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Title: Efficient colonization of the endophytes *Herbaspirillum huttiense* RCA24 and *Enterobacter cloacae* RCA25 influences the physiological parameters of *Oryza sativa* L. cv. Baldo rice.

The co-inoculation with nitrogen-fixing and indole-3-acetic acid (IAA)-producing bacterial endophytes positively affects rice (*Oryza sativa* L. *cv. Baldo.*) growth.

Running title: Effects of N-fixing and IAA-producer endophytes on rice.

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Abstract

Several important bacterial characteristics, such as biological nitrogen fixation, phosphate solubilisation, 1-aminocyclopropane-1-carboxylate deaminase activity, and production of siderophores and phytohormones, can be assessed as plant growth promotion traits. Our aim was to evaluate the effects of nitrogen-fixing and indole-3-acetic acid (IAA) producing endophytes in two *Oryza sativa* cultivars (Baldo and Vialone Nano). Three bacteria, *Herbaspirillum huttiense* RCA24, *Enterobacter asburiae* RCA23 and *Staphylococcus sp.* 377, producing different IAA levels, were tested for their ability to enhance *nifH* gene expression and nitrogenase activity in *Enterobacter cloacae* RCA25. Results showed that *H. huttiense* RCA24 performed best. Improvement in nitrogen-fixation and changes in physiological parameters such as chlorophyll, nitrogen content and shoot dry weight were observed for plants co-inoculated with strains RCA25 and RCA24 in a 10:1 ratio.

Based on confocal laser scanning microscopy analysis, strain RCA24 was the best colonizer of the root interior and the only IAA producer located in the same root niche occupied by RCA25 cells. This work shows that the choice of a bio-inoculum having the right composition is one of the key aspects to be considered for the inoculation of a specific host plant cultivar with microbial consortia.

Introduction

Nitrogen (N) is one of the primary nutrients limiting plant growth in agriculture. Despite its prevalence in the Earth's atmosphere, it mostly exists in a biologically inaccessible (inert) form. In agriculture, N limitation is circumvented by the application of chemical fertilizers, leading to environmental consequences over the years, thus producing greenhouse gases and the

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eutrophication of water systems (Stokstad, 2016). Rice is one of the main food crops for human nutrition and N availability often limits its production (Awika, 2011). Increases in cereals production are urgently needed, but the expensive and polluting chemical fertilizers have already been overused (Rasul, 2016). Biological nitrogen fixation (BNF) by diazotrophic bacteria, which reduce atmospheric N to ammonium using nitrogenase enzyme systems, accounts for 30-50% of the total nitrogen in crop fields (Wagner, 2011; Ormeño-Orrillo *et al.*, 2013). BNF is a potentially attractive source of nitrogen for cereal production (Rogers and Oldroyd, 2014). The area of BNF research has been expanded by the discovery of N-fixing bacterial endophytes in non-nodulating plants. Bacterial endophytes promote plant growth through nitrogen fixation, phytohormone production, nutrient acquisition, and by conferring tolerance to abiotic and biotic stresses (Haney *et al.*, 2015; Berg *et al.*, 2016; Yuan et al., 2016; Santoyo *et al.*, 2017). They most often inhabit the plant intercellular spaces which are abundant in carbohydrates, amino acids and inorganic nutrients (Compant *et al.*, 2011; Liu *et al.*, 2017). Bacterial endophytes initially stick to the root surface and explore the potential entry sites to access the internal plant tissues. Opening in the roots, where root hairs or lateral roots emerge, as well as wounds in the shoots, are considered the main entry points that endophytes use to enter the host plant and access its internal tissues (Mercado-Blanco and Prieto, 2012; Compant *et al.*, 2016; Kandel *et al.*, 2017). Therein, they reside for most of their life with no detrimental impact to the host plant (Mercado-Blanco and Lugtenberg, 2014; Hardoim *et al.*, 2015). The internal plant tissues constitute a favourable environment for N-fixation performed by diazotrophic endophytes: the competition with other microbes in the rhizosphere is minimized and a microaerobic environment, necessary for nitrogenase activity, is provided (Mus *et al.*, 2016; Kandel *et al.*, 2017). In the last few years a wide diversity of bacteria associated with cereals have shown to possess the *nifH* gene coding for dinitrogenase reductase (Rosenblueth *et al.*, 2018). This gene is considered the most genetically conserved within the *nif* regulon, and traditionally used as a marker gene to study the genetic diversity of diazotrophs in nature (Gaby and Buckley, 2014; Gaby *et al.*, 2017; Angel *et al.*, 2018). Functional molecular analyses in rice have also shown that not all

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these microorganisms are active in nitrogen fixation when in association with plants (Ueda *et al*., 1995; Elbeltagy and Ando, 2008). In addition, the level of ammonium, supplied to the host cereals by the most active ones, was not high enough to support the plant growth under nitrogen-limited conditions and was never similar to the one obtained with chemical fertilizers. This behaviour can be due to most diazotrophs which use concerted mechanisms for cellular N homeostasis and typically do not fix N in excess nor excrete significant amounts of N-fixation products. The factors required for nitrogen fixation are to date quite defined and therefore the use of genetically modified diazotrophs to improve plant growth has been proposed (Van Dommelen *et al*., 2009; Geddes *et al*., 2015; Ambrosio *et al*., 2017). Several studies have also highlighted that the beneficial effects of microbes on plants growth can be enhanced by co-inoculation with different microorganisms. In particular, synergistic effects on growth and yield of different plants have been observed for the co-inoculation of nitrogen-fixing and plant growth promoting bacteria (Islam *et al*., 2009; Jia *et al*., 2016; Korir *et al*., 2017; Orozco-Mosqueda *et al*., 2018).

Our previous study reported that the strain *E. cloacae* RCA25-64, engineered to produce and release 36-fold more indole-3-acetic acid (IAA) in liquid media than the wild type *E. cloacae* RCA25, showed increased *nifH* gene expression and nitrogenase activity in liquid cultures and inoculated rice plants (Defez *et al*., 2017). Therefore, the specific aims of this study were to (i) analyse the effect of purified IAA on the nitrogen-fixing ability of *E. cloacae* RCA25, (ii) select wild type IAA-producing endophytes to be used together with RCA25 for the co-inoculation of rice plants, (iii) assess whether the co-inoculated endophytes are able to colonize the intercellular spaces within the cortex of rice roots, and (iv) identify the most effective endophyte combination and analyse its effects on physiological parameters of host rice plants, with no transgenic approach.

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Results

Enterobacter cloacae *RCA25 produces very low level of IAA*

One of the first objectives of this work was the evaluation of the IAA levels produced by the selected endophytes. The highest level of IAA production was measured for strain *Staphylococcus sp.* 377 (Baldan *et al*., 2015). Strains *Enterobacter asburiae* RCA23 and *Herbaspirillum huttiense* RCA24 (32) produced similar and lower levels of auxin, while the lowest level of IAA production was measured for strain RCA25 (Table 1).

IAA affects the bacterial growth

To test the effects of IAA on *E. cloacae* RCA25 growth, different concentrations (0.01 mM, 0.1 mM, 0.72 mM and 2.86 mM) of the purified compound were added into liquid cultures and the bacterial growth was examined over time. Results showed that 0.1 mM was the maximum IAA concentration not affecting bacterial growth (Supporting Information Fig. S1). This concentration was also selected for the treatments with 2,4-dichlorophenoxyacetic acid (2,4-D) and indole (IND), compounds functionally and chemically similar to IAA, respectively. Bacterial cells treated with IAA and 2,4-D displayed very similar growth curves, whereas the growth of untreated and IND-treated cells was slightly higher (Fig. 1A).

IAA does not influence the biofilm formation of Enterobacter cloacae *RCA25*

It was earlier reported that auto-aggregation and biofilm development processes are relevant to bacterial survival and host plant colonization (Camerini *et al*., 2008). Therefore, the biofilm formation ability of strains RCA25, RCA24, RCA23, and 377 was evaluated in rich medium. The highest biofilm formation was observed for *E. asburiae* RCA23 and *H. huttiense* RCA24, while the amount of biofilm produced by the strains *E. cloacae* RCA25 and *Staphylococcus* sp. 377 was lower (Table 1). For the strain RCA25, the biofilm production in minimal medium supplemented

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with IAA, 2,4-D and IND was also tested. Results showed that RCA25 cells treated with IAA and 2,4-D produced similar level of biofilm as the untreated ones. When bacterial cells were treated with IND, a higher development of biofilm (up to 20 %) was measured compared to the untreated cells (Fig. 1B).

IAA increases the transcription level of nifH *and the activity of nitrogenase in* Enterobacter cloacae *RCA25*

To evaluate the effect of IAA, 2,4-D and IND treatments on the nitrogen fixing ability of *E. cloacae* RCA25, the expression of the *nifH* gene was measured in RCA25 treated cells and compared with the untreated ones. Results showed that the *nifH* gene transcript level significantly increased (1.6-fold) in IAA-treated cells. In contrast, *nifH* expression did not significantly change upon treatment with 2,4-D and IND (Fig. 1C). Nitrogenase activity of RCA25 cells was also measured by the acetylene reduction assay (ARA) after treatment with these compounds. When untreated cells were used as reference, the highest increase in nitrogenase activity was measured for IAA-treated cells (up to 25%) (Fig. 1D). A significant but lower induction (18%) was also observed for IND-treated cells. Finally, 2,4-D-treatment did not affect the activity of this enzyme as compared to the control.

Enterobacter cloacae *RCA25 and* Herbaspirillum huttiense *RCA24 are good colonizers of* Oryza sativa *L. cv. Baldo roots*

The ability of the selected endophytes to individually colonize rice roots has been tested by measuring the CFU number in tissues of whole inoculated *O. sativa L.* cv. Baldo plants. When the CFU number of RCA25-inoculated plants was used as reference, the highest colonization ability was observed for the strain RCA24 (Ratio = 1.32 ± 0.23). Strains RCA23 and 377 showed lower colonization ability (Ratio = 0.34 ± 0.08 and 0.04 ± 0.01, respectively).

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The strains RCA25 and RCA24 tagged with different fluorescent proteins (red fluorescent protein [RFP] and enhanced green fluorescent protein [EGFP], respectively) were then used for single inoculation of *O. sativa* L. *cvs.* Baldo and Vialone Nano. Considering that RCA24 colonized rice plants more efficiently than RCA25, single inoculation was performed using a final concentration of RCA25 (10^7 cells ml^{-1}) 10 times higher than RCA24 (10^6 cells ml^{-1}), and the CFU numbers were evaluated for both root and shoot tissues. When the root tissues of Baldo rice plants were analysed, we found that the CFU number measured for RCA25-RFP (1,757 ± 272) was 50% higher than the one estimated for RCA24-EGFP (1,123 ± 103). For 'Vialone Nano' a different behaviour was observed: the CFU numbers obtained for RCA25-RFP (130 ± 12) and RCA24-EGFP (3,439 ± 485) were less than 10% and three times higher, respectively, than the ones measured for 'Baldo' rice root tissues. Neither of the two strains showed a significant colonization of shoot tissues for both rice cultivars. The strains RCA25-RFP and RCA24-EGFP in a 10:1 ratio were also used to carry out co-inoculation of 'Baldo' rice plants. For both strains the colonization efficiency of root tissues was assessed. The CFU numbers measured for RCA24-EGFP (1,973 ± 516) was 2.5 times higher than the one scored for RCA25-RFP (808 ± 138).

