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Theme

In planta and *In vitro* assessment of certain bacterial strains on the germination and growth of some plant species

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What Allah chooses for us is always the best
To the One and Only, who was with us every step of the way
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To the light of my life,
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Sarah and Wafa
***And to their husbands** Morad and Mostafa*
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the one with the most beautiful smile
may God preserve it for you.
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to those who generously shared their sincere feelings and heartfelt advice
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I dedicate this achievement and the fruit of my hard work to you.
*All praise is due to Allah for what He has granted me and I pray that He continues to support me and make me a source of **blessing wherever I may be**.*

Dedications

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LOVE U ALL *DOUNIA*

Abstract

This study evaluates the impact of two seed pre-treatment techniques bio-priming with a consortium of plant growth-promoting microorganisms (PGP priming : C1A, C1B, G1B, and B3) and hydro-priming on the germination and growth of *Trifolium subterraneum L*. Results from both *In vitro* and *In planta* assays revealed improved germination rates, with no significant differences between PGP priming and hydro-priming. However, in the *In planta* assay, PGP priming led to a significantly enhanced root growth, along with a notable increase in chlorophyll b and total chlorophyll content. Moreover, a reduction in IC_{50} was observed, indicating greater protection capacity against oxidative stress. These findings confirm the potential of plant growth-promoting rhizobacteria (PGPR) to enhance the physiological performance of *Trifolium subterraneum*. This potential could be harnessed for the development of microorganism-based biostimulants.

Keywords : PGP priming, Hydro-priming, *Trifolium subterraneum* L., germination, growth.

Résumé

Cette étude évalue l'impact de deux techniques de prétraitement des semences le Bio-amorçage avec un consortium à base de micro-organismes promoteurs de croissance (PGP priming : C1A, C1B, G1B et B3) et l'hydro-priming sur la germination et la croissance de *Trifolium subterraneum* L. Les résultats des essais *In vitro* et *In planta* ont révélé une amélioration des taux de germination, sans différence significative entre le PGP priming et l'hydro-amorçage. En revanche, dans l'essai *In planta*, le PGP priming a entraîné une croissance racinaire significativement accrue, accompagnée d'une augmentation notable des teneurs en chlorophylle b et en chlorophylle totale. De plus, une diminution de l'IC_{50} a été observée, indiquant une meilleure capacité de protection contre le stress oxydatif. Ces résultats confirment le potentiel des rhizobactéries promotrices de croissance (PGPR) pour améliorer les performances physiologiques de *Trifolium subterraneum*. Ce potentiel pourrait être exploité dans le développement de biostimulants à base de micro-organismes.

Mots clés : PGP priming, Hydro-priming, *Trifolium subterraneum* L., germination, croissance.

الملخص

تهدف هذه الدراسة إلى تقييم تأثير تقنيتين لمعالجة البذور قبل الزراعة على إنبات ونمو *Trifolium subterraneum* L. ، وهما: المعاملة الحيوية بالبكتيريا المحفزة للنمو النباتي (PGP priming: السلالات C1A ، C1B، G1B وB3)، والمعاملة بالماء (hydro-priming). تم إجراء تجارب *In vitro* و *In planta*. أظهرت نتائج الاختبارات المخبرية وداخل النبات تحسناً في معدلات الإنبات، دون وجود فروق معنوية بين المعالجة الحيوية والمعالجة المائية. من جهة أخرى، أدت المعالجة الحيوية *In planta* الى تعزيز كبير في نمو الجذور وزيادة ملحوظة في مستويات الكلوروفيل b والكلوروفيل الكلي. أسفرت المعاملة الحيوية عن انخفاض أكبر في قيمة IC_{50} ، مما يعكس قدرة وقائية أفضل ضد الإجهاد التأكسدي. تسلط هذه النتائج الضوء على الإمكانيات الواعدة للبكتيريا المحفزة لنمو النبات (PGPR) في تحسين الأداء الفسيولوجي لنبات *T. subterraneum*، وامكانية الاستفادة منها في تطوير محسنات نمو نباتية (biostimulants) قائمة على الكائنات الحية الدقيقة.

الكلمات المفتاحية

المعاملة الحيوية، المعاملة المائية، *Trifolium subterraneum* L.، الإنبات، النمو، البكتيريا المحفزة لنمو النبات.

