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Host and tissue preferences of *Enterobacter cloacae* and *Bacillus amyloliquefaciens* for endophytic colonization

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***Enterobacter* and *Bacillus* are common endophytes in many plants. Endophytic *Enterobacter cloacae* 344 and *Bacillus amyloliquefaciens* 629 from cacao had their colonization patterns assessed in different plants and organs. The 344's colonization in all plants was ~12x higher than those observed for 629. CFU countings were 16 and 3 times lower for 344 and 629, respectively, in non-sterile than in sterile conditions. The results suggested that 344 is a better endophytic colonizer than 629, but this seems a better competitor than 344. Indigenous endophytic fungi interfered with the colonization levels of both strains in all plants. Plant species, tissue and competing microbes appear to define the levels of endophytic colonization by these bacteria.**

Key words: Cacao, endophytic bacteria, population densities.

INTRODUCTION

Beneficial microorganisms have been of great interest to researchers worldwide over the last years, especially for their potential in agricultural systems. Plant colonization is the first step and a prerequisite for the successful delivery of beneficial effects to host plants (Steenhoudt and Vanderleyden, 2000). Endophytic bacteria particularly, have been found residing latently or actively colonizing plant tissues locally as well as systemically (Hallmann et al., 1997). The intrinsic nature of the colonization within the host tissues protects endophytes from harmful environmental conditions, which constitutes an advantage over epiphytic bacteria. Endophytic bacteria have the ability to colonize an ecological niche similar to that of phytopathogens, which makes them suitable biocontrol agents (Compant et al., 2005; Leite et al., 2013). Endophytes may promote plant growth and yield, tolerance to biotic and abiotic stresses, and enhance the efficacy of phytoremediation (Leite et al., 2013; Luo et al., 2011). This group of bacteria does not visibly harm the host plant and can be isolated from surface-disinfested plant

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tissues or extracted from inside the plant (Hallmann et al., 1997). *Enterobacter* and *Bacillus* are among the most frequently isolated native endophytes found in the microbiota of several plant species (Naveed et al., 2014; Talboys et al., 2014).

Bacterial rhizosphere colonization is a relatively well-studied process (Kloepper and Beauchamp, 1992; Lugtenberg et al., 2001; Raaijmakers and Lugtenberg, 2013). Several bacterial genes involved in rhizosphere colonization have been described (Lugtenberg et al., 2001; Barahona et al., 2010; Chauhan and Nautiyal, 2010). On the other hand, endophytic colonization is relatively less studied, with only a few recent studies on the nitrogen fixing genera *Azoarcus* and *Gluconacetobacter* (Böhm et al., 2007; Alquéres et al., 2013).

The host plant influences rhizosphere colonization by beneficial bacteria, but the genetic basis of this phenomenon remains largely unknown. In one of the few existing studies, the genes responsible for supporting high populations of the beneficial bacterium *Bacillus cereus* on seeds were mapped to one specific quantitative trait locus (QTL) in the tomato genome. In addition to the contribution of the host in the selection of specific endophytes, bacteria could also produce active molecules to exclude competing microorganisms (Bergsma-Vlami et al., 2005; Smith et al., 1999).

The aim of this study was to investigate host and tissue preferences for colonization of two bacterial endophytes in four plant species grown under sterile and non-sterile conditions.

MATERIALS AND METHODS

Endophytic wild-type *Enterobacter cloacae* 344 (JQ435862 – 16S rDNA; JQ435864 – heat shock protein *hsp60*; JQ435866 – RNA polymerase beta-subunit, *rpoB*) and isolate 629, initially identified as *Bacillus subtilis* on the basis of the 16S rDNA sequence (JQ435867) were isolated from healthy adult *Theobroma cacao* trees (Leite et al., 2013). Further analyses performed with *gyr*A (LN555733) and *rec*A sequences (LN555734) revealed that isolate 629 belongs to the species *Bacillus amyloliquefaciens*. These strains are deposited in the Biological Institute Culture Collection of Phytopathogenic Bacteria - IBSBF (Campinas, São Paulo, Brazil) under accession numbers IBSBF-3105 and IBSBF-3106, respectively. This collection is registered with the World Data Centre for Microorganisms collection under number WDCM-110. Spontaneous rifampicin-resistant (rif^R) variants of 344 and 629 were used in the experiments reported herein; such mutants were individual colonies, obtained as follows: suspensions of wild-type cells at 10^5, 10^6 and 10^7 CFU ml^{-1} were plated on nutrient agar - NA (5 g peptone, 3 g beef extract, 16 g agar per liter) supplemented with 50 µg ml^{-1} rifampicin and grown for 24 h at 25 ± 2°C (room temperature); the surviving colonies were transferred to NA + 100 µg ml^{-1} rifampicin and grown in the same conditions. A single surviving colony for each strain was selected for the studies. Bacterial rif^R strains were then routinely grown on NA medium supplemented with 100 µg ml^{-1} of rifampicin. These isolates were stored in 40% glycerol at -80°C.