Co-inoculation of rice plants with the strains RCA25 and RCA24 increases nitrogenase activity

To evaluate the effect of IAA production by endophytic bacteria on the nitrogen fixing ability of *E. cloacae* RCA25, two-step inoculation and co-inoculation of *O. sativa* L. cv. Baldo were performed. Results showed that the two-step inoculation of Baldo rice plants with the strains RCA25 and RCA24, both at the concentration of 10^6 cells ml^{-1}, led to a significant increase of nitrogenase activity, regardless of the strain used in the first inoculation step (Fig. 2A and B). However, the highest increase (up to 60%) was measured when RCA25 was used first, compared to plants inoculated only with this strain (Fig. 2A). Rice plants inoculated with 377+RCA25, RCA25+377, RCA23+RCA25 and RCA25+RCA23 combinations showed a down-regulation in nitrogenase activity of 40%, 30%, 10% and 50%, respectively, when RCA25-inoculated plants were used as

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reference. Co-inoculation experiments were then carried out with the strains RCA25 and RCA24 at 100:1, 10:1, and 1:1 ratios. When the two strains were used at a 10:1 ratio (10^7 cells ml^{-1} and 10^6 cells ml^{-1}, respectively) a significant increase (up to 50%) in nitrogenase activity was observed for 'Baldo' rice as compared to RCA25-inoculated plants (Fig. 2C). In contrast, the co-inoculation with strains RCA25 and RCA24 at a 100:1 ratio (10^7 cells ml^{-1} and 10^5 cells ml^{-1}, respectively) did not significantly increase nitrogenase activity (Fig. 2C). Similar results were obtained when strains RCA25 and RCA24 were co-inoculated both at 10^6 cells ml^{-1} (data not shown). When the ARA assay was carried out on plants inoculated with the IAA-overproducing RCA25 strain (RCA25-64) at the concentration of 10^7 cells ml^{-1} the nitrogenase activity increased up to 70% (Fig. 2C).

The induction of nitrogenase activity is peculiar to IAA

To verify the specificity of the IAA effect on N-fixation in 'Baldo' rice, three-week old plants inoculated with RCA25 (10^7 cells ml^{-1}) were treated with different purified molecules, structurally (IND and ICA) and functionally (2,4-D) similar to IAA. In our conditions, none of the compound tested significantly affected the activity of the nitrogenase enzyme (Fig. 2D), thus indicating that the observed induction is a peculiarity of IAA.

Enterobacter cloacae *RCA25 and* Herbaspirillum huttiense *RCA24 coexist within the rice roots*

When two-step inoculation and co-inoculation were carried out, the use of fluorescent-tagged bacteria and CLSM enabled to demonstrate that all bacterial strains effectively colonized roots of 'Baldo' rice plants under gnotobiotic conditions. At the time of analysis, bacteria adhesion to root surface and internal colonization of the epidermal cells (including root hairs) was observed for all strains and for all plants (4 per treatment) subjected to CLSM analysis, and are exemplified in the panels of Fig. 3. In addition, the intercellular spaces within the cortex of rice root tissues were also colonized by all strains (Fig. 3). Bacteria were attached along the whole root surface, mostly at

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random distribution, displaying no preferred site for the external colonization of the plant root cells. Differences in the inner colonization pattern in sampled plants were observed for each bacteria combination. Internal colonization of rice root tissues by all bacteria strains was detected by CLSM (Fig. 3). Either single bacterial cells or bacterial microcolonies were predominantly localized in the intercellular spaces of the root cortex. Plants inoculated with strain RCA25-RFP were used as control to confirm internal colonization of the rice root tissues (Fig. 3A). Indeed, strain RCA25-RFP colonized both the root surface and the inner tissues, regardless whether being inoculated alone or in combination with the other strains (Fig. 3). Among IAA producers (RCA23, RCA24 and 377), strain RCA24-EGFP seemed to be the best colonizer, although all of them internally colonized the rice root tissues. In fact, all IAA producers could be observed both outside and inside the rice roots (Fig. 3). Only RCA24-EGFP was observed to coexist with strain RCA25-RFP in the same root regions, as clearly shown in Fig. 3B-D. Both strains can even occupy the same root niche (Fig. 3D). In contrast, strain 377-EGFP endophytically colonized the rice root when simultaneously inoculated with RCA25-RFP (Fig. 3E and inset), but co-localization of both strains in the same region of the root interior was not observed. Nevertheless, the possibility that both strains can coexist inside the rice root tissue cannot be discarded. Finally, both RCA25-RFP and RCA23-EGFP endophytically colonized rice roots (Fig. 3F). Strain RCA23-EGFP profusely colonized the root interior and was observed colonizing the same region occupied by RCA25-RFP, although these two strains were not detected mixed (coexisting), in contrast to the RCA25-RFP+RCA24-EGFP combination. The panels reported in Fig. 3 reflect representative observations of each treatment.

The effective root colonization by strains RCA25 and RCA24 positively influences physiological parameters of rice plants

To evaluate the effect of single inoculation with RCA25 and RCA24 and co-inoculation with both strains in a 10:1 ratio on rice plants growth, various physiological parameters were measured in

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shoots and leaves of *O. sativa* L. cvs. Baldo and Vialone Nano. Different results were obtained for the two cultivars (Fig. 4 and 5). At 55 days after sowing, it was verified that the roots of *O. sativa* L. cv. Baldo plants had been efficiently colonized by the selected strains, while 'Vialone Nano' plants showed a lower colonization efficiency (data not shown), consistent with the data already observed for plants grown in the growth chamber above reported. . At 90 days after sowing, a significant increase in the chlorophyll content was measured only for 'Baldo' co-inoculated with RCA25 and RCA24 as compared to the control non-inoculated plants (Fig. 4A and C). For 'Vialone Nano', a significant decrease in the chlorophyll content was measured when plants were inoculated with the strain RCA25 alone or in combination with *H. huttiense* RCA24. The flavonoid content was not significantly altered in any of the tested cultivars (Fig. 4B). For the Nitrogen Balance Index (NBI) a significant increase was measured for 'Baldo' plants both when single inoculated with RCA25 and co-inoculated with RCA25 and RCA24 compared to the non-inoculated control. An opposite result (reduction of NBI parameters) was obtained for 'Vialone Nano' plants. The data measured with the Soil Plant Analysis Development (SPAD) meter (Fig. 4D) confirmed those obtained with the Dualex and also showed a positive contribution of the single inoculation with strain RCA24 in 'Baldo' rice. When the non-inoculated control plants were used as reference, both the single and the co-inoculated 'Baldo' rice significantly enhanced height, dry weight (DW) and N content of the shoots at 90 days after sowing (Fig. 5 5). However, co-inoculated plants showed the highest increase in DW (up to 89%, $P < 0.05$) (Fig. 5B) and N content (up to 80%, $P < 0.01$) (Fig. 5C). In contrast, no positive effects on growth parameters were observed for 'Vialone Nano' rice, when the non-inoculated control plants were used as reference (Fig. 5). Similar results were observed for three-week old 'Baldo' plants co-inoculated with RCA25 and RCA24 under growth chamber conditions: co-inoculated plants showed an increase in DW (Baldo+RCA25, shoot DW = 9 ± 1 mg; Baldo+RCA25+RCA24, shoot DW = 12 ± 3 mg) and N content (Baldo+RCA25, N = 0.14 ± 0.03 g per plant; Baldo+RCA25+RCA24, N = 0.19 ± 0.02 g per plant) of 44% and 36%, respectively, when the RCA25-inoculated ones were used as reference.

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Discussion

For over a century, some nitrogen-fixing endophytic bacteria have been known to form associations with cereal crops. Although some of these bacteria have been used to produce biofertilizers, thereby supporting sustainable and non-polluting agriculture, it still remains controversial whether the enhancement of plant growth is the result of nitrogen fixation or of bacterial molecules acting as plant growth hormones. Furthermore, to overcome the limited amount of fixed nitrogen that can be achieved, the production of improved biofertilizers is required (Arora and Mishra, 2016; Sruthilaxmi and Babu, 2017; Orozco-Mosqueda *et al*., 2018). Early work from our laboratory has indicated that the endogenous IAA-overproduction in endophytic bacteria activated their nitrogen-fixing apparatus. We have shown that the biosynthesis of high levels of IAA by *E. cloacae* RCA25-64 (an engineered derivative of *E. cloacae* RCA25) led to a significant increase of both *nifH* gene expression and nitrogenase activity in liquid cultures and RCA25-64-inoculated rice plants (Defez *et al*., 2017). Here we report that the treatment of RCA25 cells with purified IAA, 2,4-D and IND, at a concentration not altering bacterial growth, led to a significant increase (1.6-fold) of *nifH* gene expression only when IAA was used. The ARA test confirmed these data: the highest up-regulation (up to 25%) of the nitrogenase activity was measured for the IAA-treated cells. These results suggested that the positive effect observed on the nitrogen-fixing ability of the *E. cloacae* RCA25 was specific to the IAA molecule. The significant but lower increase in nitrogenase activity observed for IND-treated cells, as compared to the IAA-treated ones, was probably due to its role in bacterial biofilm formation. The high level of biofilm produced by these cells could have led to the generation of a low-oxygen environment, suitable for the activity of this enzyme (Hu *et al*., 2010; Lee and Lee, 2010; Kim and Park, 2015). In this work, the bacterial strains *E. asburiae* RCA23 and *H. huttiense* RCA24 from rice (Defez *et al*., 2017) and *Staphylococcus* sp. 377 from grapevine (Baldan *et al*., 2015) were selected for their ability to produce IAA. The best IAA producer was the strain 377, which released in the growth medium IAA levels comparable to the ones produced by the strain RCA25-64. The strains RCA23 and RCA24 produced similar but lower IAA levels than