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1. Introduction

Agricultural productivity currently faces significant challenges in meeting the escalating food demand driven by a continuously growing global population. These challenges arise not only from the decline in cultivable land but also from adverse climate change (Arif *et al.*, 2020). According to estimates by the Food and Agriculture Organization of the United Nations (FAO), the world's population is projected to reach 8.6 billion by 2030 and 9.8 billion by 2050 (Goswami & Deka, 2020).Consequently, it is estimated that agricultural productivity must increase by 60% by 2050 to meet this demand(Arif *et al.*, 2020) .In this context, enhancing the physiological quality of seeds represents a crucial strategic approach to improving agronomic performance and securing sustainable crop yields. Achieving rapid and uniform seedling emergence is a key factor in crop performance. This concern dates back to antiquity, as evidenced by the writings of the ancient Greeks (Evenari, 1984).Germination, the critical first phase of the plant life cycle, determines subsequent growth and the plant's ability to adapt to environmental factors (Donohue *et al.*, 2000 ; Kırmızı, 2023). It is also of crucial importance for the ex-situ conservation of plant genetic resources (Flores *et al.*, 2008) and for understanding the evolutionary strategies of plant species. Slow or irregular germination exposes seedlings to unfavorable abiotic conditions, reducing their survival rate and hindering their development (Osburn *et al.*, 1989). Seed priming is the artificial treatment of seeds with natural or synthetic substances, which enables them to reach a specific physiological state prior to germination (Jisha *et al.*, 2013). Seed priming provides several benefits, including breaking seed dormancy,promoting uniform emergence, and increasing yields (Bruggink *et al.*, 1999 ; Fu *et al.*, 2024).Widely used across crops such as cereals, legumes, and vegetables, seed priming has significantly contributed to enhanced agricultural productivity (Fu *et al.*, 2024 ; Giri *et al.*, 2024; Rashid *et al.*, 2006).Furthermore, seed priming contributes to stress tolerance in crops. By activating stress-responsive genes and metabolic pathways, primed seeds exhibit increased resilience to environmental stresses such as drought, salinity, and temperature fluctuations (Giri *et al.*, 2024). Various approaches to seed priming such as hydro-priming, osmo-priming, nano-priming, bio-priming, phytohormone priming, matrix-priming, halo-priming, and micronutrient seed priming (Nutri-priming), are widely applied and have been extensively researched. Each crop requires specific and optimized priming technique (Pawar *et al.*, 2018).

Plants and microorganisms are believed to have co-evolved continuously, forming a complex and dynamic ecosystem in which they significantly influence each other's growth and survival. Beneficial interactions between plants and soil microorganisms, notably "Plant Growth-Promoting Rhizobacteria. PGPR are common soil-dwelling microorganisms that form mutualistic relationships with host plants, providing benefits such as improved plant vitality, increased nutrient availability, and resistance to pathogens (Das *et al*., 2022).Plant growth-promoting rhizobacteria (PGPR) have garnered considerable attention for their potential to enhance plant growth, improve soil health, and sustainably increase crop yields. Utilizing PGPR can reduce reliance on chemical fertilizers and pesticides, offering an eco-friendly alternative to traditional agricultural practices (Gupta *et al*., 2024).PGPR enhances plant growth through several biochemical pathways, such as phytohormones production (auxins, gibberellic acid, abscisic acid, cytokinins, and ethylene), solubilizing plant-inaccessible phosphate, nitrogen fixation, siderophore production, and antibiotics. Collectively, these biochemical pathways contribute to improved nutrient uptake, plant growth, and stress tolerance, underscoring the importance of PGPR in sustainable agriculture (Wahab *et al*., 2024). Bio-priming is a seed treatment technique that involves pre-soaking and simultaneous inoculation with beneficial microorganisms. In recent years, this approach has gained increasing attention and is now recognized as a promising component of the emerging "fresh green" revolution, which emphasizes sustainable and environmentally friendly agricultural practices (Fu *et al*., 2024 ; Lyu *et al*., 2020). Beneficial or plant-growth-promoting microorganisms used for seed priming include *Pseudomonas*, *Actinobacteria*, and *Bacillus*. Inoculated micro-organisms can colonize the plant's inter-root surface and support the plant's physiological growth over a long period ; thus, bio-priming promotes crop maturity (Bennett & Whipps, 2008 ; Fu *et al*., 2024). As other priming methods, bio-priming also intensifies the rate and homogeneity of seed germination, but also protects seeds against the soil and seed-borne pathogens (Fu *et al*., 2024). Subterranean clover "*Trifolium subterraneum* L." (Fabaceae family, sub-family, Papilionoideae), commonly known as burial clover or sowing clover, is a self-pollinating winter annual legume characterized by its hard seeds and its capacity to bury the reproductive structures in the soil ensuring thus, its own regeneration by self-seedling (Masson, 1997). Subterranean clover is highly valued for livestock, grains production, as a source of high-quality forage, and for its ability to fix atmospheric nitrogen (Nichols *et al*., 2013).

