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EFFECT OF 2, 4-DICHLOROPHENOXYACETATE ON THE FORMATION OF PARANODULES ROOT IN DURUM WHEAT (*Triticum durum Desf.*) INOCULATED WITH *Azospirillum brasilense* ISOLATED IN RHIZOSPHERE OF WHEAT IN ALGERIA

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Résumé

Plusieurs recherches ont montré que les PGPR ont des effets bénéfiques sur les plantes, ces rhizobactéries peuvent procurer à la plante l'azote sous forme d'ammonium par leurs associations avec les légumineuses et certaines graminées. Les souches d`*Azospirillum brasilense*, isolées préalablement de la rhizosphère du blé dur dans l`Est d`Algérie, ont la capacité de fixer l`azote cet effet a été déterminer par le dosage d`azote et la chlorophylle total des partie aériennes, ces souches peuvent coloniser des racines du blé dur (*Triticum durum* Desf.) inoculée par la formation des para-nodules au niveau des racines par l`induction du *2.4-D*. Ces bactéries possèdent aussi un effet positive sur l`augmentation du rendement qui se traduit par le poids de mille graines des plantes inoculées.
Mots clés : *Azospirillum brasilense, blé dur (Triticum durum Desf.), Paranodules, Rendement.*

Abstract

Several studies have shown that PGPR have beneficial effects on plants, these rhizobacteria may provide the plant with nitrogen as ammonium by their associations with legumes and some grasses. The strains of *Azospirillum brasilense* isolated before in rhizosphere of durum wheat, these strains can fix nitrogen improved by nitrogen content in leaves and total chlorophyll. The strains can colonized the durum wheat roots (*Triticum durum* Desf.) inocultated by formation of para-nodules in roots induced by *2.4-D*.These bacteria have also effect to improve growth yield these result was obtained by determination of thousand seeds weight.
Keywords: *Azospirillum, Durum wheat (Triticum durum Desf.), Para-nodules, Yield.*

ملخص

العديد من الدراسات قد أظهرت أن PGPR تكون لها آثار مفيدة على النباتات، قد تكون هذه بكتيريا الجذور توفير محطة مع النيتروجين والأمونيا من قبل جمعياتهم مع البقوليات و بعض الأعشاب.
PGPR(نمو النبات تعزيز بكتيريا الجذور) وتشمل عدة *Azospirillum* جنسا، عصية، و هذه البكتيريا تكون لها آثار مفيدة كثيرة على النبات المضيف: التثبيت الحيوي للنيتروجين، العود ضد مسببات الأمراض، وهرمونات النمو الاصطناعية.
تم عزل سلالات بكتيرية من ريزوسفير من القمح القاسي نمت في مجالات عدة مناطق في شرق الجزائر. تمت دراسة الاستعمار عن طريق حفز تشكيل شبه عقيدات على جذور القمح القاسي استخدام 2،4-D.
في محطات المعالجة مع2،4-D تم العثور على عدد من شبه العقيدات لكل مصنع. ومع ذلك، ومحطات التحكم (دون تلقيح)، أي تغيير في جذورها. وسيتم استغلال التفاعلات النباتية ميكروب لتحسين الإنتاج المستدام من القمح (القمح القاسي).
الكلمات المفتاحية: *Azospirillum* ، القمح القاسي. شبه العقيدات.

Diazotrophic bacteria are important in agriculture, they are known to improve the soil and increase crop productivity. Many soil bacteria have demonstrated a potential to promote plant growth and increase yields of plants. It is now well known that the use of different Diazotrophic bacteria (*Azospirillum brasilense*, *Azospirillum lipoferum*, *Azotobacter chroocaccum, Bacillus sp*., etc.), which were isolated from the rhizosphere of wheat plant can improve plant growth [1]. The production of plant hormones by *Azotobacter* and *Azospirillum* as well as changes in plant growth and development were observed [2]. In recent years, several reports have appeared describing attempts to induce the formation of new growth referred to as paranodules on the roots of several non-legume plants including rice, wheat, barley and rape seed [3, 4, 5].

