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LETTER

Bacterial endophytes and root hairs

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The crucial role that root hairs play to increase root surface area, facilitating anchorage to substrates and enabling a large interface to absorb nutrients and water, has been long known (Peterson and Farquhar 1996). Root hairs are also critically involved in shaping the composition and properties of the rhizosphere (Datta et al. 2011). Overall, most of the available studies and reviews on root hairs have been focused on their physiology, morphology and the genetic basis of cell differentiation (Dolan and Costa 2001; Knight 2007; Ishida et al. 2008; Emons and Ketelaar 2009; Datta et al. 2011). However, these specialized root epidermal structures are also involved in the interaction between plants and soil-inhabiting microbiota. In fact, the role that root hairs play in root exudation (Czarnota et al. 2003; Yan et al. 2004) can be a determinant for the growth of symbiotic bacteria and fungi (Bais et al. 2006). For instance, the involvement of root hairs in the early steps of effective formation of nitrogen-fixing nodules in legumes by symbiotic Rhizobiaceae members has been well studied (Kijne 1992; Oldroyd and Downie 2008). An increasing number of observations suggest that root hairs can also play a decisive role in plant root colonization by beneficial bacteria (Rincón et al. 2005; Mattos et al. 2008) other than some rhizobia with endophytic lifestyle.

Some soil-inhabiting bacteria do not limit their colonization to the root surface, but can also establish themselves within root tissues as non-deleterious endophytes. Endophytism can actually be considered as a universal phenomenon and it is likely that all plants harbor endophytic bacteria (Rosenblueth and Martínez-Romero 2006). Beneficial endophytic bacteria are of particular interest in promotion of plant growth and control of plant disease, since their ability to colonize plant tissues internally means that they are adapted to these ecological niches, where they may exhibit their positive effect(s). They can be considered

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as an intermediate case between endosymbiotic beneficial interactions (i.e. legumes and specific rhizobia representatives) and pathogenic interactions (i.e. *Agrobacterium* spp. infections), which eventually lead to a disease state for the plant (Mercado-Blanco and Bakker 2007; Reinhold-Hurek and Hurek 2011). Putative bacterial traits involved in endophytic colonization of plant roots as well as responses deployed by plants to this inner colonization have been reviewed (for instance, Hardoim et al. 2008; Reinhold-Hurek and Hurek 2011, and references therein). Remarkably, there are few examples in the published literature of definitive proofs of the preferential sites used by endophytic bacteria to gain entrance into root tissues. Nevertheless, root cracks are amply recognized as the main 'hot spots' for bacterial colonization (Sørensen and Sessitsch 2006; Hardoim et al. 2008). These cracks or wounds may be located at the emergence points of lateral roots or may be caused by microbial or nematode activities. Root differentiation and elongation zones and the intercellular spaces in both epidermis and cortex are also proposed to be major sites for bacterial colonization (Reinhold-Hurek and Hurek 2011). There is, however, still an important gap in our knowledge about where and why beneficial (endophytic) bacteria first attach at defined spots and then penetrate into root tissues. This information will be necessary to understand fully how and when biocontrol and/or plant growth promotion mechanisms are subsequently deployed by bacteria showing endophytic lifestyle.

