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ROOT GROWTH-PROMOTING EFFECT IN WHEAT PLANTS EXERTED BY *AZOSPIRILLUM ARGENTINENSE* REC3, ITS FLAGELLAR PROTEIN AZFLAP AND A STRAWBERRY HYDROALCOHOLIC EXTRACT IN A SIMPLE RHIZOTRON SYSTEM

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ABSTRACT

Root research often uses rhizotron systems where their main characteristic is to possess a transparent wall allowing the *in situ* observation of plants' root system. Rhizotrons can vary in size and be filled with different substrates based on soil, sand and other components. Here, a simple and low cost rhizotron system without soil substrate was developed, allowing the observation of wheat (*Triticum aestivum* L.) root system treated with bioproducts. To show the effectiveness of the rhizotrons, hydrated wheat seeds were treated with plant growth-promoting bioproducts such as *Azospirillum argentinense* REC3 (10^6 CFU.ml^{-1}), flagellin AzFlap (200 nM), or strawberry hydroalcoholic extract (0.01 mg.mL^{-1}). This rhizotron system allowed to non-destructively evaluate the roots (length, density and root hair proliferation), where the values of the bioproducts surpassed the control.

Keywords: *Azospirillum argentinense* REC3, flagellin AzFlap, strawberry hydroalcoholic extract, root growth-promotion

INTRODUCTION

Azospirillum is an alpha proteobacteria that belongs to the order Rhodospirillales and the family Azospirillaceae **(Baldani *et al.*, 2015)**. This genus is part of the well-known Plant Growth Promoting Rhizobacteria (PGPR), which are a group of bacteria that enhance plant performance through various mechanisms, such as biological nitrogen fixation and hormone production, among others **(Saad *et al.*, 2020)**; and they can also improve plant tolerance to different diseases (**Fujita *et al.*, 2017; Viejobueno *et al.*, 2021**). These bacteria are naturally present in the soil and colonize plant roots. They currently have a biotechnological application as an inoculant, commonly applied to seeds during planting to improve crop production **(Seleiman and Abdelaal, 2028)**. For example, it has been reported that the involvement of *Azospirillum brasilense* in wheat (*Triticum aestivum* L.) seed inoculation improved their productivity and plant nitrogen use efficiency **(Gaspareto *et al.*, 2023)**. Furthermore, this bacterium has the ability to alter root architecture by increasing branching and volume, which enhances water and nutrient uptake in the plant **(Cassán *et al.*, 2020; Oliveira *et al.*, 2022)**. However, in preliminary studies conducted under controlled laboratory conditions, tracking the growth of the root system can be a tough challenge due to the laborious process and complications in obtaining valid root samples without too much damage, hence, different studies use rhizotrons to *in situ* observe root system architecture of different plant species, such as soybean **(Sanada and Agehara, 2023)**, maize **(Jordan and Vonarx, 1992)**, *Arabidopsis thaliana* **(Devienne-Barret *et al.*, 2006)** or *Brassica napus* **(Feigl *et al.*, 2019)** under different growth conditions. This task is also relevant when plant growth-promoting bacteria or some other bioproducts are in association with the roots, as they can stimulate root proliferation.

Traditionally, root investigation methods included root excavation and soil core sampling **(Polomsky and Kuhn, 2002)**. But currently, root research often uses rhizotron systems and image analysis techniques that permit non-destructive and repeated root morphological analysis. Rhizotrons can vary in size and be filled with different substrates based on soil, sand and other components, depending on the aim of the experiments and the examined plant species. But in general its main characteristic is to possess a transparent wall allowing the *in situ* tracking of the development of plants' root system. Therefore, the aim of this study was to evaluate root growth in the early stages of wheat plant development treated with three plant-growth inductors (bioproducts), using a low-cost and simple assembly rhizotron system without soil substrate. The following bioproducts were applied: the bacterium *Azospirillum argentinense* REC3 (former *A. brasilense* REC3), able to promote strawberry and rice plant growth **(Pedraza *et al.*, 2009, 2010)**; the flagellin AzFlap which is the protein from the polar flagellum of REC3 **(Elías *et al.*, 2021)**, and a strawberry hydroalcoholic extract whose positive effect on germination and growth of lettuce seedlings under controlled and field conditions was recently reported **(Villalba *et al.*, 2023)**.

MATERIAL AND METHODS

Rhizotron assembly

Rhizotron was assembled into 10 cm wide and 15 cm high panels, using expanded polystyrene sheet as a base support and over that, an absorbent microfiber cloth (commonly found in the commerce) and filter paper, previously soaked in distilled water, were mounted. The front panel was made of anti-glare, 100% transparent plastic (Figure 1a). All assembled panels were held with plastic clamps to prevent its shifting or separation (Figure 1b). The microfiber cloth, filter paper and distilled water used were previously sterilized in autoclave, while the expanded polystyrene sheet and the transparent plastic (front panel) were disinfected with ethanol 70° or sodium hypochlorite 5%. In this rhizotron system, the support for plant development is constituted by the filter paper together with the absorbent cloth which retains the moisture and nutrients. The expanded polystyrene sheet gives rigidity to the rhizotron´s structure and the transparent front panel allows daily monitoring of root development.