Endophytic colonization studies were performed with seeds of (i) cucumber (*Cucumis sativus*) cv. Marketmore 76, (ii) cacao (*Theobroma cacao*) clone CEPEC-2002, (iii) maize (*Zea mays*) var. BRS Caatingueiro and (iv) common bean (*Phaseolus vulgaris*) cv. BRS Notável. Seeds were surface-sterilized by soaking in 70% ethanol for 1 min, then in 1% sodium hypochlorite for 3 min and rinsed three times with sterile distilled water. An assessment of the efficacy of surface sterilization was performed by plating the water from the third rinse on NA; no growth was observed in any treatment. Seeds were dried on sterile paper towels and transferred to Petri dishes containing 0.8% water-agar. After incubation at 25 ± 2°C, when the radicles measured approximately 1 cm long, germinating seeds were immersed for 30 min in separate bacterial suspensions containing 10^5 CFU ml^{-1} of each endophyte under study. Germinating seeds of each plant species treated with each bacterial strain were transferred to sterile tubes containing 0.8% water-agar and incubated at 25°C with a 12 h photoperiod for 7 days, except for cacao that was incubated for 14 days (sterile system). For the non-sterile system, the treated germinating seeds were transferred to 5 kg pots filled with a commercial substrate (Vivatto Slim Plus®) mixed with NPK (4-14-8) in order to aid in the development of the surrounding microbial community to generate a competition effect against the two inoculated strains. Pots were kept inside a greenhouse for the same period described for the sterile system. For non-inoculated controls, germinating seeds were immersed in distilled-sterile water for the same 30-min time (see above). After the incubation, seedlings were removed from the sterile tubes or from the pots and superficially sterilized as described above for seeds. Roots, stems and leaves were separated, weighed individually, and ground in sterilized mortars with pestles. The resulting extracts were diluted and plated in triplicates onto NA supplemented with 100 µg ml^{-1} of rifampicin. The number of CFU of *E. cloacae* and *B. amyloliquefaciens* rif^R inoculated endophytes (referred throughout only as 344 and 629, respectively) per gram of fresh tissue was determined after growing in the same conditions described.

Colonization by fungi was also estimated. Five superficially sterilized pieces of approximately 0.3 cm^2 from each part of plants grown under sterile and non-sterile conditions were plated onto PDA and observed for mycelial growth for up to 4 days.

All experiments were done at least twice and were installed in a completely randomized design with 10 replicates. Data were subjected to analysis of variance and when significant, means were compared by Scott-Knott's test ($p \leq 0.05$) using the SISVAR 5.3 software (Ferreira, 2011).

RESULTS AND DISCUSSION

Colonization levels of *E. cloacae* 344 and *B. amyloliquefaciens* 629 were on average 23 and 3 times higher, respectively, under sterile than non-sterile conditions, considering all plant species studied (Table 1). On average, when all plant species are considered under both sterile and non-sterile conditions, populations of *E. cloacae* 344 are ~12 times higher than populations of *B. amyloliquefaciens* 629. This is in agreement with recent studies that have suggested that species of the *Enterobacteriaceae* family tend to be more competent endophytic colonizers than species of the *Bacillaceae*, being more frequently found as endophytes with higher populations in some plants (Santi et al., 2012). Not unexpectedly, the fact that both strains generally colonized plants to a lesser extent in the non-sterile system (under an overall similar growing conditions, e.g. temperature, air humidity and incubation time) suggests that competition with other indigenous microorganisms likely plays an

Table 1. Populations of two bacterial endophytes in roots (R), stems (S) and leaves (L) of four plant species grown under sterile and non-sterile conditions.