Like other legumes, *Trifolium subterraneum* seeds may exhibit high hardness (generally over 90%) and/or post-harvest embryonic dormancy of varying duration or both, which hinders their germination.

To ensure effective conservation of this agronomically valuable plant resource and promote its integration into sustainable agriculture, in particular for livestock improvement, it is necessary to employ biotechnological approaches to overcome these limitations. The aim of this study is to evaluate the effect of a consortium of four rhizobacteria promoting plant growth from the rhizosphere (PGPR) to enhance both germination rate and growth parameters of *Trifolium subterraneum* in Algeria.

2. Materials and methods

2.1. Biological materials

This study employed three *Actinobacteria* isolates (designated G1B, C3A, and C1B) and one *Bacillus* strain (B3), generously provided by Fares Ramila, a PhD student in Plant Biotechnology at Abbas Laghrour University, Khenchela, in January 2024 (Fig. 1). These isolates were originally sourced from semi-arid soils surrounding Sebkhat Izmoul, located in the Ain Mlila region of Oum El Bouaghi Province. The initial isolation and characterization of these strains were carried out by (Fares and Asma , 2017) at the Laboratory of Mycology and Antibacterial Activity, University of Constantine 1. The plant growth-promoting (PGP) characteristics of these bacterial strains were determined in January 2025 and are presented in Table 1.

Table 1: Plant Growth-Promoting (PGP) characteristics of *Actinobacteria* and *Bacillus* strains

Strain	**HCN Hydrocynic acid**	**P- solubilization (μgmL^{-1})**	**IAA production (μgmL^{-1})**	**G3A Gibberillic acid**
G1B	++	35,29	10.33	++
C3A	++	32,13	13,30	++
C1B	++	30,68	12,84	+
B3	+++	37,68	15,11	+++

(+) production in normal level (++) production in medium level, (+ + +) production in high level, (-) negative for test

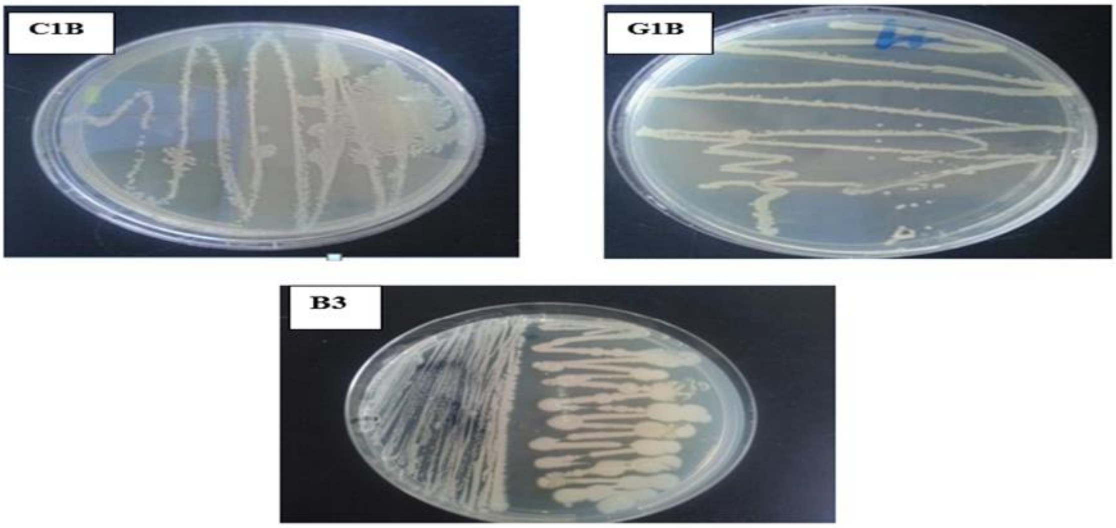

Figure 1. Macroscopic aspect of bacterial isolates.