Species of the genus *Azospirillum* are known to associate with many crops of agronomic interest. After inoculation, the bacteria absorbed the roots, proliferate at the surface and can then invade and colonize the internal tissues of roots. The factors controlling the colonization of roots by *Azospirillum* species are not yet fully understood, but it was suggested that the polar flagella, surface polysaccharides and protein lectins are involved in the process [6].

These bacteria are interesting because, first they can assimilated atmospheric nitrogen and thereby contribute to the nitrogen nutrition of plants, on the other hand, they promote the absorption of nutrients by plants and create favorable conditions for protection of the root system of pathogenic bacteria, and third, they help regulate the flow of nutrients and production of phytohormones [7]. Of the five major classes of hormones, indole acetic acid (IAA), gibberellic acid (GA3), kinetin, abscisic acid and ethylene, known *Azospirillum* produce the first three types of plant hormones. However, little information is available on the system, the nature and extent of the production of phytohormones in terms of qualitative and quantitative [6]. One of the important problems of modern microbiology is the improved supply of non-leguminous plants with available nitrogen using soil nitrogen-fixing microorganisms [8].

The artificial formation of nodules on the roots of non leguminous plants (such nodules are known as para nodules) can be induced by auxin-like substances (such as 2,4-dichlorophenoxy acetic acid) [9, 10, 11] using such an approach , paranodulation was induced in wheat, rice, maize, and rape plants[11, 12].

Studies by several researchers showed that adding small amounts of synthetic auxins such as 2,4 - dichlorophenoxyacetic acid (2,4D) may increase colonization and nitrogen fixation of Azospirillum, coinciding with pattern formation in lateral roots of modified para-nodules [2, 13, 14]. For this reason, the application of phytohormones microbiologically produced by rhizobacteria that promote plant growth (PGPR) or synthetic growth regulators and often recommended to improve the quality of performance, to change the processes of plant life [6].

MATERIALS AND METHODS

Sterilization, inoculation and germination of seeds of durum wheat: A variety of wheat (*Triticum durum*) was chosen: GTA / DUR. The seeds of durum wheat were sterilized first in 70% ethanol for 20 seconds, then emerged in a solution of 30% sodium hypochlorite for 30 minutes followed by several rinses with sterile distilled water. Finally, place the sterile seeds in sterile dishes over sterile filter, and put these boxes in sterile conditions in darkness 48 hours. After germination, the seeds are inoculated with a bacterial suspension (3 strains selected: Azo1, Azo2, Azo3) one way to put 1 ml for each seed, and leave overnight at room temperature.

Induction of para-nodules : After the phase of inoculation, place the seeds in jug containing a mixture of sterile soil (1/3soil, 1/3compost,1/3 sand) and add a solution containing n-free liquid medium (10g malat, 1g KH_2PO_4, 0.2g $MgSO_4$ $7H_2O$, 0.2g Nacl, 0.005g $FeSO_4$ $7H_2O$, 5g Mannitol, 100mg yeast extract [15]and the concentration of 2,4-D, and uninoculated seeds are irrigated also with n-free liquid medium. The test and place in the growth chamber under conditions controlled (t: 25±3°C, 16H photoperiod and humidity 30%) and monitored for 1 month and a half (45 days) to obtain the para-nodules.

The rate of total chlorophyll: Measuring the chlorophyll content of leaves was performed with a Minolta SPAD 502 chlorophyll meter. This manual is a radiometer whose measure is a ratio of leaf reflectance in the red (650nm) and near infrared (940nm). This amounts to a vegetation index measurement of the nitrogen status and leaf chlorophyll [16].