Plant species		*Enterobacter cloacae* 344		*Bacillus amyloliquefaciens* 629	
		Sterile	Non-sterile	Sterile	Non-sterile
		10^5 CFU.g^{-1}fresh weight [a]			
Cucumber	R	nd	nd	nd	nd
	S	nd	0.21±0.01Ab	6.07±4.22Aa	2.83±0.52Aa
	L	42.2±11.45Ab	7.95±2.76Ba	5.29±2.84Ba	3.00±0.95Ba
Cacao	R	9.91±1.05Ac	0.15±0.02Bb	1.36±0.43Ba	0.06±0.02Bb
	S	0.159±0.05Bc	nd	0.83±0.23Aa	0.04±0.01Bb
	L	nd	nd	4.77±3.10Aa	0.44±0.05Ab
Maize	R	20.58±5.03Ab	0.07±0.02Bb	0.96±0.21Ba	0.13±0.04Bb
	S	47.95±6.04Ab	0.50±0.10Bb	2.29±0.46Ba	0.06±0.002Bb
	L	32.28±3.89Ab	nd	1.60±0.45Ba	nd
Bean	R	28.76±7.44Ab	1.68±0.04Bb	0.12±0.05Ba	0.06±0.007Bb
	S	10.78±1.52Ac	0.41±0.15Bb	0.05±0.001Ba	0.04±0.02Bb
	L	134.28±30.55Aa	nd	nd	nd

[a]Values in the table are means of 10 replicates ± standard error. Uppercase letters allow means comparison in the same rows, whereas lowercase letters allow comparisons in the columns, for the same bacterial isolate. Means followed by the same letter are not significantly different ($P > 0.05$) according to Scott-Knott's test. '***nd***' indicates that colonies were 'not detected'.

Table 2. Percentage[a] of colonization of roots (R), stem (S) and leaves (L) of four plant species by indigenous endophytic fungi under non-sterile conditions and post-colonization by *E. cloacae* 344 and *B. amyloliquefacies* 629.

Plant spp.	Organs	Controls	*E. c.* 344	*B. a.* 629
Cucumber	R	100	100	100
	S	100	100	60
	L	60	0	20
Cacao	R	100	100	100
	S	100	60	100
	L	60	0	0
Maize	R/S/L[b]	100	100	100
Bean	R/S/L	100	100	100

[a]Each plant-tissue sample (10 replicates per organ, per plant species) was cut in five pieces that were superficially sterilized and plated out in PDA. Values in the table correspond to the proportion of those pieces showing visible mycelial growth after 4-days incubation. [b]The three organs were assessed separately, but showed the same percentage of fungal colonization.

important role in most plant-endophyte interactions. One may argue that the lack of added nutrients in sterile conditions might have been physiologically stressful for the germinating seeds, so that comparing bacterial colonization between this and the non-sterile system would not be appropriate. However, the incubation time for all treatments was not long enough to have exceeded the period of metabolic availability of the mobilized reserves from cotyledons/endosperm. Thus, a minimally 'normal' physiological state for the seedlings was assured, and so, proper conditions for colonization of inoculated endophytic bacteria. The high CFU countings recovered for both strains in these circumstances were evidence of sufficiently appropriate conditions for plant colonization by the inoculated endophytes (Table 1).