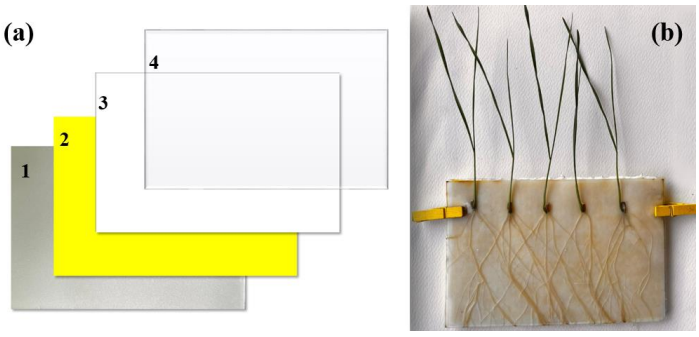


Figure 1 Rhizotron. **(a)** Components: 1, expanded polystyrene sheet; 2, absorbent microfiber cloth; 3, filter paper and 4, transparent plastic. **(b)** Wheat seedlings in the assembled rhizotron.

Preparation of bacterial inoculum

For the bacterial inoculum, a pure culture of *Azospirillum argentinense* REC3 **(dos Santos Ferreira *et al*., 2022)** was grown in NFb liquid medium **(Baldani *et al*., 2014)**, supplemented with 1% NH_4Cl for 24 h at 30°C and 120 rpm. Cells were then centrifuged at 2000 x g for 10 min and washed twice with sterile bi-distilled water at pH 7.0 to remove culture medium residues that might interfere with the assays; cells were suspended in sterile bi-distilled water and the concentration for seed inoculation was ~10^6 CFU.mL^{-1} ($OD_{560\ nm}$ 0.2).

Flagellin AzFlap

The flagellin AzFlap from *A. argentinense* REC3 was obtained as described previously **(Elías *et al*., 2021)**. After denaturing polyacrylamide gel electrophoresis, following standard procedures, the band corresponding to flagellin AzFlap (100 kDa) was excised from the gel, electroeluted in Tris–glycine buffer pH 8.3 at 40 V for 12 h, dialyzed, lyophilized, and suspended in distilled water to a 200 nM concentration.

Strawberry hydroalcoholic extract

The strawberry hydroalcoholic extract (SHE) was obtained according to **Mamaní *et al*. (2012)**. Briefly, young leaves of Camarosa strawberry plants were dried at 50°C until a constant weight was achieved. Subsequently, they were ground and mixed with 80% ethanol (v/v) at a ratio of 1/10 (dry leaf weight/solvent volume), and macerated for 24 h at 25°C with constant agitation. The alcoholic homogenate was filtered and concentrated in a vacuum rotary evaporator at 50°C until all the alcohol was completely eliminated. The resulting aqueous residue is readily soluble in water and exhibits high stability to light and high temperatures **(Mamani *et al*., 2012)**. For the rhizotron assay, the SHE was diluted with distilled water to a final concentration equivalent to 100 mg of fresh leaf weight per mL (mg FW. mL^{-1}).

Rhizotron assay

To demonstrate the effectiveness of the presented rhizotron, wheat (*Triticum aestivum* L.) seeds were disinfected using 70% ethanol and 5% sodium hypochlorite. The seeds were then rinsed with sterile distilled water and tested for the presence of microbes by placing them onto trypticase soy agar medium (TSA) (Difco-BBL, Sparks, MD) and incubating them for 72 hours at 30°C. Once the absence of microbial growth was confirmed, the seeds were hydrated with physiological solution for 24 h at 30°C and then were treated by immersion during 2 h at 30°C with a 10^6 CFU.mL^{-1} suspension of *Azospirillum argentinense* REC3, 200 nM of flagellin AzFlap or 0.01 mg.mL^{-1} of strawberry hydroalcoholic extract (SHE). Seeds treated only with sterile physiological solution were used as control. Each treatment was applied independently. Then, the treated seeds were transferred to the rhizotron system, placing each seed on the filter paper at one centimeter below the edge, and then the plastic frontal was placed over the filter paper (Figure 1b). For each treatment, three rhizotrons were made with 5 seeds each. Seedlings were hydrated with 10 mL of 50% Hoagland nutrient solution **(Hoagland, 1920)** every three days.

