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Calcofluor white, an Alternative to Propidium Iodide for Plant Tissues Staining in Studies of Root Colonization by Fluorescent-tagged Rhizobia

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Authors' contributions

This work was carried out in collaboration between all authors. Authors EM, JDFF and RR designed the study, wrote the protocol and wrote the first draft of the manuscript. Authors JDFF, MMG and LCL performed microscopy assays and obtained images. Authors EM and JDFF managed the literature searches. All authors read and approved the final manuscript.

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Method Article

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ABSTRACT

Aims: To study multiple bacterial colonization *In vitro*, several limitations are obvious. One of these limitations is the plant autofluorescence generally between green and red fluorescence depending on the plant sections. The most important limitation is the bacterial fluorescence labelling, compromised by different kind of variables. Here we report the use of a secure stain, Calcofluor White, in rhizobial and other kind of beneficial bacteria colonization studies.
Study Design: Root colonization assays were designed to confirm the stability of Calcofluor White stain (Sigma®) in root cell walls.
Place and Duration of Study: Every assay developed in this method article was performed using the technical resources at the Department of Microbiology and Genetics in the University of Salamanca (Spain) in 2013.
Methodology: We have labelled rhizobia with two different kinds of fluorescent protein genes (gfp

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and rfp). We have co-inoculated *Lactuca sativa* and *Daucus carota* seedlings with two rhizobia: GFP-tagged *Rhizobium* sp. PEPV16 and RFP-tagged *Mesorhizobium* sp. CSLC01. Colonization assays were perfomed in several days post-inoculation, staining inoculated lettuce and carrot roots with Calcofluor White stain (Sigma®). Samples were monitorized for several days, using a fluorescence microscope (NIKON Eclipse 80i).

Results: Bacterial attachment to plant tissues is observed by fluorescence microscopy after their labelling with fluorescent proteins. Our results show how Calcofluor White staining for plant tissues improves bacterial visualization in contrast with tissues stained with propidium iodide, a carcinogenic agent that cannot be used when bacteria are tagged with red fluorescent proteins such as RFP or mCherry.

Conclusion: Calcofluor white is a non-carcinogenic and low toxic compound that has been classically used to stain fungi and plant tissues for different uses. Due to its low wavelength, calcofluor white may be used in combination with several fluorophores. In the present work we showed that this compound is a reliable alternative to propidium iodide for plant tissues staining in multiple rhizobial/bacterial colonization studies.

Keywords: Calcofluor; carrot; lettuce; gfp; rfp; Rhizobium; root colonization; epifluorescence.

1. INTRODUCTION

Several soil bacteria can attach to plant roots, producing beneficial or deleterious effects. Attachment is the first step of root colonization, followed by the establishment of microcolonies by clonal propagation, which is the initial stage for biofilm formation [1]. The attachment step, one of the most important stages for establishing a competitive interaction between microorganisms and plants, determines the evolution of bacterial colonization. In the last years, several studies showed the high diversity associated with root plant environment, involving bacteria of different phylum, such as α-, β-, γ-Proteobacteria, *Firmicutes* or Actinobacteria. Due to the influence of the Plant Growth Promoting Rhizobacteria (PGPR) and plant relationships, new challenges for research rhizosphere interaction have appeared, such as the necessity of labelling rhizobia and plant structures separately. Rhizobial attachment to plant roots can be observed after transformation of bacterial cells with fluorescent proteins such as GFP [2,3, 4]. To enhance the visualization of GFP-labelled bacteria in roots surfaces, propidium iodide is commonly used [5,6]. This molecule is a DNA intercalant agent and therefore, a potential mutagenic agent that should be avoided in experimentation. Moreover, the plants stained with propidium iodide have a red fluorescence that hampered the visualization of bacteria labelled with red fluorescent proteins (RFP). Although propidium iodide toxicity is well known, this compound is still commonly used in plant–bacterial interactions. Thus, alternative fluorescent dyes are necessary to stain the plant tissues in order to minimize the use of hazardous compounds and to improve the visualization of bacteria attached to them including those labelled with red fluorescent proteins.