Two recent studies (Paungfoo-Lonhienne et al. 2010; Prieto et al. 2011) have provided evidence of a new site through which some endophytic bacteria enter into the plant roots - the root hairs. It is notable that, unlike studies involving some rhizobia (i.e. Duodu et al. 2009), the vast majority of reports dealing with root hair colonization by bacteria only refer to attachment and/or colonization of root hair surface, but not to internal colonization of these structures (for instance, Hansen et al. 1997; Bianciotto et al. 2001; Rediers et al. 2003; Rothballer et al. 2003; Rincón et al. 2005; Buddrus-Schiemann et al. 2010). But recently Prieto et al. (2011) have clearly demonstrated that *Pseudomonas fluorescens* PICF7 and *P. putida* PICP2 are able to internally colonize olive (*Olea europaea* L.) root hairs. These bacterial strains are natural inhabitants of the olive rhizosphere and display effective biocontrol activity against the soil-borne fungal pathogen *Verticillium dahliae* Kleb. (Mercado-Blanco et al. 2004; Prieto et al. 2009; López-Escudero and Mercado-Blanco 2011). Unequivocal confirmation of inner colonization of root hairs and subsequent establishment in the intercellular spaces of the cortex by both *Pseudomonas* spp. was demonstrated using fluorescently tagged bacteria and confocal laser scanning microscopy (CLSM) in both a non-gnotobiotic system, which resembles natural plant growth situations (Prieto et al. 2009), and in vitro propagated olive plants (Prieto et al. 2011). Modern microscopy technologies combining CLSM with fluorescent tagging of bacteria offers several advantages. There is no need for plant tissue manipulation since CLSM provides an optical section of the sample avoiding plant structure disruption, and the use of non-diffusible fluorescent markers also allows their in situ location without any manipulation techniques. Moreover, profound colonization of plant tissues by bacterial endophytes, sometimes difficult to demonstrate and visualise even with the use of CLSM, due to the limitations on obtaining images deeper than 100 μm, can be demonstrated using dissection or sectioning to increase accessibility to inner cell layers (Prieto and Mercado-Blanco 2008). Therefore, observations of internal root hair colonization and the subsequent ingress into root tissues by indigenous *Pseudomonas* spp. in olive represent consistent phenomena taking place in different plant growth conditions. Moreover, colonization of a given olive root hair is not a consequence of a single penetration event and later bacterial multiplication, but of multiple penetration episodes. This was demonstrated by simultaneously detecting the presence of different fluorescently tagged bacteria, either from the same species or from two different ones (Prieto et al. 2011). This observation can explain how endophytic strains of *P. fluorescens* and *P. putida* enter and coexist in the same root hair, and eventually colonize the same sub-epidermal tissues, apparently without competing each other. Although internal olive root hair colonization by *Pseudomonas* spp. has been demonstrated unequivocally, the question of how bacteria ultimately reach the intercellular spaces of the cortical root tissue remains to be elucidated, as does determination of the precise moment at which a bacterial cell enters the root hair. Do these bacteria penetrate by an active mechanism entering intact cells? Or, do they enter passively? Are they simply taken up by this specialized cell? Indeed, it remains to be determined whether these native inhabitants of the olive rhizosphere should be considered either as ‘competent endophytes’ (possessing the genetic machinery required to colonize and persist in the endosphere), ‘opportunistic endophytes’ (competent rhizosphere colonizers but only entering root tissue coincidentally), or ‘passenger endophytes’ (those that in the absence of any machinery for efficient root colonization or entry, might enter plants by chance), according to the definitions proposed by Hardoim et al. (2008). So far, none of the bacterial traits thought to be involved in endophyte-plant interaction (see Reinhold-Hurek and Hurek 2011, and references therein) has been implicated in the endophytic colonization, including root hairs, of olive roots by these *Pseudomonas* spp. strains. On the other hand, since the number of root hairs colonized by strains PICF7 and PICP2 is rather low (< 2%, Prieto et al. 2011), their internal colonization might be simply an artefact or, alternatively, that these endophytic bacteria may only be able to enter a small fraction of the root hairs through micro-injuries present at the surface (Lugtenberg 2011). Nevertheless, the fact that inner colonization of root hairs has been demonstrated under different experimental conditions and in several independent experiments argues against artefact (Prieto et al. 2009, 2011). Moreover, internal colonization of root hairs by microorganisms other than *Pseudomonas* spp. has been also demonstrated in other plant species (see below).