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the 377 one. We have here demonstrated that the strains RCA25, RCA24, RCA23 and 377 were able to endophytically colonize 'Baldo' rice plantlets and that the strains RCA25 and RCA24 seemed to be the best colonizers, when single inoculation was performed. Bacterial cells of all strains were randomly attached along the whole root surface and colonized the intercellular spaces of the root cortex. We also verified that the colonized roots contained mixed population of RFP- and GFP-tagged bacteria when double bacterial inoculation was carried out. However, differences in the inner colonization pattern were observed. The CFU number measurement in homogenates, deriving from plants inoculated with individual bacteria, revealed that the strain RCA24 had the highest colonization ability, while the 377 the lowest one, probably due to its slow growth on swarm agar plates. The ability to move towards or on the host plant is important for colonization by many plant-associated bacteria (Compant *et al*., 2010). Furthermore, an interaction among endophytes takes place naturally in the endophytic environment, due to physical barriers of plant tissues (Czajkowski *et al*., 2012; Liu *et al*., 2017; Kandel *et al*., 2017). The analysis of mutual interaction and antagonism by swarm agar plates showed that the nitrogen-fixing *E. cloacae* RCA25 did not exhibit antibacterial activity against all the IAA producers (Supporting Information Fig. S2). The simultaneous inoculation of rice plants with the strains RCA25 and RCA24 in a 10:1 ratio led to the highest increase in nitrogenase activity. This result was probably due to the fact that, although all the IAA-producers were able to internally colonize the rice root tissue, RCA24-EGFP colonized the host plants more efficiently and coexisted with RCA25-RFP in the same root regions, as confocal microscopy analysis showed. This colonization pattern was similar to the one already described for other plant growth promoting bacteria (Prieto *et al*., 2011) and demonstrated that previously colonized roots did not prevent further bacterial attachment and penetration. A better ability of the strain RCA24 to colonize rice roots could be due not only to its ability to produce higher amount of biofilm, which in turn promotes the adhesion of bacteria to plant surfaces, but also to the lack of competitive action for the niche occupancy inside the plant (Kawarai *et al*., 2009; Qurashi and Sabri, 2012; Bogino *et al*., 2013). It is reported that many species of beneficial soil

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bacteria, including rhizobia, form micro-colonies or biofilms when colonizing roots (Rinaudi and Giordano, 2010; Sorroche *et al.*, 2012). The increase of nitrogenase activity measured for co-inoculated plants was significant,but lower than the one observed for 'Baldo' plants inoculated with the engineered IAA-overproducing strain RCA25-64 and with the RCA25-inoculated plants treated with the purified IAA. We thus here propose an alternative approach to the use of genetically modified endophytes for the improvement of nitrogen fixation in rice plants. 'Baldo' plants co-inoculated with strains RCA25 and RCA24 under greenhouse conditions also showed higher chlorophyll, nitrogen content of shoot tissue and biomass as compared to RCA25-inoculated plants. We cannot rule out the possibility that the co-inoculation of 'Baldo' rice with RCA25 and RCA24 could have stimulated the activity of other components undetectable when the inoculated endophytes were re-isolated from these plants. Further specific analyses will be carried out to verify whether the nitrogen fixed by the RCA25 diazotroph was directly transferred to the host plants. The absence of significant variations for the various physiological parameters analysed in shoots and leaves of inoculated 'Vialone Nano' rice plants was consistent with the lower colonization efficiency measured for this cultivar as compared to 'Baldo' rice. However, although the identification of the morphological, physiological and molecular traits linked to this lower association efficiency requires further investigation, our results reinforce the concept that a host-specificity can also exist for endophytic bacteria.

In recent years a large deal of literature has been produced on co-inoculation (consortium) of agriculturally beneficial microorganisms and in many cases benefits have been observed compared to the single inoculation (Akintokun and Taiwo, 2016; Sahu *et al.*, 2016; Molina-Romero *et al.*, 2017; Lally *et al.*, 2017; Orozco-Mosqueda *et al.*, 2018; Sahu *et al.*, 2018). This present study shows that the beneficial endophytes *E. cloacae* RCA25 and *H. huttiense* RCA24 can be promising strains to improve, directly or indirectly, nitrogen fixation in rice plants under greenhouse conditions. Challenging projects are trying to genetically modify cereals to enhance diazotroph colonization or to engineer bacterial competitiveness and ammonium release (Rosenblueth *et al.*,

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2018; Geddes *et al*., 2015; Fox *et al*., 2016). The data here reported highlight that the assessment of location and distribution of the individual microbial components within the plant tissues is fundamental for the selection of bio-inoculants, able to enhance nitrogen-fixing ability of the host plants. Furthermore, if the nitrogen fixed by the bacteria was effectively transferred to the host plants, such an approach could also improve the biological capabilities of the plant and might have a major impact on sustainable agriculture.

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Experimental procedures

Bacterial strains and growth conditions

The endophytic bacteria used in this work were *E. cloacae* RCA25 and RCA25-64, *E. asburiae* RCA23, *H. huttiense* RCA24 (Defez *et al*., 2017), and *Staphylococcus sp.* 377 (Baldan *et al*., 2015). Strains RCA25, RCA23 and RCA24 were grown in Luria Bertani (LB) medium whereas strain 377 was grown in TY-CM medium (3g L^{-1} yeast extract, 5g L^{-1} tryptone, 2.5 mM $CaCl_2$ and 2.5 mM $MgSO_4$). All strains were incubated at 30°C on a shaker at 200 rpm. The following antibiotics were included in the media: vancomycin (Sigma-Aldrich) (20 μg ml^{-1}) for strains RCA25, RCA23 and RCA24, and penicillin G (Sigma-Aldrich) (40 μg ml^{-1}) for *Staphylococcus sp.* 377.

Strain tagging with fluorescent proteins (FP)

Plasmids pMP4655 (harbouring the enhanced green fluorescent protein [EGFP] marker) and pMP4662 (harbouring the red fluorescent protein [RFP] marker) (Bloemberg *et al*., 2000; Prieto and Mercado-Blanco, 2008) were purified from *E. coli* DH5α host cells using the PureLink™ HiPure Plasmid Filter Midiprep Kit (Invitrogen), according to the manufacturer's instructions. Electrocompetent cells of strains RCA25, RCA24 and 377 were prepared as follows: bacterial cells were grown up to the exponential phase (OD_{600} =0.5) in LB medium, washed twice with pre-cooled sterile water and once with 10% glycerol, resuspended in ice-cold 10% glycerol, aliquoted and frozen at -80°C. Aliquots were thawed on ice, and the pMP4662 purified plasmid DNA (500 ng) was added to the RCA25 cells and mixed quickly while the pMP4655 purified plasmid DNA (500 ng) was used to transform RCA24 and 377 cells. The mixtures were incubated on ice for 1 min and transferred to a sterile pre-chilled cuvette (0.1 cm interelectrode gap), and placed in the Gene Pulser II apparatus equipped with a Pulser Controller (BioRad Laboratories, California, USA). The electroporation unit was used at the following settings: 2.5 kV, 25 μF, 200 Ω, and pulse time 4–5 ms. The cells were immediately diluted with 1 ml of LB broth, transferred to sterilized tubes, and

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incubated at 30°C for 4 h. From each tube, aliquots (100 µl) were plated onto selective LB agar plate containing 20 µg ml^{-1} tetracycline (Tc) and incubated at 30°C. Colonies from each plate were randomly selected and the presence of the plasmids pMP4655 or pMP4662 was verified by using fluorescence microscopy. Transformation of RCA23 cells with the pMP4655 purified plasmid DNA was performed by triparental mating. Cells of the donor strain (*E. coli* DH5α with the pMP4655 plasmid), the helper strain (*E. coli* HB101 with the pRK2013 plasmid), and the recipient strain (*E. asburiae* RCA23) were grown in LB medium until late exponential phase. Aliquots (2 ml) of the cultures were centrifuged a 6000 rpm for 3 min at room temperature. The supernatant was removed and the pellet washed once with 1 ml of 10 mM $MgSO_4$ and resuspended in 350 µl of 10 mM $MgSO_4$. 100 µl of the RCA23 cells were mixed with 20 µl of both the helper and donor cells, spotted on LB agar plate and incubated overnight at 30°C. Two negative controls were set up: one by mixing RCA23 and helper cells and another by mixing donor and helper cells. After 24 h of incubation at 30°C the bacterial colonies grown on each spot were streaked onto LB agar plates containing Tc (20 µg ml^{-1}). This procedure was carried out 3–4 times to isolate purified colonies. The colonies were randomly chosen and the presence of the plasmids verified by using fluorescence microscopy. Selected transformants were cryopreserved at −80°C in LB broth (OD_{600}= 0.7-0.8) with a solution containing 16% (v/v) dimethyl sulfoxide (DMSO) and 10% (v/v) glycerol.

Effect of IAA, 2,4-D and IND on bacterial growth and biofilm formation in minimal medium

Enterobacter cloacae RCA25 was grown in 1X M9 minimal medium (50) for 24 h at 30°C on a rotary shaker (200 rpm). Different concentrations of IAA (0.01 mM, 0.10 mM, 0.72 mM, 2.86 mM) were included into 15 mL cell cultures (OD_{600} = 0.5) and the absorbance at 600 nm was measured at different times. The compounds 2,4-D and IND, functionally and chemically similar to IAA, respectively, were solubilized in 50% EtOH and added into liquid cultures of strain RCA25 at 0.1 mM final concentration. To avoid solvent interference the control cultures were treated with similar

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amount of EtOH solution. The IAA-, 2,4-D and IND-treated cultures were also used for biofilm formation analysis. The bacterial cultures (10 ml) treated for 4 hours were washed 3 times with 2 ml 1X M9 minimal medium without NH_4Cl and then centrifuged at 13.000 rpm for 10 min at room temperature. The cell pellets were re-suspended in 10 ml of minimal medium containing 1 mM ammonium chloride and incubated at 30°C in polystyrene microtiter plates (100µl/well). After 20 h of incubation under static condition, unbounded cells were removed by inversion of the microtiter plate, followed by vigorous tapping on adsorbent paper. Subsequently, adhered cells were fixed, stained and quantified as described by Bianco *et al.* (2006a).

Biofilm formation analysis in rich medium

For biofilm analysis liquid cultures of the selected strains were grown in LB medium and incubated at 30°C in polystyrene microtiter plates (100 µl/well. After 20 h of incubation under static condition, unbounded cells were removed by inversion of the microtiter plates and the adhered cells were fixed, stained and quantified as previously described (Bianco *et al*., 2006a).

qRT-PCR analysis

The bacterial cultures (2 ml) were treated with IAA, 2,4-D and IND for 4 h, as described above. After the treatment the cultures were centrifuged at 13.000 rpm for 5 min at 4°C, frozen in liquid nitrogen and used for RNA extraction and purification as previously described (Imperlini *et al*., 2009). Residual DNA present in the RNA preparations was removed by using the RNAse-free TURBO DNase I Kit (AMBION, California, USA) according to the manufacturer's instructions. After purification and quality checking by 1.5% agarose gel electrophoresis, RNA concentration was determined by absorbance at 260 nm using a NanoDrop ND-1000 spectrophotometer and the RNA was stored at −20°C until further use. Synthesis of cDNA was carried out from 1 µg of total RNA, using the QuantiTect® reverse transcription kit (QIAGEN, Hilden, Germany) following the

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manufacturer's instructions. Specific primer pairs for the *nifH* gene were those reported in Defez et al. (2017). The primer pair for *dnaA* (constitutively expressed gene), 5'-GTAAAGGCCCTGCAAAACAA-3' and 5'-AACGATCCGAGGTCAAAATG-3', were included in the qRT-PCR analyses for data normalization. After normalizing for *dnaA* gene expression, data of five biological replicates were recorded as relative gene expression changes and computed using the C_T method ($2^{-\Delta\Delta CT}$) as previously described (Bianco *et al*., 2006b).