Typically, Calcofluor staining is used as whitening agent by the paper industry and also, is used to detect fungal parasites [7,8]. Some of them are also biofilm-producer pathogens [9], due to its capacity for selectively binding toβ 1-3 and β 1-4 bonds, present in cellulose and chitin. Calcofluor White fluoresces when exposed to UV light and offers a very sensitive method for direct microscopic examination of samples from different kinds [10]. Its emission wavelength is in a range of 300-412nm and has an absorbance peak at 347nm.

In plants, Calcofluor White is able to bind cellulose in cell walls and it is used for the microscopic observation of plant structures [11,12]. Moreover, it is classically used in rhizobial studies to observe exopolysaccharide formation defects [13] and to monitor biofilm development in different *Rhizobium*-related genera [1,14,15]. Rhizobial strains are able to form mature biofilms, colonizing effectively plant roots. Here we report the isolation of two rhizobial strains, which were labelled with GFP or RFP and tested their effectivity to colonize vegetable plant root surfaces, using Calcofluor White to stain those roots. In this work, we have proven the usefulness of this non-hazardous dye in multiple rhizobial colonization assays.

2. MATERIALS AND METHODS

2.1 Bacterial Strains

Strains used in this study were isolated from different legume effective nodules. *Rhizobium sp.*

PEPV16 strain was isolated from *Phaseolus vulgaris* nodules and *Mesorhizobium sp.* CSLC01 strain was isolated from *Lotus corniculatus* nodules, both in yeast-mannitol agar (YMA) plates as described [16]. To isolate rhizobial strains, root nodules were surface-sterilized using $HgCl_2$ (2.5%, w/v) for 1min and rinsed five times with sterile distilled water. Surface-sterilized nodules were crushed, homogenized and spread on YMA plates, which were incubated at 28ºC for 3-5 days.

GFP-tagged *Rhizobium sp*. PEPV16 and RFP-tagged *Mesorhizobium sp.* CSCL01 were obtained as described [3,17]. Plasmid pHC60 [18] was introduced in *Rhizobium sp*. PEPV16 by biparental mating and plasmid pBHR-mRFP [19] was introduced to *Mesorhizobium sp*. CSLC01 by triparental mating using pRK2013 as a helper plasmid. Transconjugants were selected on TY medium plates supplemented with tetracyclin at 10µg/ml. The labelled strains were routinely grown for 3 days at 28ºC in YMA suplemented with tetracycline (10µg/ml).

2.2 Phylogenetics Analysis of 16s Gene

The amplification and sequencing of rrs gene was carried outas described [20]. The sequences obtained were compared in EzTaxon-e server [21]. Sequences were aligned using Clustal X [22]. The distances were calculated according to Kimura's two-parameter model [23]. The phylogenetic trees were inferred using the Neighbor-joininganalysis [24]. MEGA6 software [25] was used for all analyses.

2.3 Plant Root Colonization Assays

For root plant colonization assays, we selected two different species of vegetable plant, *Lactuca sativa* var. romana and *Daucus carota* var. nantesa. Plant seeds were surface sterilized by immersion in 70% ethanol for 30 seconds followed by 5% sodium hypochlorite aqueous solution during 5min. Seeds were rinsed five timesand were germinated in agar plates. The plates were placed in darkness at 24ºC until seedling roots were 1–2cm.Seedlings with 2-3 days-old were inoculated with 250µL/plant of the two tagged rhizobia suspensionof 10^7 cells/mland incubated for 3 to 7 days in growth chamber controlled conditions. Uninoculated controls were also included in the study. Workflow diagram of the assay setting up is included in Supplementary material (Supplementary Fig. 1).