2.2. Plant material

Seeds of *Trifolium subterraneum* used in this study (Fig. 2) were collected in July 2010 from the Souk Ahras region, located in North-East Algeria. The collection was carried out by Ms. ISSOLAH Rachida, a researcher in the plant genetic resources division (National Institute of Agronomic Research of Algeria (INRAA).

Figure 2. Samples of the studied species *Trifolium subterraneum* L.

2.3 Methods

2. 3.1. Preparation of the bacterial suspension

Each bacterial strain was individually cultured in liquid broth under continuous agitation at 30°C for 24 hours to reach the exponential growth phase, optimal for inoculation. Following incubation, equal volumes (3 mL) of each of the four strains were aseptically combined to prepare a mixed bacterial suspension. The final volume of the suspension was adjusted according to the seed quantity and size to ensure complete immersion and uniform exposure during inoculation (Fig. 3).

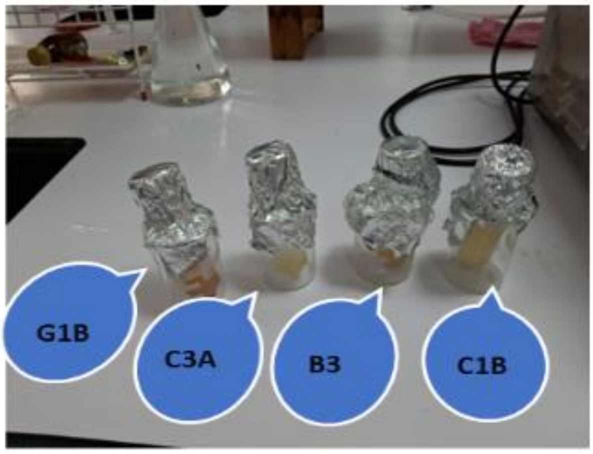

Figure 3. Preparation of the mixed bacterial suspension using four PGPR strains (C1B, C3A, G1B, and B3).

2.3.2. Seeds sterilization and priming

Seeds were surface-sterilized by immersing them in 70% ethanol for 1 minute, followed by treatment with a 20% (v/v) sodium hypochlorite solution for 15 to 20 minutes. After sterilization, seeds were rinsed thoroughly three times with sterile distilled water to remove any residual sterilizing agents. The seeds were then air-dried under a laminar flow hood for a minimum of 3 hours. Three groups were established for the experiment: the first group, a control, consisted of seeds without any treatment; the second group underwent hydropriming, consisting of seeds soaked in warm sterile water for 1 hour; and the third group involved seeds inoculated with a mixed bacterial consortium (Fig.4). For the inoculated group, surface-sterilized seeds were immersed in the bacterial suspension and subjected to agitation for 1 hour to promote effective seed-bacteria interaction (Costa-Gutierrez *et al*., 2020). All seeds, both control and treated, were divided into two groups in order to perform the in vivo and in planta tests.

Figure 4. Seed priming of *T. subterraneum* with bacterial consortium (PGP priming) and water Hydropriming).

2.3.3. *In vitro* assay

Under aseptic conditions in a laminar flow hood, seeds of *Trifolium subterraneum* were carefully transferred and uniformly sown onto Murashige and Skoog (MS) medium (Murashige *et al*., 1962)(Appendix 1), Seedlings were subsequently cultivated under identical conditions in an *In vitro* culture chamber, with a controlled temperature range of 22–25 °C and a 16-hour photoperiod (Fig. 5).

Figure 5. Petri dishes containing MS medium with treated and control seeds in controlled conditions.

2.3.4. *In planta* assay

To assess the effectiveness of the seed priming treatments, an *In planta* assay was performed using sterilized alveolate trays, each disinfected with 70% (v/v) ethanol and filled with sterile soil. One treated seed was sown per cell under aseptic conditions. Seedlings were grown under ambient conditions, with soil moisture carefully maintained using tap water applied via syringe to ensure precise irrigation. This setup minimized contamination risks and enabled accurate evaluation of germination responses to the priming treatments. (Fig. 6).

Figure 6. *In planta* experimental setup for studying seed germination under different treatment.

2.3.5. Measured parameters

2.3.5.1. Germination and seedling emergence

The seed germination rate for the different priming applied treatments was determined as the ratio of the number of germinated seeds to the total number of seeds tested (Come, 1970).

$$\text{Germination (\%)} = \frac{\text{Number of germinated seeds}}{\text{Total number of seeds tested}} \times 100$$

For in vitro assey germination was defined as the initial protrusion of the radicle through the seed coat, whereas seedling emergence corresponded to the visible appearance of the shoot above the surface of the soil.