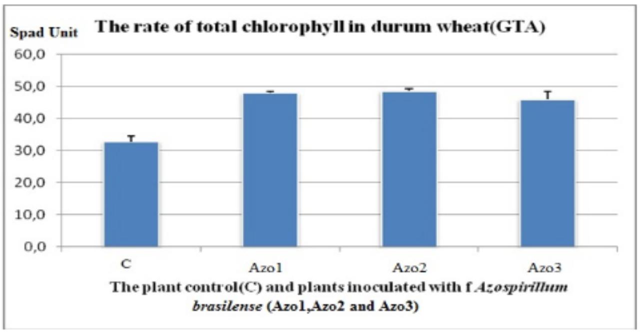
Determination of nitrogen content in leaves of durum wheat: Total leaves nitrogen determination was done using kjeldahl method for soil and plant analysis as described by Sertsu and Bekele (2000) [17]. Dried leaves samples from each pot were milled into powder using a coffee grinder and sieved through 0.5 mm sieve. From each sample 0.2 g was weighed and subjected to Kjeldahl digestion for the determination of total nitrogen.

Microscopy : Observe the morphology of the nodules and the location of bacteria, why the nodules of plants inoculated cultures were harvested after 45 days of growth oriented and then included in the agarose to 30%. The microscopic sections are performed in Vibratome to a thickness of 30 microns and then stained with Syto 13 (diluted 1 / 1000) for 15 minutes followed by rinsing with

distilled water for 15 minutes. The stained sections were mounted between slide and coverslip in PBS 10% glycerol (phosphate buffered saline). A first observation is carried out cuts macroscope (white light or epifluorescence) to detect cuts interest. Then microscopy (epifluorescence) is used for imaging of our cut. This part was realized in LSTM laboratory in Montpellier, France.

RESULTS AND DISCUSSION

The rate of total chlorophyll: Total chlorophyll content of plants of durum wheat (GTA) inoculated with *Azospirillum brasilense* (3 strains: Azo1, Azo2 and Azo3) and the uninoculated was presented in Histogram 1.

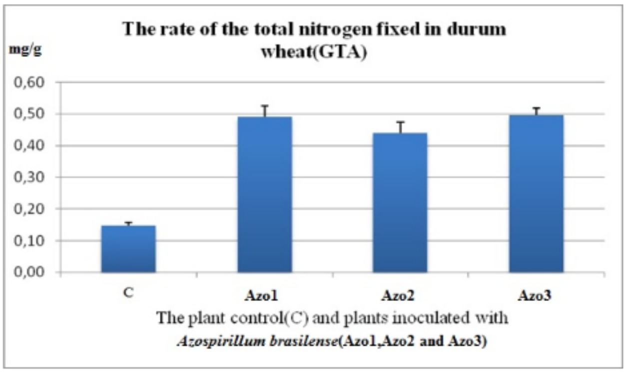


Histogram 1: Total chlorophyll content of durum wheat plant uninoculated (C) and durum wheat plant inoculated with strains *Azospirillum brasilense* (Azo1, Azo2 and Azo3)

The results obtained are different; we note that chlorophyll content is higher in plants inoculated with *Azospirillum*. Note that the highest content was recorded in plants inoculated with strain Azo2 of *Azospirillum brasilense*, followed by plants inoculated with the strain Azo3, and a plant inoculated with strains Azo1 them or less the same level as other plants inoculate. However, the minimum content was recorded in plants controls (controls without inoculation).

Determination of nitrogen content in leaves of durum wheat

The total nitrogen fixation by different parameters (uninoculated plants, plants inoculated with 3 strains (Azo1, Azo2 and Azo3) of *Azospirillum brasilense* was estimated by the Kjeldahl method. The results obtained are presented in Histogram 2. The content of total nitrogen fixation varies between 0.15 mg and 0.50 mg, whichever is higher of the fixed nitrogen was obtained in plants inoculated with strains Azo3, followed by plants inoculated with strain Azo2 and plants inoculated with strain Azo1. However, uninoculated plants and the nitrogen calculated low.