2.4 Root Tissues Staining and Microscopy

Unbound bacteria were removed by gently washing three times in sterile distilled water before root staining. Seedlings were placed in slides and stained with 50µl of Calcofluor White solution 50mg/L (Calcofluor White stain, Sigma®) and 50µl of 10% potassium hydroxide solution for improving resolution, as recommended by the manufacturer. Preparations were covered and incubated during 1 min before observation with an epifluorescence microscope (Nikon Eclipse 80i). Calcofluor White solution is combined with Evans Blue 25µg/L, which reduces considerably fluorescence background. For propidium iodide staining, seedlings were placed in slides and stained with 50µl of propidium iodide 10µM (Propidium Iodide, Sigma®), dissolved in distilled water. Preparations were covered and directly observed as described above.

3. RESULTS AND DISCUSSION

Plant-microbe interactions study in a global scenario of food deficiency and continuous environmental conditions impoverishment is becoming crucial. The genus *Rhizobium*, classically associated to the root-nodule symbiotic interactions in legumes, is described as a good PGPR endophyte in several crops, including cereals and vegetables [3,20,26,27]. The genus *Rhizobium* and related beneficial genera are considered as safe microorganisms for human, animal and plant health, becoming excellent candidates for novel biofertilizer formulations [3,28].

Biofertilizer composition is usually based in multiple bacteria involving phosphate solubilizer bacteria, phytohormone and siderophore producer bacteria and biocontrol agents, among others. Formulation of multiple effective PGPR biofertilizers based in its mode of action is a challenge for scientists and for the industry [29].

In vitro co-inoculation studies are essential in the development of an effective PGPR-based biofertilizer. Nevertheless, several limitations are present for the correct *in situ* visualization of different strains colonization in plant roots. Many plasmids carrying reporter genes are described [18,19,30]. Those plasmids are essential for labelling bacteria when is required for colonizing and infection assays, helping to localize those labelled bacteria outside or inside plant tissues. In this work, we have labelled both of the isolated

rhizobial strains and tested their natural ability to colonize non-legume plants such as *Lactuca sativa* and *Daucus carota*. The first isolate, PEPV 16, belonging to the species *Rhizobium leguminosarum*, was previously tested successfullyon both vegetables [17]. The second isolate, CSCL01 strain, belongs to *Mesorhizobium huakuii*, as revealed by phylogenetic analyses (Supplementary Fig. 2). *Mesorhizobium* is a common endosymbiont in *Lotus corniculatus* nodules, confirming that this isolate is a secure microorganism, susceptible of being used for biofertilizer formulation.

Plant roots have natural autofluorescence, a problem for rhizobial visualization in co-colonization studies. Usually, propidium iodide root stainingis performed but, since this compound is hazardous and one of the bacteria is labelled in red, another compounds with adequate features are required.
In this work, we have observed roots stained with Calcofluor White in combination with gfp- and rfp-tagged rhizobial strains. Calcofluor, RFP and GFP emit fluorescence at different emission peaks (387nm, 510nm and 472nm, respectively). Recently, some authors have reviewed the use of Calcofluor White staining in other microscopy techniques and other kind of plants [31], remarking its benefits not only for the particular technique we have described here. Therefore, this compound has a low emission peak that does not overlap with GFP and RFP, granting a clear imaging of each fluorophore (as showed in Fig. 1). The advantage of using this fluorescent dye means an improvement of the resolution in microscopy techniques and reduces the risk for the human health.

The epifluorescence images presented in Fig. 1 show the staining technique in carrot (A, D) and lettuce (B) co-inoculated roots after the treatment with Calcofluor White, showing blue-white fluorescence that allowed a clear visualization of bacterial attachment to the root surface either GFP and RFP-labelled rhizobia, avoiding inner plant root autofluorescence. In contrast, Propidium Iodide staining (C, E) showed less definition in bacterial imaging and did not permit the visualization of RFP-labelled rhizobia.

