3.0/18, pCelE, pEglA and pGFP), was inoculated into *Citrus sinensis* seedlings under greenhouse conditions. The impact of this on the indigenous bacterial endophytic community was studied by analyses of 2 different morphologic groups. The germination rates of inoculated seeds were evaluated in greenhouse, and plasmid stability under *in vitro* conditions. Results demonstrated a great and diverse endophytic community inside plants, and specialization in tissue colonization by some bacterial groups, in different treatments. Shifts in seed germination rate were observed among treatments: in general, the PR2/7 harboring pEglA bacterial clone significantly reduced seed germination, compared to the PR2/7 harboring pEC3.0/18 clone. This suggests that the presence of the pEglA plasmid changes bacteria-seed interactions. The endophytic community of citrus seedlings changed according to treatment. In seedlings treated with the PR2/7 with pEglA clone, the population of group II decreased significantly, within the context of the total endophytic community. These results indicate that the application of GMEs induces shifts in the endophytic bacterial community of citrus seedlings.**

Key words: endophytic bacteria, *Enterobacter cloacae*, genetically modified endophyte, citrus

Endophytic bacteria are defined as bacteria, which have been isolated from surface-disinfected plant tissues, or extracted from inner plant parts (Hallmann *et al.*, 1997). Endophytes include both commensal microorganisms, which have no direct effect on the host plant, and mutualistic symbionts, which can be used in the biological control of pathogens and for the promotion of plant growth. The role of the endophytic community in endophyte-plant associations has been intensively discussed (Hallmann *et al.*; 1997; Azevedo *et al.*, 2000; Sturz *et al.*, 2000; Araújo *et al.*, 2002; Peixoto-Neto *et al.*, 2002). In citrus plants, endophytic bacteria have been isolated from healthy plants, where *Methylobacterium* spp., *Pantoea agglomerans*, *Enterobacter cloacae*, *Curtobacterium flaccumfaciens*, *Acromobacter* spp., *Acinetobacter baumanii*, *Acinetobacter iwoffii*, *Alcaligenes-Moraxella*, *Arthrobacter* spp., *Bacillus* spp., *Burkholderia cepacia*, *Citrobacter freundii*, *Corynebacterium* spp., *E. aerogenes* and *Pseudomonas* spp. have been reported as the main bacterial species associated with this host (Gardner *et al.* 1982, 1985; Araújo *et al.*, 2001; Araújo *et al.*, 2002).

There is an increasing interest in the possible commercial applications of genetically modified endophytes (GME). This interest is currently centered on the biological control of pests and plant diseases, and the promotion of plant growth. The introduction of heterologous genes in endophytic bacteria may confer new characteristics, which may be useful in biocontrol of diseases and pests that harm the host plant. For example, the endophytic bacteria *Clavibacter xyli* subsp. *cynodontis*, which colonizes the xylem of different plant species, was genetically modified to express the gene *cryA(c)* from *Bacillus thuringiensis*, encoding a protein that controls the larvae of *Ostrinia nubilalis* (Fahey *et al.*, 1991; Lampel *et al.*, 1994). Recently, the genes *cry1Ac7* of *Bacillus thuringiensis* and *chiA* of *Serratia marcescens* were introduced into sugarcane-associated bacteria, in an attempt to exert biocontrol over *Eldana saccharina* (Downing *et al.*, 2000). Inoculation of wheat seeds with a genetically modified *Pseudomonas putida* WCS358r, codifying an antifungal compound, results in a reduction of fungal biodiversity and population in the soil, reducing the populations of some pathogenic fungi,

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such as *Fusarium* spp. (Glandorf *et al.*, 2001). The purpose of this work was to determine whether a GME, obtained by the transformation of *E. cloacae* PR2/7 with model plasmids, might affect the germination of citrus seeds, and alter the indigenous endophytic community in citrus seedlings.

Materials and Methods

Bacterial strain, plasmids and growth conditions

Enterobacter cloacae strain PR2/7, isolated from *Citrus reticulata,* was grown in LB medium at 28°C for 24 h. Four plasmids (pEC3.0/18, pGFP, pCelE, and pEglA) were used to obtain the GMEs. These plasmids were used in order to evaluate the effect of heterologous endoglucanase (plasmids pCelE and pEglA), heterologous gene (pGFP), or antibiotic resistance markers (all plasmids) on plant development and interactions between the plant and the endophytic bacteria. Transformant clones denominated as PR2/7 : pEC3.0/18, PR2/7 : pCelE, PR2/7 : pEglA, and PR2/7 : pGFP carried the plasmids pEC3.0/18, pCelE, pEglA and pGFP (Table 1), respectively.