Assessment of nitrogenase activity in bacterial cultures by the acetylene reduction assay (ARA)

The nitrogen-fixing ability of the strain RCA25 treated for 4 hours with IAA, 2,4-D and IND as above described was determined by the ARA test. Cultures of strain RCA25 treated with 0.125% EtOH were used as control. The cell cultures (4 ml) were washed twice with 2 ml 1X M9 minimal medium without NH_4Cl and then centrifuged at 13.000 rpm for 7 min at room temperature. The cell pellets were re-suspended in 2 ml of minimal medium without ammonium chloride, transferred into airtight glass tubes with serum cap, incubated under a hypoxic atmosphere (2% O_2) and 10% acetylene for 18 h at 30°C. A sample (1 ml) from each tube was used to evaluate the amount of the ethylene (C_2H_4) produced by using a gas chromatograph GC Clarus®580 (PerkinElmer, Shelton, USA) equipped with a hydrogen flame detector (FID) and a TG-IBOND Alumina (Na_2SO_4 deactivate) column (30 m × 0.53 mm × 10 μm, Thermo Scientific). The flow rate of the carrier (helium) was 48 cm s^{-1}, and the oven programmer was 130°C isocratic for 3 min (Defez *et al*., 2017). Inoculated tubes without injected acetylene were used as a negative control.

IAA production

Bacterial IAA production was quantified by using the Salkowski reagent (Salkowski *et al*., 1885). Bacterial cultures (15 ml) were grown at 30°C overnight in rich medium containing 5 mM L-tryptophan and then centrifuged at 12.000 rpm for 5 min to collect the bacterial cells. The cells were dried and weighed for data normalization. The culture supernatants were mixed with the reagent

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(35% $HClO_4$ 50 ml, 0.5 M $FeCl_3$ 1 ml) in a 1:1 ratio. After 30 min of incubation at room temperature, absorbance at 530 nm was measured. The concentration of IAA from each culture medium was calculated from a pure IAA standard curve.

Plant growth and inoculation methods

Dehulled seeds of *O. sativa* L. cvs. Baldo (breeder Ente Nazionale Risi, Milan, Italy) and Vialone Nano (breeder CREA-CI, Research Centre for Cereal and Industrial Crops, Vercelli, Turin, Italy) were surface sterilized with 70% ethanol for 7 min, rinsed with sterile water, and shaken in 5% sodium hypochlorite solution for 25 min at room temperature. Seeds were then washed several times with sterilized distilled water, positioned onto the surface of 0.8% water-agar plates and incubated at 21°C in the dark for germination. After 5 days, the roots of the germinated seeds were cut (0.5 cm from the bottom) with a sterile lancet to facilitate the uptake of bacteria, incubated with bacteria for the infection and then transferred into plastic pots containing sand and perlite soil in 1:1 ratio, as previously described (Defez *et al*., 2017), or into hydroponic units (plastic tubes of 15 cm in length and 5 cm in diameter) containing Jensen nitrogen-free nutrient solution (Jensen, 1942). For the inoculation of rice plants, three different methods were used. Method 1 (single inoculation): seedlings were incubated with each selected strain at 10^6 cells ml^{-1} in 1× PBS solution under constant gentle stirring for 4 h at room temperature. Method 2 (two-step inoculation): seedlings were incubated during 2 hours with 10^6 cells ml^{-1} of one strain, the cell suspensions were then removed and seedlings were treated with the second one at the same concentration for other two hours. No washing was performed between the incubation with the two bacteria. Method 3 (co-inoculation): the seedlings with the cut roots were incubated for 4 h with a two component bacterial mixture containing the RCA25 strains at 10^7 cells ml^{-1} and each of the other strain at 10^5 or 10^6 cells ml^{-1}. To analyse the effect of purified IAA on the nitrogenase activity of RCA25 in plant, rice plants inoculated with RCA25 were grown in hydroponic conditions as described above for three weeks. The growth medium was then replaced with a PBS solution containing IAA at final

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concentration of 4 μM and the plants were kept in the growth chamber for 4 hours. After the IAA-treatment the plants were carefully removed from the pots and used for the ARA assay. To verify the specificity of IAA, purified molecules, functionally (2,4-D) or structurally [IND and indole-3-carboxylic acid (ICA)], were solubilized in 50% EtOH and tested at a final concentration of 4 μM. To avoid solvent interference the control plants were treated with similar amount of EtOH solution. For confocal microscopy experiments the two-step inoculation and co-inoculation were used, while the co-inoculation method was used to perform greenhouse experiments. Inoculated seedlings were then transferred into plastic pots, as described above. Each planting unit was kept in the growth chamber under long daylight (16 h), 19–23°C temperature and 75% relative humidity.

Re-isolation of endophytic bacteria from inoculated plants

The plant colonization was assessed at 15 days after *O. sativa* L. cv. Baldo infection. The surface of the whole plant was sterilized using the following procedure: 1 min in 70% EtOH, 1 min in 5% sodium hypochlorite solution and 30 seconds in 70% EtOH. The tissues of the whole plant were then washed several times with sterilized distilled water and homogenized using a sterile mortar and pestle in 5 ml of 1× PBS. Dilutions of the homogenates were spread onto rich medium agar plates containing the specific antibiotics. After 24 h at 30°C the CFU number was counted. The colonization efficiency was presented as CFU ratio based on RCA25 strain. To determine the extent of bacterial population inside plants co-inoculated with RFP-tagged RCA25 and EGFP-tagged RCA24 at 10^7 cells ml^{-1} and at 10^6 cells ml^{-1}, respectively, the CFU numbers were counted. The colonies were distinguished according to the different colours (red and green) produced on LB agar plates. The CFU numbers of plants inoculated with the single strains at the same concentrations were used as reference.

Assessment of nitrogenase activity in inoculated plants by ARA

Three-week-old rice plants (*O. sativa* L. cvs. Baldo and Vialone Nano), grown as above described, were carefully removed from the pots and the roots were rinsed with water. The plants were then

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transferred into 20 ml glass tubes and analysed for the ARA assay as described by Defez *et al.* (2017).

Microscopy analysis

Oryza sativa L. cv. Baldo plants inoculated with the fluorescent-tagged strains (RCA25-RFP, RCA24-EGFP, RCA23-EGFP and 377-EGFP) were analysed by confocal microscopy to verify their ability to colonize rice roots endophytically. Inoculation with strain RCA25-RFP and the double bacteria combinations (RCA25-RFP+RCA23-EGFP, RCA25-RFP+RCA24-EGFP, RCA25-RFP+377-EGFP) as well as growth conditions were described above. Root samples (4 per treatment and per assay) were analysed on the confocal microscope 13 days after bacterial inoculation to monitor bacteria colonization of root tissues. Two different experiments (two-step inoculation and co-inoculation) were carried out and four plants per treatment were analysed in each experiment. Rice plants were carefully uprooted from tubes and the root systems washed by dipping them in tap water. Then, roots from each plant were exhaustively analysed by confocal microscopy. Confocal optical stacks were collected using an Axioskop 2 MOT microscope (Carl Zeiss Inc., Jena GmbH, Germany) equipped with a krypton and an argon laser, controlled by Carl Zeiss Laser Scanning System LSM5 PASCAL software (Carl Zeiss Inc.). EGFP-tagged bacterial cells were excited with the 488-nm Ar laser line and were detected in the 500–520-nm window. RFP-tagged bacterial cells were excited with the 568-nm Kr laser line and detected in the 580–620-nm window. The microscope data were recorded and then transferred for analysis to Zeiss LSM Image Browser version 4.0 (Carl Zeiss Inc.). Bacterial colonization of rice roots was analysed from three dimensional (3D) confocal data stacks. Projections from adjacent confocal optical sections were made for building up the images shown in Fig. 3 3. Final images were processed for brightness and contrast with PHOTOSHOP 4.0 software (Adobe Systems Inc., San Jose, CA).

Greenhouse experiments and plant growth-promotion activity in inoculated rice plant

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Dehulled seeds of *O. sativa* L. cvs. Baldo and Vialone Nano were surface-sterilized and germinated as above described. For the single inoculation, seedlings were separately incubated with strains RCA25 (10^7 cells ml^{-1}) and RCA24 (10^6 cells ml^{-1}), while for the co-inoculation, seedlings were incubated with a bacterial mixture containing RCA25 and RCA24 at the same concentration as the one used for the single-inoculation. For each experimental sample, five non-inoculated (control) and inoculated (RCA25, RCA24 and RCA25 + RCA24) seedlings were sown into 12 plastic pots (23 cm x 21 cm) filled with non-sterile paddy field soil (47.8% sand, 9.4 % clay, 42.8% silt, pH 6.4, organic matter 1.45%) taken from the experimental rice field of CREA-CI in Vercelli (VC) in April 2018. Plants were watered every day with tap water, and kept in a greenhouse for 90 days, following the natural photoperiod. At 55 days after sowing, the inoculation efficiency was measured as described in the Methods section "Re-isolation of endophytic bacteria from inoculated plants". At 90 days after sowing (i.e. heading-flowering stage), the Soil Plant Analysis Development (SPAD) meter (KONICA MINOLTA, Milan, Italy) was used for the estimation of leaf chlorophyll content. The ratio of chlorophyll (CHL) and flavonoid (FLA) concentration, which is termed Nitrogen Balance Index (NBI=CHL/FLA), was also recorded as an indicator of the plant nitrogen status at the beginning of flowering stage (Tremblay *et al*., 2012) using the DUALEX 4 Scientific (Dx4) chlorophyll meter (Force-A, Paris, France) (Goulas *et al*., 2004). Measurements were carried out on both adaxial and abaxial faces of the panicle leaf for each plant. Plants were then removed from pots, submerged in tap water to remove soil from the root system and the height and weight of the shoots measured. Plants were then dried for 48 h at 70 °C and weighed to measure the shoot dry mass. Total nitrogen content from 5 mg dried material was determined by using an elemental analyser (Flash EA 1112, Thermo Finnigan) at 950°C.