Histogram 2: The total nitrogen content of durum wheat plant uninoculated (C) and durum wheat plant inoculated with strains *Azospirillum brasilense* (Azo1, Azo2 and Azo3)

Effect of isolates on the growth of wheat (*Triticum durum* var: GTA / DUR) and training of para-nodules: The effect of selected isolates (Azo1, and Azo2 Azo3) on growth of wheat (*Triticum durum* var: GTA / DUR) was studied in plants grown for 6 weeks. Different concentrations of 2,4-D were tested to determine which produced an optimal number of para-nodules.

Effect of concentration of 2,4-D and *Azospirillum* inoculation on the growth and number of para-nodules on plant roots: Results in Table 1 show that treatment of seedlings with 2,4-D influenced the growth of plant shoots and roots and induced formation of para-nodules. Root elongation and lateral root formation was compared with control plants. Application of Azospirillum significantly enhanced shoot and root weights as well as plant height. It was also noted that better results were obtained by adding 2,4-D after the root systems had reached a length of about 5cm to allow enough space for para-nodules induction [18]. Inoculation of cereals with *Azospirillum* increased the total dry weight of the plant, the amount of nitrogen, the number of seeds per spike, seed weight and the surface of the sheet, with a high germination rate. Azospirillum also affects root growth of wheat and increased yield. Bashan and Levanony raporter that these bacteria have a direct influence on root length, number of secondary roots and increase the elongation zone and root volume and dry weight.

In plants treated with 2.4 D, a variation in the number of para-nodules per plant was found; these results are similar to those reported by Zeman et *al* (1992) [18]. This training of para-nodules was described in the literature. These were found in a medium containing low concentration of 2.4D [7, 9, 19].Wheat after inoculation with Sp7 and *A.brasilense A.brasilense* Sp7.S mutant who is deprived of the synthesis of polysaccharides, they observed that all *A.brasilense* Sp7colonisé the root surface, whereas treatment with strains mutant shows an accumulation of bacteria only on the sides of the roots [20]. All of the strains

Table 1: Effect of inoculation with *Azospirillum brasilense* on the different parameters durum wheat plant treated with 2, 4-dichlorophenoxyacetate (2, 4-D)

Treatments	Plant length (cm)*	Plant weight (g)	Root length (cm)	Root weight (g)	Thousand seeds weight (g)
Control+2,4-D	73.554±0.826	1.720±0.040	36.000±0.500	0.560±0.020	64.483±0.172
Plants inoculated S1+2,4-D	82.500±0.500	1.917±0.015	39.033±0.550	0.630±0.020	64.840±0.176
Plants inoculated S2+2,4-D	85.000±0.500	2.047±0.054	40.533±0.208	0.683±0.011	65.490±0.738
Plants inoculated S3+2,4-D	83.667±0.577	1.963±0.028	41.800±0.264	0.736±0.025	68.026±0.309

***the average of 3 replication**

examined in this work colonized both the crevices surrounding the sites of the lateral root emergence and those crevices associated with the para-nodules appearing in 2, 4-D treated roots.

Pereg-Gerk *et al.*(2000) [21] found that the strains of *Azospirillum brasilense*, colonized both the crevices surrounding the sites of lateral root emergence in wheat and those crevices associated with the paranodules appearing after 2,4-dichlorophenoxyacetic acid (2,4-D) treatment. However, the colonization of paranodules was more extensive than that of lateral root emergence sites in plants that were not treated with 2,4-D [22].

Azospirillum is another much studies diazotroph especially the species *Lipoferum* and *brasilense*, which have been shown to infect a number of cereal plants including wheat, maize and sorghum [23, 24, 25, 26, 27, 28, 29].

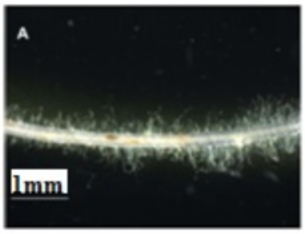
In general, these diazotroph are reported to improve root growth and function, often leading to increased uptake of water and mineral nutrients. Plant inoculation with *Azospirillum brasilence*, for example, promoted greater uptake of NO_3, K and H_2PO_4 in corn, sorghum, wheat and setaria [30, 31, 32, 33].