Transformation of E. cloacae strain PR2/7

Electro-competent cells of *E. cloacae* (strain PR2/7) were obtained by the following method: an overnight culture was diluted in a new fresh medium (1 : 50), and development was monitored by spectrophotometry until OD_{600}= 0,6 to 0,8. Cells were harvested by centrifugation (1800 x g, 5 min, 4°C) and washed 3 times with ice-cold sterile water. The cell suspension was concentrated 50-fold in 10% glycerol, aliquoted in 50 µl aliquots, and kept at -80°C.

For bacterial transformation, electro-competent cells (50 µL) were mixed with DNA solution (500 ng) in an ice-chilled 0.1cm cuvette. Electroporation was carried out using a Gene Pulser (Bio-Rad, USA) with the following parameters: 2.5 kV, 25 µF and 200 Ω. Immediately after pulsing, 1 ml of LB media was added to the cuvette, the cell suspension was transferred to a test tube, and then was incubated for 1 h at 28°C. Transformed clones were selected on solid LB medium with ampicillin (100 µg · ml^{-1}).

Detection of plasmid gene expression in PR2/7 strain

Heterologous gene expression was verified using specific media. Bacteria carrying the pEglA and pCelE plasmids were grown on plates containing solid LB media with 1% CMC (carboxy-methyl-celullose) and 100 µg · ml^{-1} ampicillin (Sigma, USA). After cultivation, the CMC degradation halo was visualized by staining with Congo Red 1% solution and washing with 5 M NaCl. Transformants expressing the *gfp* gene (pGFP) fluoresced green under UV light, and those containing plasmid pEC3.0/18 were ampicillin-resistant.

In vitro plasmid stability

Plasmid stability (Table 1) in endophytic *E. cloacae* was assessed as follows. Clones containing plasmids were grown in LB supplemented with ampicillin (100 µg · ml^{-1}) to log phase at 28°C, diluted to 10^{-5} bacteria per 50 ml of LB medium without antibiotics, and further grown for 72 h. Bacterial culture was diluted and plated onto LB and LB+ampicillin (100 µg · ml^{-1}) plates. The percentage of patched colonies, grown on these plates, was recorded. This experiment was performed twice for each tested bacterium.

Plant inoculation

Colonization of seedlings by GMEs and wild strain PR2/7 was assessed in *Citrus sinensis* Osbeck, var. Natal. The GMEs were inoculated by shaking seeds (30 per treatment) in a bacterial suspension (10^6 CFU · ml^{-1}) in PBS buffer (NaCl 0.8%, KCl 0.02%, Na_2HPO_4 0.14%, KH_2PO_4 0.024 %) for 20 h, before sowing. After this, seeds were planted in appropriate substrates, under greenhouse conditions. Germination rates were evaluated after seedlings reached 2 leaves (~30 days).

Isolation of endophytic bacteria

Colonization of citrus by endophytic bacteria was assessed in roots, stems and leaves of 30-day old seedlings of *C. sinensis* inoculated with GMEs. As a control, non-inoculated plants were used, and five plants per treatment were evaluated. Seedlings were washed in running tap water, and surface-disinfected with stepwise washes: 70% ethanol for 5 min, sodium hypochloride solution (2% available Cl^-) for 5 min, 70% ethanol, and twice with sterile distilled water. To confirm the efficiency of the disinfection process, aliquots of sterile distilled water used in the last washing were spread over plates of 5% Tryptic Soy Agar (TSB, Merck) media, and examined for surface contaminants after 3 days' incubation at 28°C.

Table 1. Plasmids used to transform *E. cloacae* PR2/7

Plasmid	Description*
pEC3.0	Cryptic plasmid
pEC3.0/18	pEC3.0 cloned in pUC18, Amp^r
pGFP	pBADMycHis with *gfp* gene, Amp^r
pCelE	pBADMycHis; *celE* gene, which codify a endoglucanase enzyme from *Pseudomonas celullosa,* Amp^r
pEglA	pBADMycHis *eglA* gene, which codify a endoglucanase enzyme from *Bacillus pumilus,* Amp^r

*amp^r: resistance to ampicillin

Surface-disinfected samples were macerated in a PBS buffer, and appropriate dilutions were plated onto 5% TSB, with 50 $\mu g \cdot ml^{-1}$ of benomyl added to inhibit fungal growth. Samples were also inoculated in TSB 5% media supplemented with 100 $\mu g \cdot ml^{-1}$ of ampicillin, in order to estimate the bacterial community resistant to this antibiotic in each treatment. Incubation was carried out at 28°C for 1 to 8 days to allow the growth of endophytic bacteria, and to determine the number of CFUs $\cdot g^{-1}$ of tissue. Representative bacterial isolates were picked out, purified and identified by biochemical traits, as described by Holt *et al.* (1994).