Statistical analysis

Statistical analysis of data was performed by applying one-way analysis of variance (ANOVA), followed by Tukey's HSD post-hoc test, using the VassarStats ANOVA program available at:

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http://faculty.vassar.edu/lowry/VassarStats.html. The results were considered statistically significant when $P \leq 0.05$. Five biological replicates have been carried out for the measurement of biofilm, gene expression, nitrogenase activity, IAA production, nitrogen content, and endophytes re-isolated from inoculated plants. The evaluation of physiological parameters was performed on forty biological replicates.

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References

Akintokun, A. K., and Taiwo, M. O. (2016) Comparison of single culture and the consortium of growth-promoting rhizobacteria from three tomato (*Lycopersicon esculentum Mill*) varieties. *Adv Plants Agric Res* **5**: 167. doi: 10.15406/apar.2016.05.00167.

Ambrosio, R., Ortiz-Marqueza, J. C. F., and Curattia, L. (2017) Metabolic engineering of a diazotrophic bacterium improves ammonium release and biofertilization of plants and microalgae. *Metabolic Engineering* **40**: 59-68.

Angel, R., Nepel, M., Panhölzl, C., Schmidt, H., Herbold, C. W., Eichorst, S. A., Woebken, D. (2018) Evaluation of primers targeting the diazotroph functional gene and development of NifMAP – A bioinformatics pipeline for analyzing *nifH* amplicon data. *Front Microbiol* **9**: 703.

Arora, N. K., and Mishra, J. (2016) Prospecting the roles of metabolites and additives in future bioformulations for sustainable agriculture. *App Soil Ecol* **107**: 405-407. doi: 10.1016/j.apsoil.2016.05.020.

Awika, J. M. (2011) Major cereal grains production and use around the world. *ACS Implications to Food Processing and Health Promotion* **1**: 1-13. doi: 10.1021/bk-2011-1089.ch001.

Baldan, E., Nigris, S., Romualdi, C., D'Alessandro, S., Clocchiatti, A., Zottini, M. (2015). Beneficial bacteria isolated from grapevine inner tissues shape *Arabidopsis thaliana* roots. *PLoS ONE* **10**. doi:10.1371/journal. pone.0140252.

Berg, G., Rybakova, D., Grube, M., and Köberl, M. (2016) The plant microbiome explored: implications for experimental botany. *J Exp Bot* **67**: 995-1002.

Bianco, C., Imperlini, E., Calogero, R., Senatore, B., Amoresano, A., Carpentieri, A., *et al.* (2006a) Indole-3-acetic acid improves *Escherichia coli's* defences to stress. *Arch Microbiol* **185**: 373-382. doi:10.1007/s00203-006-0103-y.

Accepted Article

This article is protected by copyright. All rights reserved

Accepted Article

Bianco, C., Imperlini, E., Calogero, R., Senatore, B., Pucci, P., and Defez, R. (2006b) Indole-3-acetic acid regulates the central metabolic pathways in *Escherichia coli*. *Microbiology* **152**: 2421-2431.

Bloemberg, G. V., Wijfjes, A. H. M., Lamers, G. E. M., Stuurman, N., and Lugtenberg, B. J. J. (2000) Simultaneous imaging of *Pseudomonas fluorescens* WCS365 populations expressing three different autofluorescent proteins in the rhizosphere: new perspectives for studying microbial communities. *Mol Plant Microb Interact* **13**: 1170-1176.

Bogino, P. C., Oliva, M. M., Sorroche, F. G., and Giordano, W. (2013) The role of bacterial biofilms and surface components in plant-bacterial associations. *Int J Mol Sci* **14**: 15838-15859. doi: 10.3390/ijms140815838.

Camerini, S., Senatore, B., Lonardo, E., Imperlini, E., Bianco, C., Moschetti, G., *et al.* (2008) Introduction of a novel pathway for IAA biosynthesis to rhizobia alters vetch root nodule development. *Arch Microbiol* **190:** 67-77.

Compant, S. Clément, C., and Sessitsch, A. (2010) Plant growth-promoting bacteria in the rhizo- and endosphere of plants: their role, colonization, mechanisms involved and prospects for utilization. *Soil Biol Biochem* **42:** 669-678.

Compant, S., Mitter, B., Colli-Mull, J. G., Gangl, H., and Sessitsch, A. (2011) Endophytes of grapevine flowers, berries, and seeds: identification of cultivable bacteria, comparison with other plant parts, and visualization of niches of colonization. *Microb Ecol* **62**: 188-197. doi: 10.1007/s00248-011-9883-y.

Compant, S., Saikkonen, K., Mitter, B., Campisano, A., and Mercado-Blanco, J. (2016) Editorial special issue: soil, plants and endophytes. *Plant Soil* **405**: 1–11. doi: 10.1007/s11104-016-2927-9.

Czajkowski, R., de Boer, W. J., van Veen, J. A., and Van der Wolf, J. M. (2012) Characterization of bacterial isolates from rotting potato tuber tissue showing antagonism to *Dickeya sp.* biovar 3 in vitro and in planta. *Plant Pathol J* **61**: 169–182. doi: 10.1111/j.1365-3059.2011.02486.x.

This article is protected by copyright. All rights reserved

Accepted Article

Defez, R., Andreozzi, A., and Bianco, C. (2017) The overproduction of indole-3-acetic acid (IAA) in endophytes upregulates nitrogen fixation in both bacterial cultures and inoculated rice plants. *Microb Ecol* **74**: 441-452. doi:10.1007/s00248-017-0948-4.

Elbeltagy, A., and Ando, Y. (2008) Expression of nitrogenase gene (*nifH*) in roots and stems of rice, *Oryza sativa*, by endophytic nitrogen-fixing communities. *Afr J Biotechnol* **7**:1950-1957. doi: 10.5897/AJB2008.000-5042.

Fox, A. R., Soto, G., Valverde, C., Russo, D., Lagares, A. Jr., Zorreguieta Á., *et al.* (2016) Major cereal crops benefit from biological nitrogen fixation when inoculated with the nitrogen-fixing bacterium *Pseudomonas protegens* Pf-5 X940. *Environ Microbiol* **18**: 3522-3534. doi: 10.1111/1462-2920.13376.

Gaby, J. C., and Buckley, D. H. (2014). A comprehensive aligned *nifH* gene database: a multipurpose tool for studies of nitrogen-fixing bacteria. *Database: the journal of biological databases and curation*, *2014*, bau001. doi: 10.1093/database/bau001.

Gaby J. C., Rishishwar, L., Valderrama-Aguirre, L. C., Green, S. J., Valderrama-Aguirre, A., Jordan, I. K. and Kostka, J. E. (2018). Diazotroph community characterization via a highthroughput *nifH* amplicon sequencing and analysis pipeline. *Appl Environ Microbiol* **84**: 4. doi:10.1128/AEM.01512-17.

Geddes, B. A., Ryu, M. H., Mus, F., Garcia-Costas, A., Peters, J. W., Voigt, C. A., and Poole, P. (2015) Use of plant colonizing bacteria as chassis for transfer of N_2-fixation to cereals. *Curr Opin Biotechnol* **32**: 216-222. doi: 10.1016/j.copbio.2015.01.004.

Goulas, Y., Cerovic, Z. G., Cartelat, A., and Moya, I. (2004) Dualex: a new instrument for field measurements of epidermal ultraviolet absorbance by chlorophyll fluorescence. *Appl Opt* **43**: 4488–4496. doi: 10.1364/AO.43.004488.

Haney, C. H., Samuel, B. S., Bush, J., and Ausubel, F. M. (2015) Associations with rhizosphere bacteria can confer an adaptive advantage to plants. *Nat Plants* **1**:15051.

This article is protected by copyright. All rights reserved

Accepted Article

Hardoim, P. R., van Overbeek, L. S., Berg, G., Pirttilä, A. M., Compant, S., Campisano, A., *et al.* (2015) The hidden world within plants: ecological and evolutionary considerations for defining functioning of microbial endophytes. *Microbiol Mol Biol Rev* **79**: 293-320.

Hu, M., Zhang, C., Mu, Y., Shen, Q., and Feng. Y. (2010) Indole effects biofilm formation in bacteria. *Indian J Microbiol* **50**: 362-368. doi: 10.1007/s12088-011-0142-1.

Imperlini, E., Bianco, C., Lonardo, E., Camerini, S., Cermola, M., Moschetti, G., and Defez, R. (2009) Effect of indole-3-acetic acid on *Sinorhizobium meliloti* serviva and on symbiotic nitrogen fixation and stem dry weight production. *Appl Microbiol Biotechnol* **83**: 727-738. doi: 10.1007/s00253-009-1974-z.

Islam, M. R., Madhaiyan, M., Deka-Boruah, H. P., Yim, W., Lee, G., Saravanan, V. S., *et al.* (2009) Characterization of plant growth-promoting traits of free-living diazotrophic bacteria and their inoculation effects on growth and nitrogen uptake of crop plants. *J Microbiol Biotechnol* **19**: 1213-1222.

Jensen, H. L. (1942). Nitrogen fixation in leguminous plant. General characters of root-nodule bacteria isolated from species of Medicago and Trifolium. *Proc Linn Soc NSW* 66: 98-108.

Jia, X., Liu, C., Song, H., Ding, M., Du, J., Ma, Q., and Yuan, Y. (2016) Design, analysis and application of synthetic microbial consortia. *Synth Syst Biotechnol* **1**: 109-117. doi: 10.1016/j.synbio.2016.02.001.

Kandel, S. L., Joubert, P. M., and Doty, S. L. (2017) Bacterial endophyte colonization and distribution within plants. *Microorganisms* **5**: 77. doi: 10.3390/microorganisms5040077.

Kawarai, T., Furukawa, S., Narisawa, N., Hagiwara, C., Ogihara, H., and Yamasaki, M. (2009) Biofilm formation by *Escherichia coli* in hypertonic sucrose media. *J Biosci Bioeng* **107**: 630-635. doi: 10.1016/j.jbiosc.2009.01.018.

Kim, J., and Park, W. (2015) Indole: a signaling molecule or a mere metabolic byproduct that alters bacterial physiology at a high concentration? *J Microbiol* **53**: 421-428. doi: 10.1007/s12275-015-5273-3.

This article is protected by copyright. All rights reserved

Accepted Article

Korir, H., Mungai, N. W., Thuita, M., Hamba, Y., and Masso, C. (2017) Co-inoculation effect of rhizobia and plant growth promoting rhizobacteria on common bean growth in a low phosphorus soil. *Front Plant Sci* **8**: 141. doi:10.3389/fpls.2017.0014.