Initially, the treated plants and plants inoculated and treated differ in root. Infection of *Azospirillum sp* on durum wheat results in a poorly developed root system: the roots are short and few or no hairs with para-nodules protruding round distributed at a regular interval along the roots. The roots of wheat infested Azo1 are shorter and we note the absence of hairs (Fig1 B1 and B2) the same is observed with Azo2 (Fig1 d1 and d2).

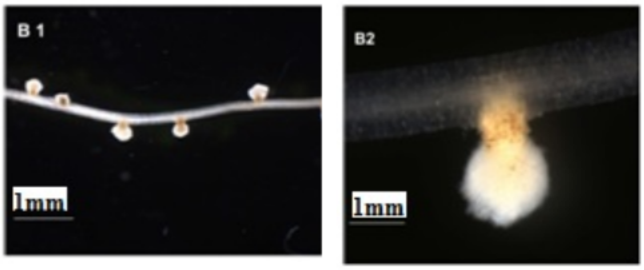
Instead, the root systems invaded by Azo3 has a large number of root hairs (Fig1:C1 and C2). Azo2 As for these para-nodules was larger and well distributed on a regular basis throughout the roots. In the case of control treated with the 2.4D (concentration 0.4μg/ml) and not inoculated, there is a significant formation of root hairs. Similar results were found by Katupitiya and collaborators (1995) [8] with the mutant Sp7.S compared to wild-Sp7.De over an observable effect on the development of the morphology of the roots of wheat (*Triticum durum* var: GTA / DUR) resulted in a decrease in root length, but an increase in root hairs.

This effect was achieved in the case of inoculation of germinated seeds of durum wheat with selected bacterial strains (Azo1, Azo2 and Azo3) and treatment with 2.4D (concentration 0.4μg/ml).

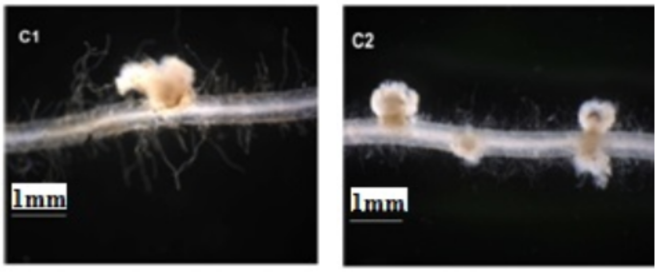
The 2.4D is known to facilitate the entry of bacteria into the lateral roots of the plant, causing the formation of round structures. However, plants control (without inoculation) shows no change in their roots. This was observed visually with a dissecting microscope (Fig1). We also noticed that some bacteria are more efficient in terms of inoculation and training para-nodules (Azo2), was observed by the obtaining of large structures (para-nodules) (Fig1,d3).



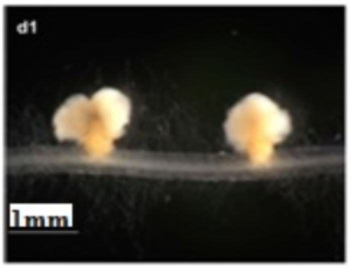
A: Control

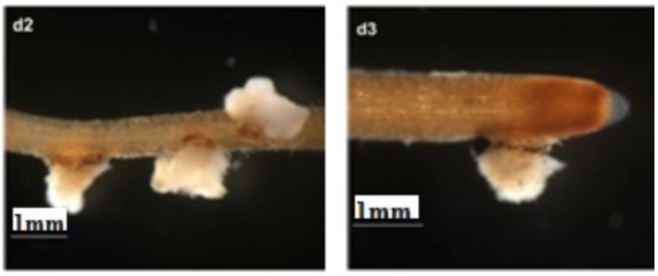


(B1, B2) durum wheat inoculated with Azo1+2.4 D



(C1, C2) durum wheat inoculated with Azo3





(d1, d2, d3) durum wheat inoculated with Azo2

Figure 1: Para-nodules in roots induced by the report of 2.4-D on durum wheat (*Triticum durum*) inoculated with *Azospirillum* brasilense (Azo1, Azo2 and Azo3) observed under a binocular (×40)