2.3.5.2. Growth parameters

- **Root length**

  Root lengths were measured using a calibrated caliper.

- **Shoot length**

  Shoot lengths were measured using a calibrated caliper.

- **Fresh weight of root (FW)**

  The fresh weight of the root was measured immediately after harvest using an analytical balance, with precision recorded in milligrams (mg).

- **Fresh weight of shoot (FW)**

  The fresh weight of the shoot was measured immediately after harvest using an analytical balance, with precision recorded in milligrams (mg).

- **Dry weight of shoot**

  The dry weight of the roots (mg) was determined using an analytical balance after drying to a constant weight.

- **Ratio calculation**: Root length/Shoot length, and Root fresh weight/Shoot fresh weight.

2.3.5.3. Chlorophyll extraction and quantification

The concentrations of chlorophyll a (Chl a), chlorophyll b (Chl b), and total chlorophyll (Chl total), were determined following the method described by Arnon & Whatley (1949). Approximately 0.5 for stem tissue from each sample were excised and homogenized in 10 mL of 80% acetone.

The homogenates were kept in the dark at 10°C for 12–24 hours to ensure full pigment extraction (Fig. 7). The absorbance of the resulting supernatant was measured at 663 nm and 645 nm using a spectrophotometer. Chlorophyll concentrations were then calculated using standard equations, and the results were expressed as milligrams per 100 grams of fresh weight (FW^{-1}) (mg 100g FW^{-1}).

$$Chl_a(\text{mg L}^{-1}) = 12.41\ (\text{OD } 663) - 2.59\ (\text{OD } 645)$$

$$Chl_b(\text{mg L}^{-1}) = 22.9\ (\text{OD } 645) - 4.68\ (\text{OD } 663)$$

$$Chl_{tot}(\text{mg L}^{-1}) = Chl_a + Chl_b$$

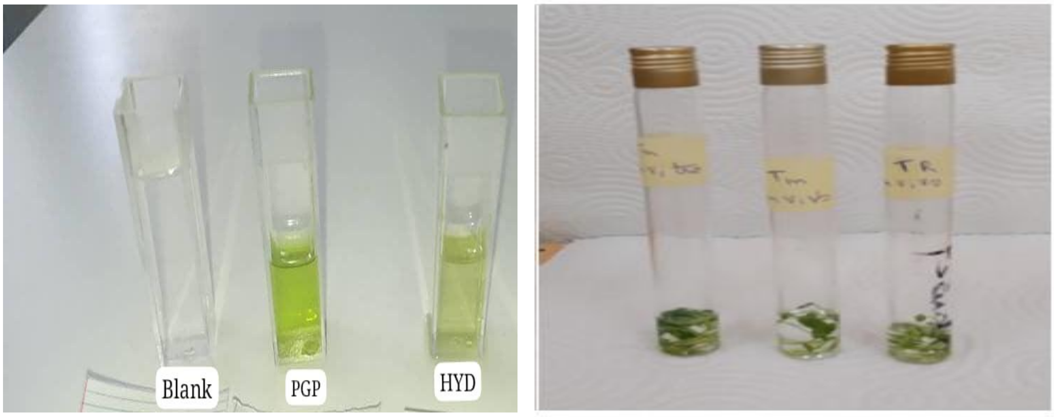

Figure 7. Chlorophyll extraction from *T. subterraneum* leaves.

2.3.5.4. Antioxidant activity

The antioxidant activity of *Trifolium subterraneum* extracts was evaluated using the 2,2-diphenyl-1-picrylhydrazyl (DPPH.) radical scavenging assay. Plant extracts prepared in acetone were tested by mixing 200 µL aliquots with 2 mL of DPPH. solution. The mixtures were incubated in the dark at ambient temperature for a specified duration to prevent photodegradation of the DPPH radical. Absorbance was measured at 517 nm using a UV-Visible spectrophotometer (Pellegrini *et al*., 2018).The percentage inhibition of the DPPH. radical was calculated using the formula:

$$\text{IC50\%} = \left(\frac{A_{control-}A_{sample}}{A_{control}}\right) \times 100$$

where $A_{\mathbf{control}}$ is the absorbance of the DPPH. solution without extract, and $A_{\mathbf{sample}}$ is the absorbance in the presence of plant extract. The IC$_{50}$ value, representing the extract concentration required to scavenge 50% of the DPPH. radicals, was also determined to compare antioxidant potency among treatments (Fig. 8).