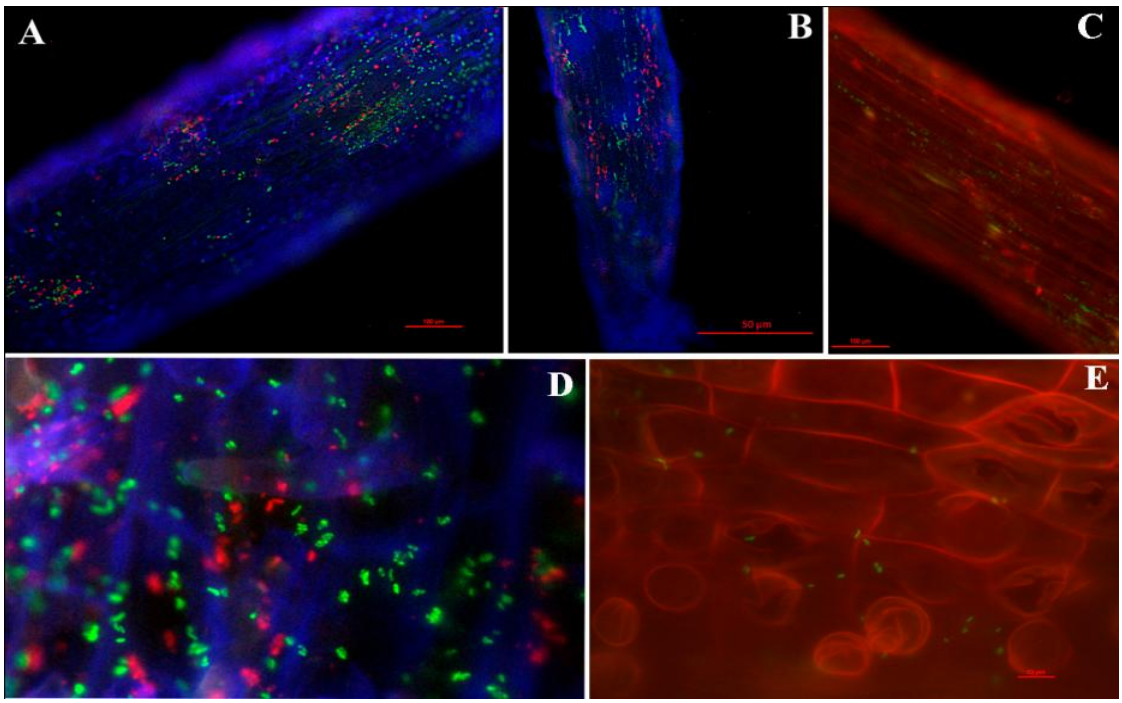

Fig. 1. Calcofluor white vs propidium Iodide staining techniques. epifluorescence micrographs of carrot (A, C, D, E) and lettuce seedling root (B) 9 days after inoculation with GFP-tagged *Rhizobium sp.* PEPV16 and RFP-tagged *Mesorhizobium sp*. CSLC01. The micrographs show the ability of both strains to colonize root surfaces. Roots were stained with Calcofluor White (A, B, D) and Propidium Iodide (C, E). Size bars represent 100µm (A, C), 50µm (B) and 10µm (D, E)

4. CONCLUSION

The isolation of new PGP bacteria suitable for being part of an efficient biofertilizer composition is continously occurring due to the high biodiversity contained in local rhizosphere. Development of new techniques for in vitro evaluations of colonization and interaction of the isolates are completely necessary. Co-inoculation assays are required for testing either positive or negative interactions of predicted good PGPR strains, suitables for being included in multiple PGPR-based biofertilizer formulations. The selection of an adequate root staining technique represents an issue and depends on the technique used to label the strain.

Our results showed that the staining of plant tissues with Calcofluor White is an excellent tool for microscopy studies of bacterial colonization. Since it permits the visualization of bacteria labelled with fluorescent proteins of different colours, including red, this methodology can be used instead of propidium iodide staining in competition assays and root colonization and adhesion studies.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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