Lally, R. D., Galbally, P., Moreira, A.S., Spink, J., Ryan, D., Germaine, and K.J., Dowling, D. N. (2017) Application of endophytic *Pseudomonas fluorescens* and a bacterial consortium to *Brassica napus* can increase plant height and biomass under greenhouse and field conditions. *Front Plant Sci* **8**: 2193. doi: 10.3389/fpls.2017.02193

Lee, J. H., and Lee, J. (2010) Indole as an intercellular signal in microbial communities. *FEMS Microbial Rev* **34**: 426-444. doi: 10.1111/ j.1574-6976.2009.00204.x.

Liu, H., Carvalhais, L. C., Crawford, M., Singh, E., Dennis, P. G., Pieterse, C. M. J., and Schenk, P. M. (2017) Inner plant values: diversity, colonization and benefits from endophytic bacteria. *Front Microbiol* **8**: 2552. doi: 10.3389/fmicb.2017.02552.

Mercado-Blanco, J., and Prieto P. (2012) Bacterial endophytes and root hairs. *Plant Soil* **361**: 301-306. doi: 10.1007/s11104-012-1212-9.

Mercado-Blanco, J., and Lugtenberg B. J. J. (2014) Biotechnological applications of bacterial endophytes. *Curr Biotech* **3**: 60-75.

Molina-Romero, D., Baez, A., Quintero-Hernández, V., Castañeda-Lucio, M., Fuentes-Ramírez, L. E., and Bustillos-Cristales, M. D. R. (2017) Compatible bacterial mixture, tolerant to desiccation, improves maize plant growth. *PLoS ONE* **12**. doi: 10.1371/ journal.pone.0187913.

Mus, F., Crook, M. B., Garcia, K., Garcia-Costas, A., Geddes, B. A., and Kouri, E. D. (2016) Symbiotic nitrogen fixation and the challenges to its extension to nonlegumes. *Appl Environ Microbiol* **82**: 3698-3710.

Ormeño-Orrillo, E., Hungria, M., and Martínez-Romero, E. (2013) Dinitrogen-fixing prokaryotes. In: Rosenberg, E., De Long, E. F., Lory, S., Stackebrandt, E. and Thompson F. (eds) The Prokaryotes. Springer, Berlin, Heidelberg, pp. 427-451. doi: 10.1007/978-3-642-30141-4_72.

This article is protected by copyright. All rights reserved

Accepted Article

Orozco-Mosqueda, M. D. C., Rocha-Granados, M. D. C., Glick, B. R., and Santoyo, G. (2018) Microbiome engineering to improve biocontrol and plant growth-promoting mechanisms. *Microbiol Res* **208**: 25–31. doi: 10.1016/j.micres.2018.01.005.

Prieto, P., and Mercado-Blanco, J. (2008) Endophytic colonization of olive roots by the biocontrol strain *Pseudomonas fluorescens* PICF7. *FEMS Microbiol Ecol* **64**: 297-306. doi: 10.1111/j.1574-6941.2008.00450.x.

Prieto, P., Schilirò, E., Maldonado-González, M. M., Valderrama, R., Barroso-Albarracín, J. B., and Mercado-Blanco, J. (2011). Root hairs play a key role in the endophytic colonization of olive roots by *Pseudomonas spp.* with biocontrol activity. *Microbial ecology* **62**: 435-45. doi: 10.1007/s00248-011-9827-6.

Qurashi, A. W., and Sabri, A. N. (2012) Bacterial exopolysaccharide and biofilm formation stimulate chickpea growth and soil aggregation under salt stress. *Braz J Microbiol* **43**: 1183-1191. doi: 10.1590/S1517-838220120003000046.

Rasul, G. (2016) Managing the food, water, and energy nexus for achieving the sustainable development goals in South Asia. *Environ Dev* **18**: 14-25. doi: 10.1016/j.envdev.2015.12.00.

Rinaudi, L.V., and Giordano, W. (2010) An integrated view of biofilm formation in rhizobia. *FEMS Microbiol Lett* **304**: 1-11.

Rogers, C., and Oldroyd, G. E. D. (2014) Synthetic biology approaches to engineering the nitrogen symbiosis in cereals. *J Exp Bot* **65**: 1939-1946. doi: 10.1093/jxb/eru098.

Rosenblueth, M., Ormeño-Orrillo, E., López-López, A., Rogel, M. A., Reyes-Hernández, B. J., and Martínez-Romero, J. C. (2018) Nitrogen fixation in cereals. *Front Microbiol* **9**: 1794. doi: 10.3389/fmicb.2018.01794.

Sahu, P. K., Lavanya, G., Gupta, A., and Brahmaprakash, G. P. (2016) Fluid bed dried microbial consortium for enhanced plant growth: a step towards next generation bio formulation. *Vegetos* **29**: 4. doi: 10.5958/2229-4473.2016.00093.8.

This article is protected by copyright. All rights reserved

Accepted Article

Sahu, P. K., Shivaprakash, M. K., Mallesha, B. C., Subbarayappa, C. T., and Brahmaprakash, G. P. (2018) Effect of bacterial endophytes *Lysinibacillus sp.* on plant growth and fruit yield of tomato (*Solanum lycopersicum*). *Int J Curr Microbiol App Sci* **7**: 3399-3408. doi: 10.20546/ijcmas.2018.705.397.

Salkowski, E. (1885) Ueber das verhalten der skatolcarbonsaure im organismus. *Z Physiol Chem* **9**: 23-33.

Santoyo, G., Hernández-Pacheco, C., Hernández-Salmerón, J., and Hernández-León, R. (2017) The role of abiotic factors modulating the plant-microbe-soil interactions: toward sustainable agriculture. *Span J Agric Res.* **15**:1. doi: 10.5424/sjar/2017151-9990.

Sorroche, F. G., Spesia, M. B., Zorreguieta, Á., and Giordano, W. (2012) A Positive correlation between bacterial autoaggregation and biofilm formation in native *Sinorhizobium meliloti* isolates from argentina. *Appl Environ Microbiol* **78**: 4092-4101. doi: 10.1128/AEM.07826-11.

Sruthilaxmi, C.B., and Babu, S. (2017) Microbial bio-inoculants in indian agriculture: ecological perspectives for a more optimized use. *Agr Ecosyst Environ* **242**: 23-25. doi: 10.1016/j.agee.2017.03.019.

Stokstad, E. (2016) The nitrogen fix. *Science* **353**: 1225–1227. doi: 10.1126/science.353.6305.1225.

Tremblay, N., Wang, Z., and Cerovic, Z. G. (2012) Sensing crop nitrogen status with fluorescence indicators. A review. *Agron Sustain Dev* **32**: 451–464. doi: 10.1007/s13593-011-0041-1.

Ueda, T., Suga, Y., Yahiro, N., and Matsuguchi, T. (1995) Remarable N_2-fixing bacterial diversity detected in rice roots by molecular evolutionary analysis of *nifH* gene sequences. *J Bacteriol* **177**: 1414–1417. doi: 10.1128/jb.177.5.1414-1417.1995.

Van Dommelen, A., Croonenborghs, A., Spaepen, S., and Vanderleyden, J. (2009) Wheat growth promotion through inoculation with an ammonium-excreting mutant of *Azospirillum brasilense*. *Biol Fertil Soils* **45**: 549–553. doi: 10.1007/s00374-009-0357-z.

Wagner, S. C. (2011) Biological nitrogen fixation. *Nature Education Knowledge* **3** (10): 15.

This article is protected by copyright. All rights reserved

Accepted Article

Yuan, Z., Druzhinina, I. S., Labbé, J., Redman, R., Qin, Y., Rodriguez, R., *et al.* (2016) Specialized microbiome of a halophyte and its role in helping non-host plants to withstand salinity. *Sci Rep*. **6**: 32467. doi: 10.1038/srep32467.

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Table 1. IAA production and biofilm formation in rich medium.

Strain	**IAA production**[a] (µmol mg^{-1} cells)	**Ratio**	**Biofilm**[b] (OD_{570nm})	**Ratio**
RCA25	0.005 ± 0.001		0.20 ± 0.03	
RCA24	0.036 ± 0.011	7.2*	0.69 ± 0.02	3.4*
RCA23	0.040 ± 0.002	8.0*	0.71 ± 0.02	3.5*
377	0.114 ± 0.007	22.8*	0.15 ± 0.01	0.75*

Ratio calculation was based on RCA25 cultures. The values reported in the table are the averages ± standard deviation (SD) of at least four independent bacterial cultures.

*The significance ($P < 0.01$) of the data was confirmed by Student's t test.

[a]Stationary phase cultures treated with L-tryptophan were used in this assay.

[b]Crystal violet staining quantification.

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Figure Legends

Fig. 1. IAA specifically induces the expression of *nifH* and the activity of nitrogenase in *H. huttiense* RCA25 but does not significantly affect its growth or biofilm formation. To verify the specificity of IAA effects, IAA and its functional analogue 2,4-D and its structural analogue IND were dissolved in 50% EtOH and exogenously added to RCA25 bacterial cultures. Cultures non-treated with these compounds were used as reference. A. Absorbance at 600 nm over time. B. Microtiter plate biofilm assay. Surface attached biofilm of treated and not-treated cells were stained using crystal violet after 20 h of incubation at 30°C. C. Quantitative RT-PCR analysis of *nifH* gene expression. Gene expression was presented as a fold change in expression between the RCA25 cells treated with the selected compounds and the untreated ones. D. Nitrogenase activity by ARA test. After treatment with the selected compounds the cultures were centrifuged, the cell pellets re-suspended in minimal medium without ammonium chloride and transferred into glass tubes for the assay. Values are the means ± standard deviation (SD) of at least five bacterial cultures. The asterisk in all panels indicates significant difference ($P < 0.05$, Tukey's post-hoc test) between control and treated cells.

Fig. 2. Two-step inoculation and co-inoculation induce nitrogenase activity in inoculated rice plants. Three-week-old rice plants (*Oryza sativa* L. cv. Baldo) inoculated with different combinations of the selected endophytes were carefully removed from the pots, washed with tap water and transferred into glass tubes for ARA assay. A. Nitrogenase activity in rice plants inoculated first with the RCA25 strain and then with each of the other strains (377, RCA23 and RCA24, respectively) all at the concentration of 10^6 cells ml^{-1}. B. Nitrogenase activity in rice plants inoculated with the same strains used in A but with a reverse order. C. Nitrogenase activity in rice plants inoculated only with RCA25 or RCA25-64, both at the concentration of 10^7 cells ml^{-1}, and co-inoculated with RCA25 and RCA24 mixed in two ratios (100:1 and 10:1). D. Effect of purified

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molecules, structurally (indole, IND; indole-3-carboxylic acid, ICA) or functionally (2,4-dichlorophenoxyacetic acid, 2,4-D) similar to IAA, on the activity of nitrogenase enzymes in RCA25-inoculated rice plants. ARA data are presented as means ± SD of at least five plant replicates. Asterisks in all panels indicate significant difference ($P < 0.05$, Tukey's post-hoc test) between RCA25-inoculated plants and each treatment.