Bacteria able to establish as root endophytes may also take advantage of processes occurring during the growth and elongation of root hairs. We speculate that active endocytosis occurring at the tip of growing hairs and subsequent trafficking of the endocytosed membranes (Ovečka et al. 2005) may provide a way for the passive entry of bacteria into the root hair. Prieto et al. (2011) show that inner colonization takes place not only in fully-elongated root hairs, the most frequent observation, but also in cells only showing initiating root hairs (Fig. 1). It would be interesting to investigate whether some of the cytological, physiological and genetic events occurring during root hair initiation and elongation (presence of the organelle-rich zone in the sub-apical region of the growing root hair, ion movement, synthesis and modification of the cell wall, auxin transport, etc.) (reviewed by Datta et al. 2011) have a major influence on bacteria penetration, either when a root hair is fully elongated or when it is just at the beginning of its formation.

Another study also showing internal colonization of root hairs provides a different and interesting perspective (Paungfoo-Lonhienne et al. 2010). This work proves that, as well as bacteria, yeasts can also be found inside root hairs. The authors elegantly demonstrate internal colonization of *Arabidopsis thaliana* and tomato (*Solanum lycopersicum* Mill) root hairs by *Escherichia coli* and *Saccharomyces cerevisae*.

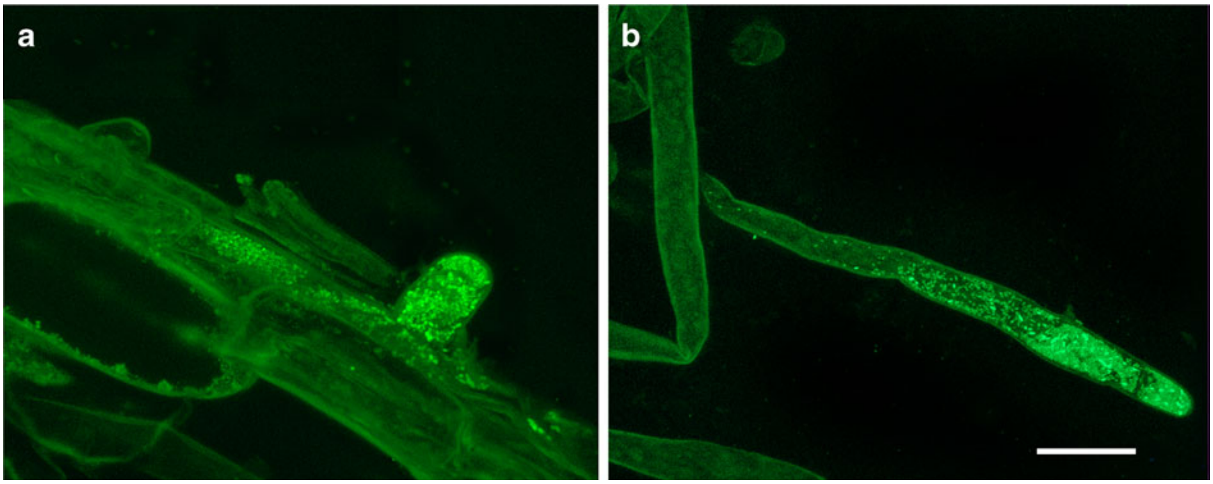

Fig. 1 Confocal laser scanning microscopy images of olive root hairs colonized by *Pseudomonas* spp. fluorescent-tagged cells. Confocal analysis was performed on whole representative olive roots to show internal root hair colonization by *Pseudomonas fluorescens* PICF7 and *Pseudomonas putida* PICP2. **a** A developing root hair internally colonized by EGFP-tagged *P. fluorescens* PICF7. **b** A full-expanded root hair internally colonized at the tip by EGFP-tagged *P. putida* PICP2. Scale bar represents 15 μm in panel (**a**) and 20 μm in panel (**b**). For technical details see Prieto et al. (2009, 2011)