It is known that it is the root elongation zone that primarily responds to the action of auxins by changing the proprieties of the cells of epidermis, cortical, parenchyma, endodermis, pericycle, and the central vascular cylinder [14]. Light microscopic studies revealed that *Azospirillum brasilense* successfully colonized para-nodules, externally both their basal connection with the root and on the epidermal root surfaces. This may lead to the penetration of bacteria to conducting plants tissues and eventually to their colonization by the bacteria. In the other words, the type of nodulating agent influenced not only the formation time of paranodules and their morphology but also the distribution of microorganisms in the plant tissues (Fig 2, Fig 3).

However, if seeds or seedlings can be effectively inoculated in growing field crops such as wheat, it may not be important for the bacteria to survive in the soil during the prolonged periods between crops. In this case, the development of inoculation techniques that allow sufficient exposure of the roots to *Azospirillum* strains would be necessary [21]. Efforts at extending N_2 fixing ability to important non-leguminous crops such as cereals has long been a major goal of workers in the field of biological nitrogen fixation

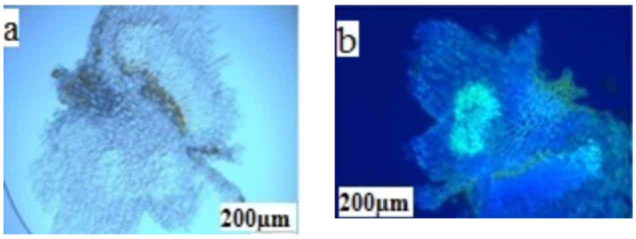


Figure2: Nodules sections (30µm) of durum wheat observed under the microscope without staining (a) and (b) with color (syto13) (×100)

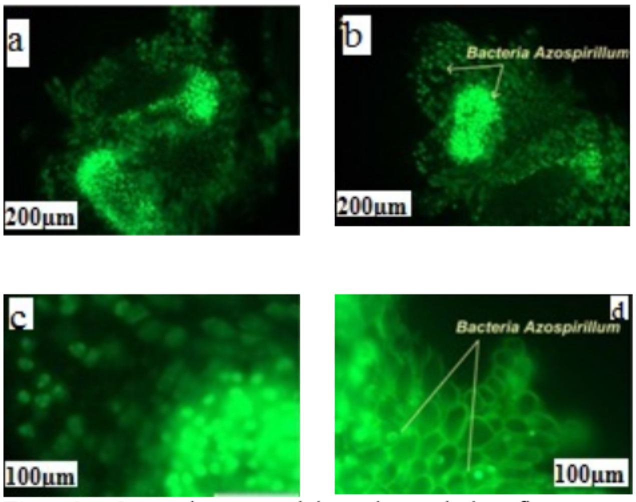


Figure3: Cups durum nodules observed by fluorescence microscopy(a and b) (×100) and (c and d) (×2000)

CONCLUSION

The probability of an effective association of grain appears to be low at present. However, further studies on these relationships will promote the practical application of paranodules for improving the nitrogen nutrition of cereals. In the other word, intensification of research on the interaction microorganism's plants using labeled bacteria is required to follow the path of the bacteria in the para-nodules.

Tools such as genetic analysis by PCR-based markers for the study of parameters that can provide insight into the evolution of symbiotic systems in cereals (wheat). This knowledge will aid in the development of technology that will exploit a favorable plant-bacterial interaction in the production of durum wheat (*Triticum durum* Desf.).

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