Under the conditions of a more 'natural' microbial competition with the inoculated strains (non-sterile system), we assessed the levels of indigenous fungal colonization within each bacterial endophyte treatment, that is, 344 and 629 (Table 2). There seemed to be an inverse relationship between the population of endophytic bacteria and the colonization of plants by culturable fungi. For example, higher levels of both 344 and 629 colonization occurred for stems and leaves of cucumber and cacao (Table 1) whenever the levels of fungal colonization were below 100% (Table 2). Overall, maize and bean were not well colonized by both strains, with exception of 344 in roots of bean (Table 1). These results were probably due to a higher endophytic fungal population in all the three organs tested in these two plant species (Table 2). Endophytic culturable fungi were not observed under sterile conditions (data not shown), thereby confirming they were all natural colonizers in non-sterile treatments. Interestingly, null or very low colonization results for fungal endophytes were only obtained for leaves from cucumber and cacao (Table 2), which coincided with the highest colonization rates of inoculated bacterial endophytes 344 and 629 in non-sterile conditions (Table 1). This not only further strengthens

the hypothesis of an inverse relationship between endophytic colonization of fungi and bacteria, but also suggests that these two bacterial endophytes seem to be more efficient colonizers/residents/competitors in leaf tissues, at least for these two plant species. Similar studies comparing the richness of culturable fungi and bacteria in fruit seeds of cacao have demonstrated the same inverse relationship between these two taxa (Silva and Loguercio, in prep.). In this study, colonization by other indigenous microorganisms besides culturable fungi was not taken into account; nevertheless, their influence in the final endophytic-colonization outcomes obviously cannot be discarded.

Since the inoculation treatments with 344 and 629 were subjected to same nutritional settings within each growth condition (sterile or non-sterile), a direct comparison between the colonization behaviour of these strains was assessed by taking together their individual CFU countings in both conditions. Populations of *E. cloacae* 344 were 16 and 2 times higher than populations of *B. amyloliquefacies* 629 under sterile and non-sterile conditions, respectively. The sharper decreases in 344 countings when sterile and non-sterile conditions were compared suggest this isolate has a lower competitive ability than 629. Indeed, several studies show that species of *Bacillus* produce more antimicrobial compounds than species of *Enterobacter* (Xu et al., 2013). Our previous greenhouse studies with these two bacteria in cacao seedlings have also shown higher growth-promotion and stress-reduction effects for 629 than for 344 (Leite et al., 2013). Nevertheless, further studies are still necessary to confirm this hypothesis of an overall lower competitive ability for *E. cloacae* than for *B. amyloliquefaciens* strains.

Concerning the endophytic colonization results obtained among the four plant species under study, cucumber was the most colonized one by both bacterial strains under both experimental conditions. While 344 seemed to show a preference for colonization of cucumber leaves, 629 colonized leaves and stems to the same extent (Table 1). Bean plants grown in the sterile system supported the highest populations of 344, mainly in leaves, and the second highest countings in non-sterile conditions; however, this species was the least colonized by *B. amyloliquefaciens* 629 under sterile conditions (Table 1). Little is known about the molecular determinants of endophytic colonization in both plants and bacterial genomes. We are initiating studies with these two isolates as models to determine which genes are important for endophytic colonization.

Although the 344 and 629 endophytes were originally isolated from inside healthy cacao trees, the colonization of this plant species was not preferred by these bacterial strains, at least at the conditions and timeframe tested. This indicates that these endophytes are likely not specifically adapted to the plant of origin, which seems to be a common feature among various beneficial microbes (Zinniel et al., 2002). Because of their close contact with host cells, endophytes probably need to avoid plant defense mechanisms in a manner similar to what was reported for biotrophic plant pathogens and other types of beneficial microbes such as mycorrhyzae. These organisms secrete proteins and other metabolites called ‘effectors’ that interfere with plant defenses, allowing them to colonize host plants (Corradi and Bonfante, 2012). Nevertheless, the fact that plant-associated micro-organisms are the rule in nature, their multi-faceted interactions can lead to beneficial, neutral or detrimental effects to the host, depending clearly on the environ-mental status of the surroundings (Partida-Martinez and Heil, 2011). In fact, the recent view of a ‘holobiont’ (the combination of a host and all its interacting microbial cells) serving as the genetic unit subjected to evolutionary forces (Zilber-Rosenberg and Rosenberg, 2008) brings an alternative theoretical framework for hypothesis-driven research and data interpretation regarding plant-microorganisms association. The results of this study show that bacterial endophytes tend to preferentially colonize certain plant species and tissues within these plants, and that, among various interfering factors, the levels of individual colonization seems to be significantly influenced by the indigenous microbial content of the host plant.

Conflict of interest

The authors have no conflict of interest to declare.

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