This study offers some fascinating conclusions. First, inner colonization of these two plant species was assessed for non-pathogenic (*E. coli*) and non-symbiotic (*S. cerevisae)* microorganisms. Second, several lines of evidence suggest that these microorganisms are actually taken up by *Arabidopsis* and tomato, with root hairs playing an important role in the process. Microbes are then confined into the root cortex where they seem to be degraded by the plant. The authors suggest that plants can coordinate the entry of *E. coli* and yeast into root cells, most likely because they can be used as nutrient source. This could be a consequence of the absence of true pathogenic or symbiotic relationships (Paungfoo-Lonhienne et al. 2010). Alternatively, it might well be that this behaviour could just be exacerbated by the specific experimental conditions used (i.e. axenic hydroponic or axenic agar cultivation). This study proposes that mixotrophy—the use of nutrients derived from photosynthesis and organic sources—may also occur in higher plants as well as in photosynthetic phytoplankton (Raven et al. 2009). This study also offers some clues as to how these microorganisms gain entry into intact root cells. Thus, *E. coli* induces plant cellulase (s), plant cell wall-like outgrowth, and changes in the expression of genes involved in cell wall modification (Paungfoo-Lonhienne et al. 2010). Similarly to the olive-*Pseudomonas* spp. interaction, nothing can be concluded about the moment that bacteria gain access into the root hairs. Neither can we say whether the structural and genetic alterations observed only occur in root hair cells or whether they also take place in the additional spots of the rhizodermis that are also colonized by these microorganisms.

Previous findings may also have important implications for food security issues. Some enterohaemorrhagic *E. coli* (EHEC) outbreaks have been associated with the consumption of fruits and vegetables, including fresh sprouts of different edible plants, contaminated with faeces from domestic or wild animals at some stage during cultivation (http://www.who.int/mediacentre/factsheets/fs125/en/). Irradiation, pressure sterilization and a wide range of chemicals such as hydrogen peroxide, calcium hypochlorite, sodium hypochlorite and ethanol have been tested for surface disinfection of seeds and sprouts (Lang et al. 2000; Beuchat et al. 2001; Bari et al. 2003), in combination with good agriculture practices, but no single treatment completely eliminates the pathogens under experimental conditions. Moreover, if some EHEC strains are eventually taken up by plants, root hairs playing a key role in such ingress, and they remain alive within root tissues for some time (or they can move to edible organs), attention should be paid to the effectiveness of sanitation protocols of fresh sprouts, since they may potentially harbour noxious endophytes due to the use of contaminated water and/or cultivation substrates.

In summary, recent findings raise the question of whether entry of bacteria into roots *via* root hairs could be a common phenomenon that has been previously overlooked. If root hairs are one of the main routes for inner colonization of beneficial bacteria, and some biocontrol and/or plant growth promotion mechanisms are deployed once the bacteria are inside root tissues, the fitness of the root hairs could be of decisive importance. Hence, it is necessary to be aware of factors influencing root hairs that may subsequently affect their interaction with bacteria. Genetic factors, soil acidity and humidity, aluminum, iron, nitrate, phosphate, potassium levels in soil, etc., greatly influence root hair density, number, length, physiology and development (Haling et al. 2010; Römheld and Kirkby 2010; Datta et al. 2011, and references therein). Moreover, some plant growth promoting rhizobacteria (Lugtenberg and Kamilova 2009) may stimulate cellular division in roots and increase root hair numbers under soil drought conditions (Michiels et al. 1989). This then leads to improved water and nutrient uptake, particularly from deeper soil layers (Compant et al. 2010). It is clear that long root hairs can be a desirable trait for plants in modern crop breeding programmes (Datta et al. 2011). This aim should be pursued not only because of the benefits discussed at the beginning of this letter, but also because of the important influence of root hairs in the interaction with endophytic bacteria. The current powerful biotechnological and microscopy tools will help to determine whether entry through root hairs is utilized by other soil inhabitants showing an endophytic lifestyle, and whether this phenomenon also takes place in other plant species. The same methods should uncover whether plants use root hairs to take up soil microorganisms, whether non-symbiotic, non-competent or non-beneficial endophytes, under natural conditions as additional sources of nutrients. We expect that the answers to these exciting questions will be available very soon.

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