Fig. 3. CLSM images of colonization events of *Oryza sativa* L. cv. Baldo roots by *Enterobacter cloacae* RCA25, *Enterobacter asburiae* RCA23, *Herbaspirillum huttiense* RCA24 and *Staphylococcus sp.* 377 fluorescent-tagged cells. Rice plants were inoculated with the RCA25-RFP strain alone and with RCA25-RFP+RCA23-EGFP, RCA25-RFP+RCA24-EGFP, RCA25-RFP+377-EGFP combinations and analysed by CLSM 13 days after the two-step inoculation. Confocal analysis was performed on 3-4 cm long rice roots to show bacteria colonization. Images are projections of 10 adjacent confocal optical sections in panels B, C, E and F and single confocal images in panels A and D. Focal step between confocal optical sections was 0.5 μm. The panels reported in this Figure are representative images of four plant replicates for each treatment.

A. Epidermal cells including a root hair internally colonized by RCA25-EFP bacterial cells. B. Simultaneous localization within the cortical rice root tissue of RCA25-RFP + RCA24-EGFP bacterial cells. C. A different rice root region internally colonized by the same RCA25-RFP + RCA24-EGFP combination, and D a close up image (inset in C) showing both bacteria coexisting within the same root cell. E. CLSM image of a rice root internally colonized by the RCA25-RFP + 377-EGFP combination. Both bacteria were not simultaneously observed within the same root area but 377-EGFP was also able to internally colonize the root tissue in different root regions where RCA25-RFP was not detected (inset). F. Simultaneous localization of both RCA25-RFP and RCA23-EGFP bacterial cells within the rice root cortex. A root hair colonized by RCA23-EGFP cells is also shown. The asterisk in all panels represents internal bacteria colonization and

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colonization of the rice root surface is shown by arrow. rh, root hair; co, cortex. Scale bars represent 50 μm in all panels except in D where represents 20 μm.

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Fig. 4. Co-inoculation affetcs chlorophyll and nitrogen content in 'Baldo' rice leaves. *Oryza sativa* L. cvs. Baldo and Vialone Nano were single- and co-inoculated with RCA25 (10^7 cells ml^{-1}) and RCA24 (10^6 cells ml^{-1}), and at 90 days after inoculation two leaf-clip devices were used to assess the chlorophyll content and the N nutritional status of rice plants. The chlorophyll content (chl), the polyphenols concentration as flavonol (fla) and the Nitrogen Balance Index (nbi), calculated as the ratio between chl and fla, were measured on fully opened leaves by a Dualex clip sensor (A, B and C). The chlorophyll content was also measured on fully opened leaves by using a SPAD clip sensor (D). The average of results obtained from at least forty plant replicates is reported in this Figure. The error bars representing SD are shown in each case. Asterisks indicate significant difference ($P < 0.05$, Tukey's post-hoc test) between control and inoculated plants. The statistical analysis was carried out separately for the two cultivars.

Fig. 5. Co-inoculation with *Enterobacter cloacae* RCA25 and *Herbaspirillum huttiense* RCA24 influences physiological parameters of *Oryza sativa* L. cv. *Baldo*. At 90 days after sowing plants single- and co-inoculated with RCA25 (10^7 cells ml^{-1}) and RCA24 (10^6 cells ml^{-1}) were removed from pots and the height (A), dry weight (B) and nitrogen content (C) of the shoots measured. The calibration curves for the elemental analyser used for nitrogen content analyses were determined by using atropine standard to different known concentration of nitrogen contents (LAR, regione Piemonte, Italy). Data are presented as mean ± SD. The height and dry weight values were collected from forty plant replicates, while at least five plant replicates were used for measurements of total nitrogen content. The box plots reported in panels A and B show where 50% of the samples lie. The black line inside the box represents the median; the upper and lower boxes represent the first and third quartile; the whiskers indicate variability out of the first and third quartile. Asterisks indicate

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significant differences ($P < 0.05$, Tukey's post-hoc test) between RCA25-inoculated plants and two-step and inoculated plants. Statistical analyses of Baldo and Vialone Nano cultivars were carried out independently.

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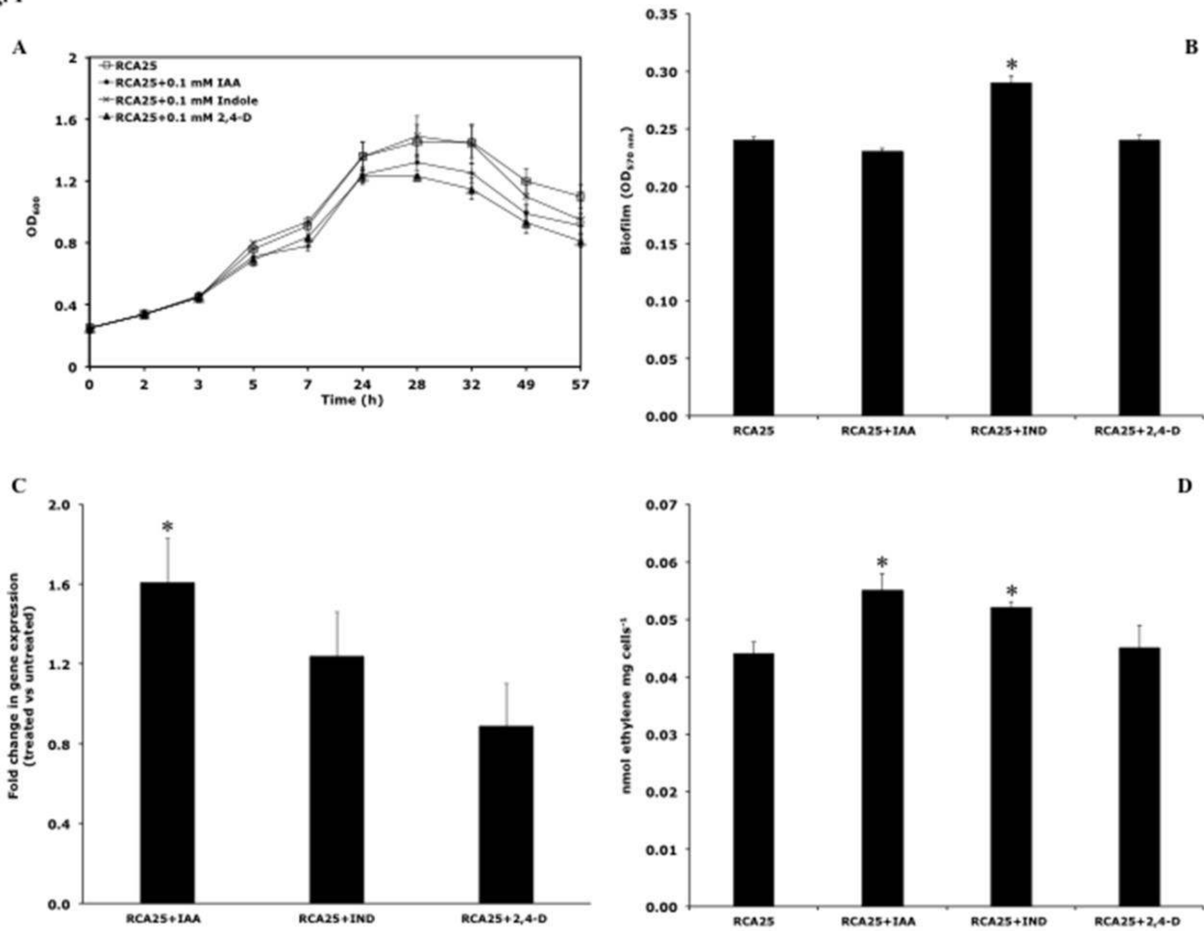
Fig. 1



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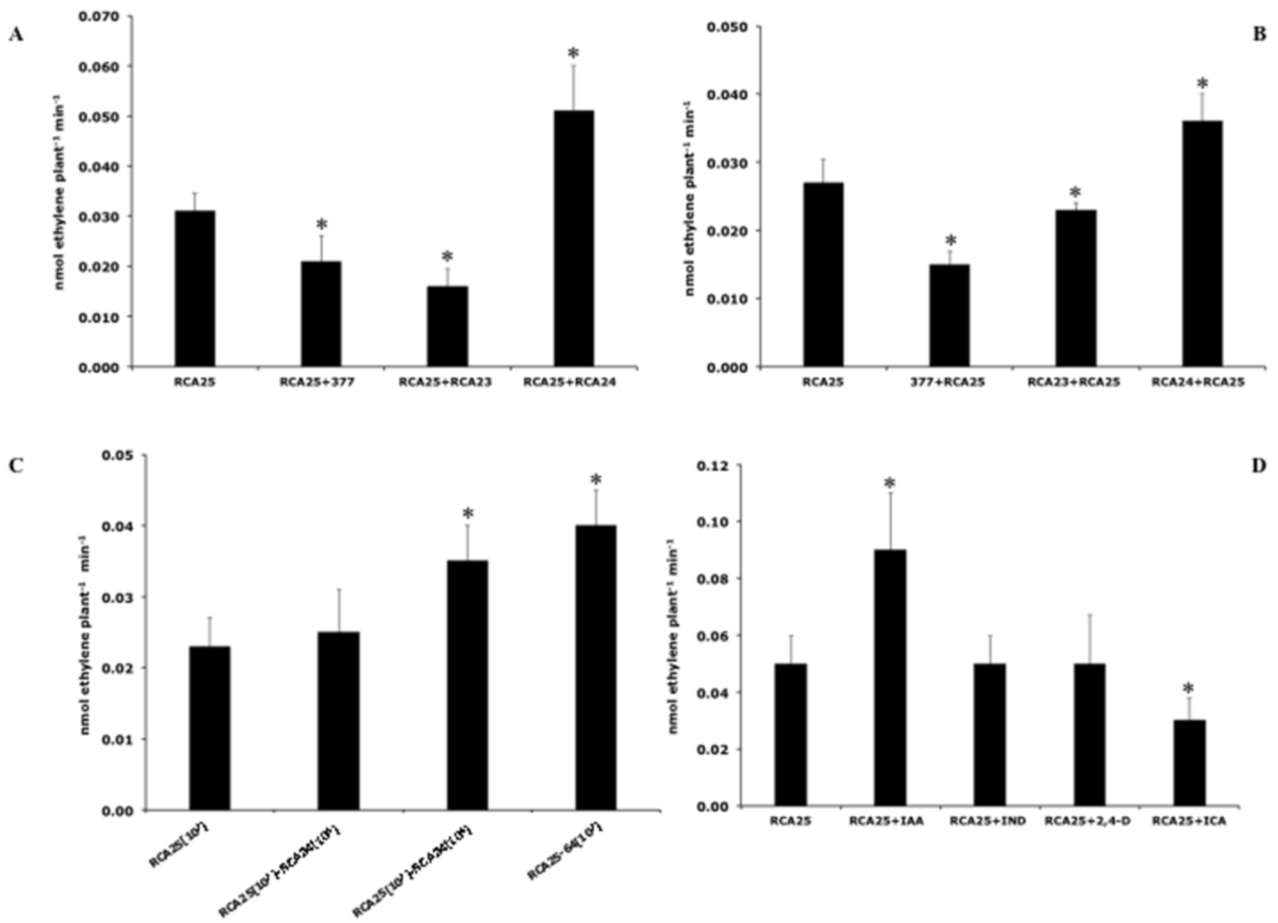