Differences in total and groups isolation frequencies among seedlings were determined by performing Tukey tests at 5% significance.

Results and Discussion

Transformation of E. cloacae strain PR2/7 and plasmid stability

An essential factor in achieving expression and stability of heterologous genes in endophytic bacteria is the use of suitable vectors. Used plasmids were derived from commercial pUC18 (pEglA, pGFP, and pCelE), or from an *E. cloacae* cryptic plasmid pEC3.0 (plasmid pEC3.0/18). Fig. 1 shows plasmid stability after 72 hours of bacterial growth in LB medium without antibiotic (Fig. 1). Overall, plasmid stability was high, except for plasmid pCelE, which showed a 25% loss after 72 h. Results suggest that plasmids pEglA, pGFP and pEC3.0/18 are kept inside the host cell during citrus endophytic colonization, allowing their use in expression of heterologous genes inside plant tissues.

The constitutive expression of heterologous genes was confirmed in *in vitro* assays. Media LB+CMC+ampicillin showed endoglucanase activity (strains PR2/7 : pCelE and PR2/7 : pEglA), LB+amp was used to verify plasmid presence in strains PR2/7:pEC3.0/18 and PR2/7 : pGFP, (PR2/7 : pGFP presented green fluorescence under UV light). Plasmids obtained from the pBAD vector were tested in media containing arabinose (promoter inductor), and no difference was observed in media without it. These observations support that the effect of the GMEs, expressing these genes on indigenous endophytic community inside the plants, may be evaluated.

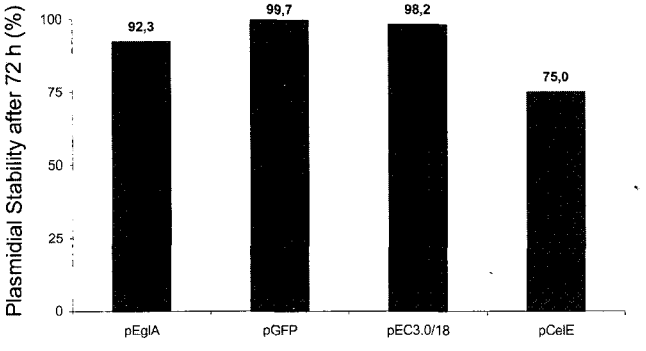

Fig. 1. *In vitro* plasmidial stability of *Enterobacter cloacae* (strain PR2/7) transformed with pEglA, pGFP, pEC3.0/18 or pCelE plasmids (average of three repetitions).

Effect of GME in germination rate

Seed germination was analyzed every 10 days, for up to 30 days after inoculation with GMEs (PR2/7 : pCelE, PR2/7 : pEGLA, PR2/7 : pEC3.0/18, and PR2/7 : pGFP) or the indigenous PR2/7 strain. Seeds treated with the PR2/7 : pEC3.0/18 strain showed a statistically significant increase in germination rate when compared to PR2/7 : pEglA (Fig. 2), suggesting that the expression of the endoglucanase gene in PR2/7 : pEglA may exert a negative effect on seed physiology. This negative effect could be due to a degradation of plant tissues by endoglucanase (EglA), which is codified by pEglA. Conversely, the presence of PR2/7 : pEC3.0/18 may confer some advantages carried by the pEC3.0 plasmid; in fact, pEC3.0 cloned in a pUC18 vector considerably increases the copy numbers of this plasmid in host cells. This increase could be related to an inhibition of soil pathogens. Although differences in germination rate had been observed, the GMEs had no effect on seedling development or health status of the plants (data not shown).

Effects on the composition of indigenous endophytic bacterial community

The endophytic community of GME-treated and non-treated citrus seedlings was analyzed by isolation in 5% TSB medium. 2 major groups (*E. cloacae* and *P. agglomerans*) were observed, as were a few other minor groups (*Bacillus* spp., *Streptomyces* spp., *Pseudomonas* spp.), which were not studied due to their low frequencies. In orchard-cultivated citrus plants, the bacterial community is composed of different bacterial groups, such as *Bacillus* sp., *E. cloacae, P. agglomerans, Methylobacterium* spp., *Curtobacterium* sp., *Streptomyces* sp. and *Xanthomonas* sp. (Araújo *et al.*, 2001, 2002). However, in the present

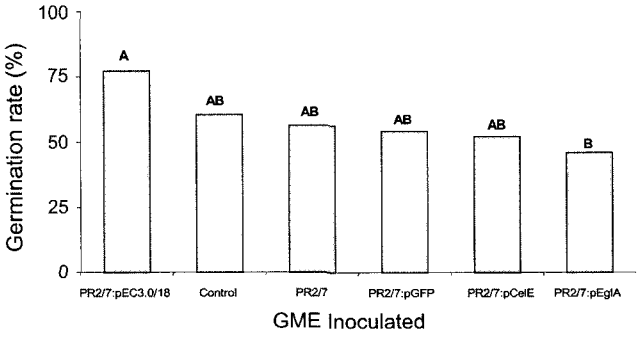

Fig. 2. Effect of *E. cloacae* strain PR2/7 carrying different plasmids on the germination rate of seeds of *Citrus sinensis*. Results are based in number of germinated seeds from 30 that were sowed. Bars with the same letters are not statistically ($p>0.05$) different by Tukey test.