The rhizotrons were placed upright in a plastic tray and the seedlings were cultivated in phytotron at photon flux density of 150 µmol m^{-2} s^{-1} (12/12 h light/dark cycle) at a relative humidity of 60-70% and 25 ± 2°C for 10 days.

Root length was determined after 10 days of the experiment, also, roots adhered to the filter paper were stained with 1% Crystal Violet (Figure 2a) and observed under magnification (4x) to compare root hair proliferation between treatments. The rhizotrons were scanned to obtain images and determine root density, as described previously **(Tognacchini *et al*., 2020)**, with Image-J software **(Schroeder *et al*., 2021)**. For this purpose, the images were converted to binary, where only black ("0") and white ("255") pixels were displayed (Figure 3b). In the binary images, "0" (black/roots) and "255" (white/filter paper) pixels in the rhizotrons were counted with the "pixel count" function of the Image-J program. Root density was measured as the percentage (%) of black pixels in the total area and expressed as % pixel counts.

Statistical analysis

After verifying the normal distribution of the data, the general linear ANOVA model was applied using Infostat statistical software version 2028 **(Di Rienzo *et al*., 2020)**. Significant differences between mean values were determined by Fisher's pairwise comparisons ($p< 0.05$) **(Di Rienzo *et al*., 2020)**.

RESULTS AND DISCUSSION

Root growth-promoting effect in wheat plants treated with three bioproducts

In previous studies, the growth-promoting ability of *A. brasilense* REC3 **(Pedraza *et al*., 2007)**, recently reclassified as *A. argentinense* REC3 **(dos Santos Ferreira *et al*., 2022)**, was reported (Pedraza *et al*., 2009; 2010). It was also informed that the flagellin AzFlap obtained from *A. brasilense* REC3 elicits defense responses against the phytopathogenic fungus *Macrophomina phaseolina*. Nevertheless, its growth-promoting capacity for roots has not been evaluated until now. Additionally, the positive effect of SHE on lettuce germination and growth is known **(Villalba *et al*., 2023)**, but its specific impact on root development has not been assessed so far.

This rhizotron system, free from microbial contamination, allowed us to clearly observe the differences between the treatments, where the development of the plants treated with bioproducts surpassed the control. After staining roots with Crystal Violet, a large proliferation of root hairs was observed in the treatments with bioproducts (Figure 2d-2f), compared to the control (Figure 2c).

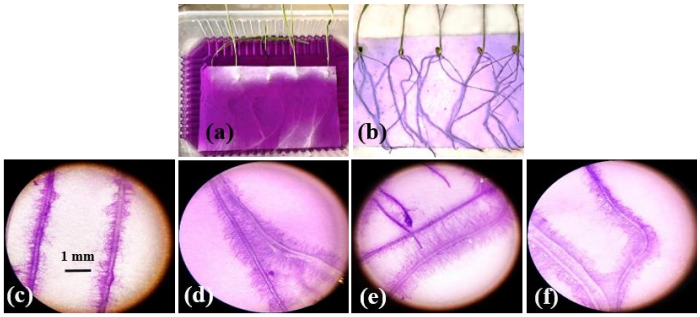
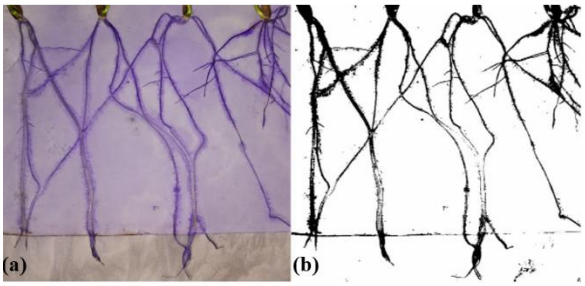


Figure 2 Wheat seedlings treated with plant-growth bioproducts. **(a)** Roots attached to the rhizotron filter paper panel during crystal violet staining. **(b)** Roots after crystal violet staining. Representative optical images of roots from seedling**: (c)** treated with physiological solution (control); **(d)** treated with Flagellin AzFlap (200 nM); **(e)** treated with strawberry hydroalcoholic extract (SHE) (0.01mg. mL^{-1}) and **(f)** treated with *Azospirillum argentinense* REC3 (10^6 CFU.ml^{-1}). Scale bar = 1 mm.

The root density measurements confirmed the significant increase in root hairs observed through staining: AzFlap (13.96 ± 1 % pixel counts), followed by SHE (13.11 ± 0.81 % pixel counts), REC3 (10.85 ± 0.53 % pixel counts), and the control (10.17 ± 0.32 % pixel counts) (Figure 3c). In addition, the root length measurements were greater with AzFlap (12.08 ± 1.05 cm), followed by SHE (10.76 ± 0.84 cm) and REC3 (10.48 ± 0.34 cm), while the control was 9.38 ± 0.50 cm (Figure 3c).