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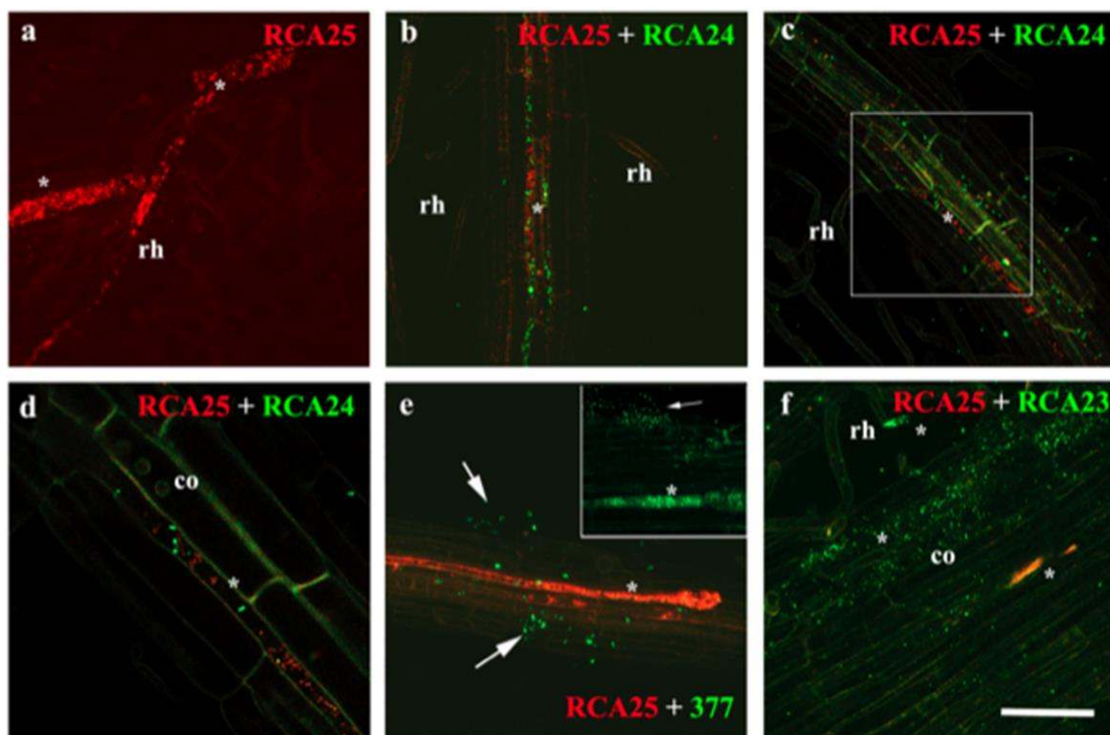
Fig. 3



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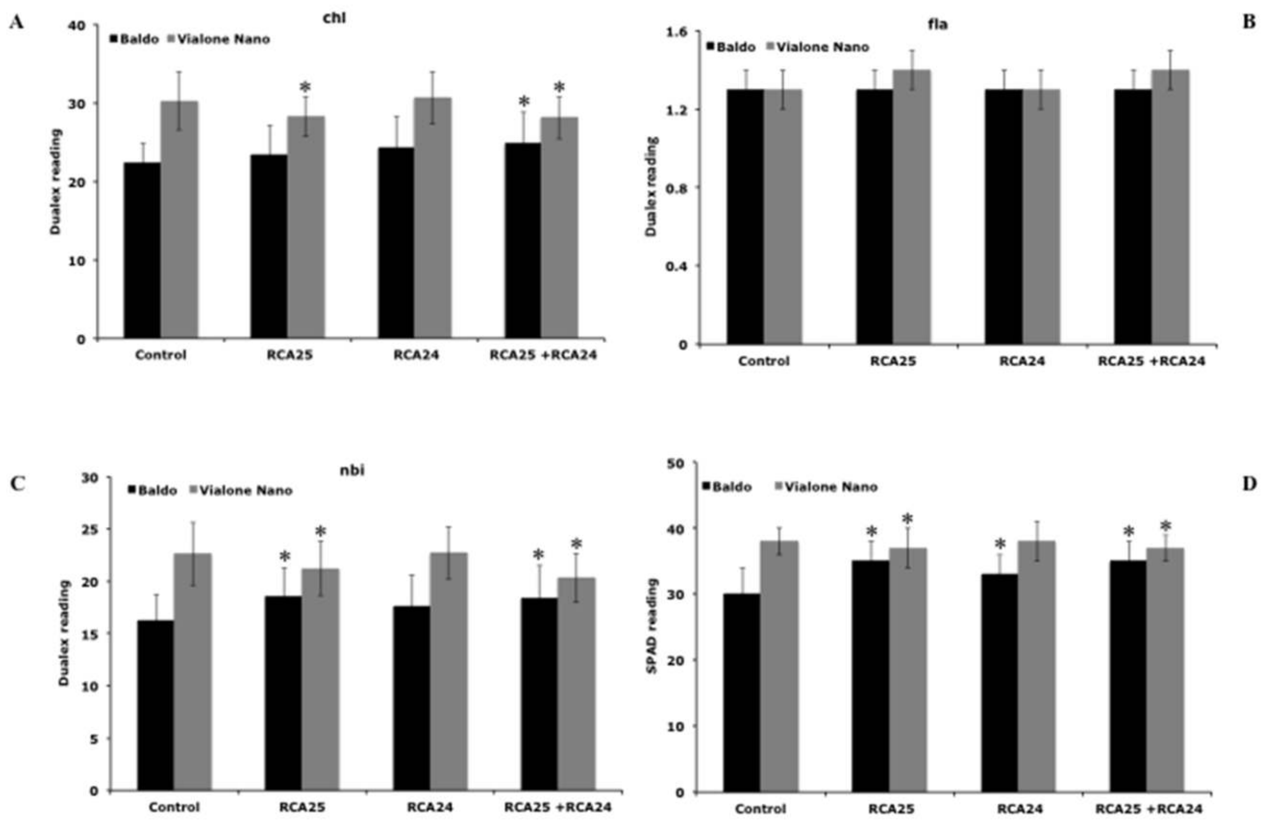

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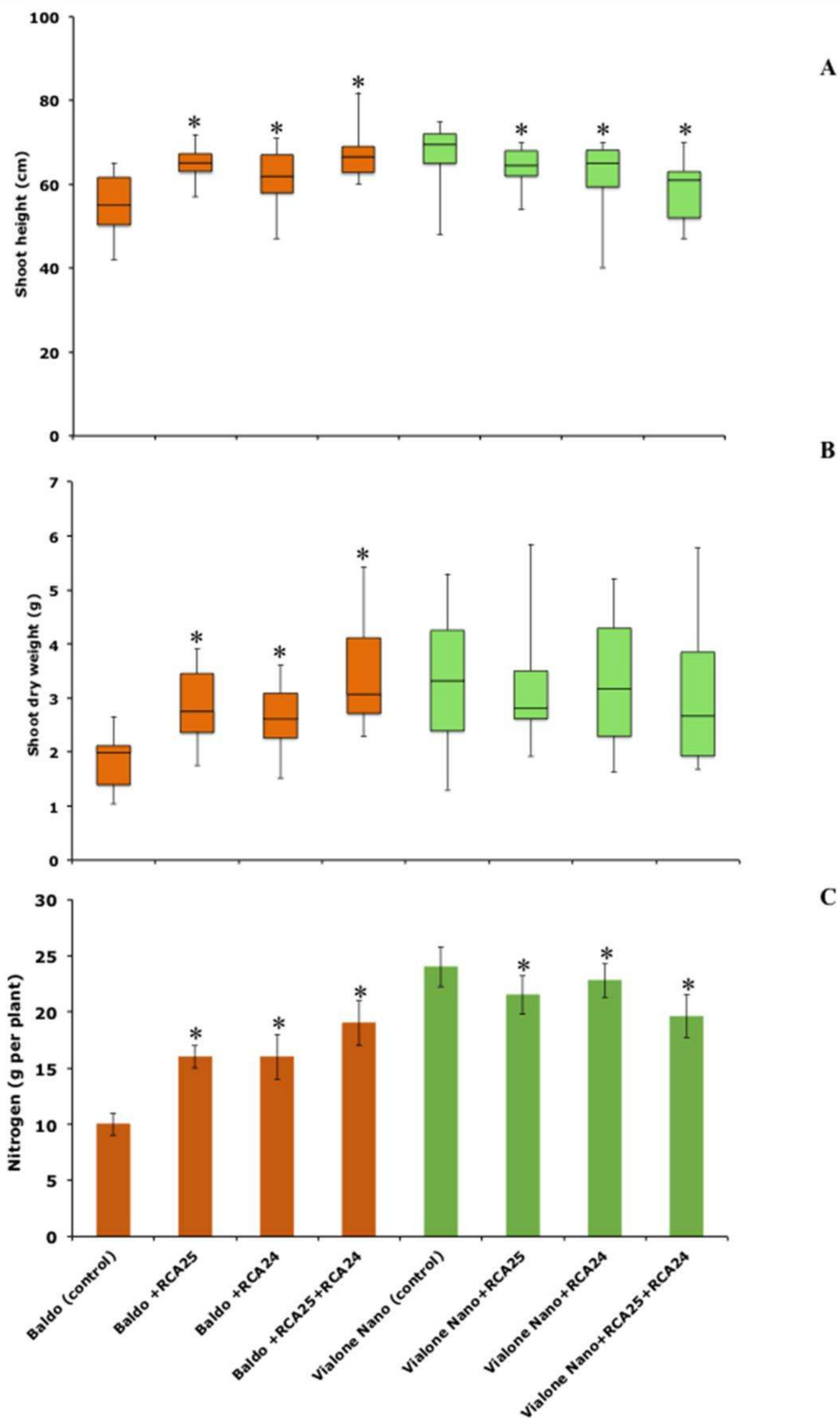

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