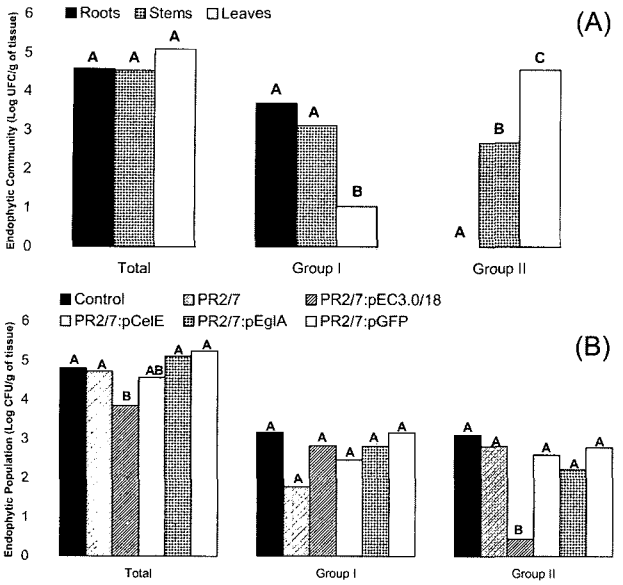

Fig. 3. Isolation frequency of endophytic bacterial groups in citrus seedlings inoculated with GME. A) Total, group I (*E. cloacae*) and group II (*P. agglomerans*) isolation frequency in roots, stems and leaves of inoculated seedlings; B) Isolation frequency of endophytic bacteria from different seedlings treated with GME. Bars with the same letters are not statistically ($p<0.05$) different by Tukey tests that were made separately for each group.

study, using greenhouse conditions, diversity was considerably lower. As shown, *P. agglomerans and E. cloacae* had already been isolated from citrus plants (Araújo *et al.*, 2001; 2002), and their role in plant-fungal-bacteria interaction had previously been proposed (Araújo *et al.*, 2001).

Application of GMEs and the PR2/7 strains to seeds caused a shift in the bacterial endophytic population of citrus seedlings (Fig. 3). The development of the bacterial endophytic community in the GME-treated seedlings appeared to be different from that observed in the control seedlings, in details such as the different density of the group II population.

The density and diversity of the bacterial population varies in plant tissues, according to the adaptations of each endophytic species. Group I (*E. cloacae*), which is present in the whole plant, preferentially colonizes the roots, and the population decreases in the upper parts of the plant (Fig. 3A). In contrast, group II was isolated in high density from leaves, lower density in stems, and was not observed in the roots (Fig. 3A). The major site of endophytic penetration in the host plant has been demonstrated to be the secondary root emission zone and the stomata areas (Hallmann *et al.*, 1997), suggesting that differences in bacterial densities in citrus seedlings may be associated, first, with the initial colonization site, followed by interactions with the host plant and with the indigenous endophytic bacterial community.

Analyzing total population frequencies, and groups I and II in treated plants, a minor bacterial community was verified to colonize the inner tissues of seedlings treated with transformant PR2/7:pEglA (Fig. 3B). The reduced population of *P. agglomerans* (group II) observed in this treatment can explain this decrease in total bacterial density in these plants (Fig. 3B), suggesting that *EglA* gene activity may interfere with plant-bacteria interactions.

Recent studies have examined natural bacterial community variations, concluding that environmental factors, such as soil type, field location, and annual climate variation, play a major role in determining the profile of rhizosphere-colonizing bacteria in the sugar beet (*Beta vulgaris*) (Schmalenberger and Tebbe, 2003). Variation in population densities can be attributed to several factors, such as changes in species that encourage this community or host, and seasonal changes, as described by Mocali *et al.* (2003), who studied fluctuations in the endophytic bacterial community in the roots and branches of elm trees (*Ulmus* spp.), by ARDRA analysis.

Future research will focus on the identification of culturable and unculturable bacterial species which are affected by the introduction of GMEs. In the present study, it was observed that the bacterial community, endophytically associated with citrus seedlings, is affected by the introduction of GMEs expressing different heterologous genes. Further studies will also focus on the nature of the induced effects, and on the question of whether this shift could play a role in the health status of citrus groves.

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