(c) Treatment	Root lenght (cm)	Root density (% pixel counts)
Control	9.38 ± 0.50^{c}	10.17 ± 0,57^{b}
REC3	10.48 ± 0.34^{b}	10.85 ± 0,32^{b}
SHE	10.76 ± 0.84^{b}	13.11 ± 0.81^{a}
AzFlap	12.08 ± 1.05^{a}	13.96 ± 1.00^{a}

Figure 3 Wheat seedlings treated with plant-growth inductors. **(a)** Representative picture of the roots attached to the rhizotron filter paper panel after crystal violet staining. **(b)** Original photo scanned to convert to "bits" ("o" and "255" pixels) with the program Image-J in order to count black and white pixels. **(c)** Root length and density values after 10 days of growth. Results are given as mean values ± SE. Different letters indicate statistically significant differences ($p< 0.05$).

In the case of bacterial treatment, it is known that the production of auxins, especially indole acetic acid by *Azospirillum* **(Pedraza *et al*., 2007, 2010)**, is one of the mechanisms promoting root growth that would be involved in the increased development of wheat roots compared to the control observed in this study. Similarly, it was also reported that the flagellin of *A. argentinense* would be another mechanism promoting root development, but independent of the production of indole acetic acid, as observed by **Mora *et al*. (2023)** in *Arabidopsis thaliana*. This work demonstrates that the flagellin AzFlap from *A. argentinense* REC3 not only functions as an elicitor of plant defense mechanisms against phytopathogens, as reported by **Elías *et al*. (2021)**, but also contributes to root growth. In the case of SHE, the mechanism of promoting root growth is still to be

elucidated; and in this work, it is the first time that the effect on root elongation and hair proliferation has been tested. It was previously reported that one of the compounds present in SHE induces auxin synthesis in *Arabidopsis thaliana*, which could be related to the changes observed in root structure (**Grellet Bournonville *et al.*, 2020**).

Effectiveness of the rhizotron

The results obtained showed the effectiveness of the rhizotron system used to non-destructively evaluate the early root development of wheat plants through root length and density, along with root hair proliferation, allowed an easy sampling for microscopic or other analyzes. Due to the ease of assembling this type of rhizotron, its size may vary depending on experimental requirements; also to get a better contrasting view of the roots, the white filter paper can be replaced for black color (Figure 4a). Considering the size of the rhizotron presented here, which constitutes a small experimental surface that takes up little space, several rhizotrons can be made and assembled simultaneously (Figure 4b), according to the needs of the experiment to be carried out. Besides, as the rhizotron is very easy to transport, it can be located under different environmental conditions.

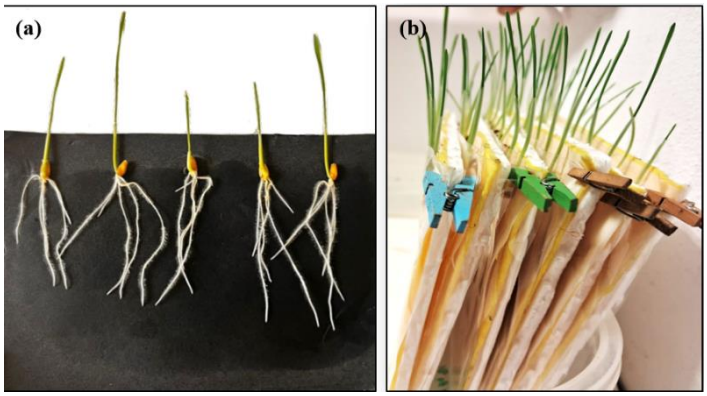


Figure 4 (a) Contrasting view of the roots assembled with black filter paper. **(b)** Representative picture of several rhizotrons assembled simultaneously.

Also, it is possible to place the rhizotron in a container with the liquid used in the test, keeping the microfiber cloth wet and without the need to rehydrate it frequently. Additionally, as the roots were not destroyed for evaluation, the filter paper containing the attached roots can be dried and kept for later reference about the assays. Although here we highlight the usefulness of the rhizotron to evaluate root development and architecture, the system also allows evaluating the development of the aerial part.

CONCLUSION

In this work we have evaluated root growth in the early stages of wheat plant development treated with *Azospirillum argentinense* REC3, its flagellar protein AzFlap, or a strawberry hydroalcoholic extract in a simple assembly rhizotron system without the use of soil as a plant substrate. This rhizotron system allowed us to clearly observe the differences between the treatments, where the development of the plants treated with bioproducts surpassed the control. The results obtained showed the effectiveness of the rhizotron system used to non-destructively evaluate the root development of wheat plants through root length and density, along with root hair proliferation. Further studies are needed to evaluate other phenomena such as the plant interaction with co-inoculated microorganisms, effect of different water or nutrient availability, or movement of particles or compounds from the root to the aerial part or vice versa, among others.

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