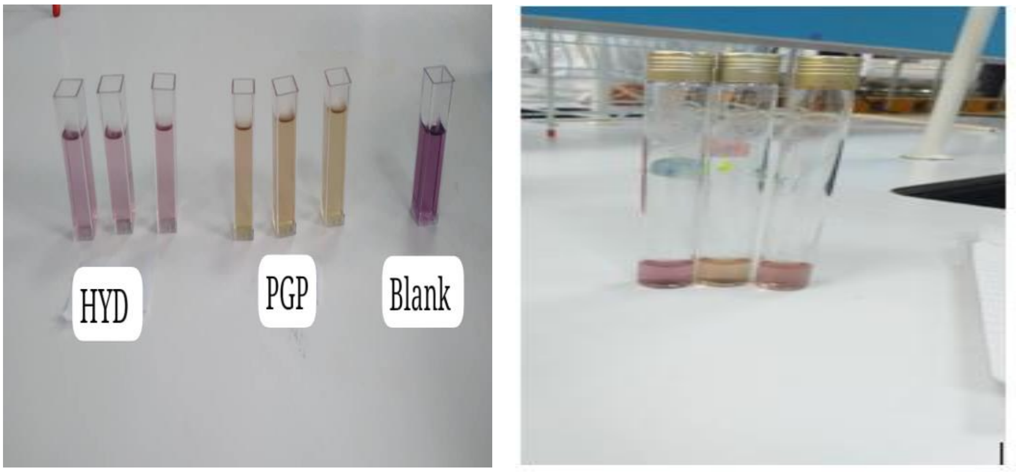

Figure 8. Preparation of *T. subterraneum* extracts for antioxidant activity.

2.4. Statistical analysis

All experimental data are represented as the means of four independent replicates. Germination percentage (*in vitro*) and emergence rate (*in planta*) were subjected to arcsin square root transformation$\sqrt{(X/100)}$. All parameters were analyzed using one-way analysis of variance (ANOVA) at a significance level of $p < 0.05$. When significant differences were observed, Tukey's multiple comparison test was applied to determine statistically homogeneous groups. Statistical analyses were performed using Minitab® version 16.

3. Results

3.1. *In vitro* assay

3.1.1 Percentage of germination (%)

For the *In vitro* test, the germination behavior of *T. subterraneum* seeds was evaluated under controlled conditions, with a focus on microscopic observations to assess the interaction between the bacteria and the roots of the species. Due to contamination in the control samples (CTL), the comparison of germination rates was restricted to the two priming methods, hydro-priming (HYD) and bacterial priming (PGP). The germination percentage results showed similar rates (50%) for both priming methods tested (Fig. 9 and 10). ANOVA analysis revealed no significant difference between the two treatments (p = 0.900) (Appendix), suggesting that both hydropriming and bacterial priming had a similar effect on seed germination.

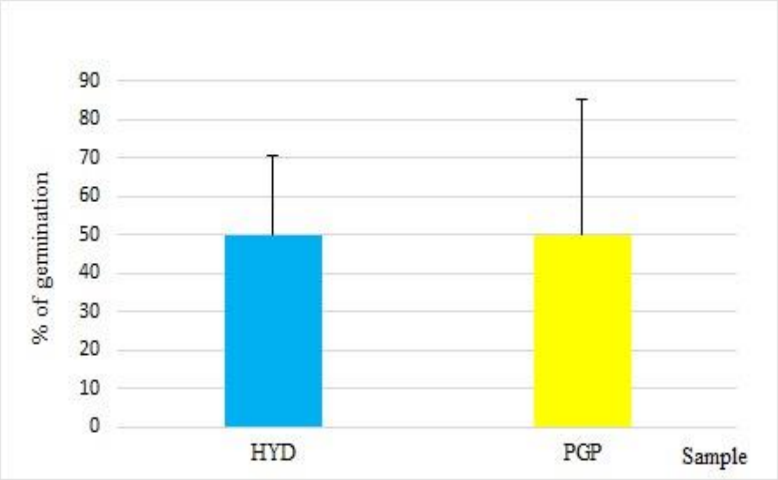

Figure 9. *In vitro* germination rate of *Trifolium subterraneum* seeds under hydropriming (HYD) and bacterial priming (PGP) treatments.

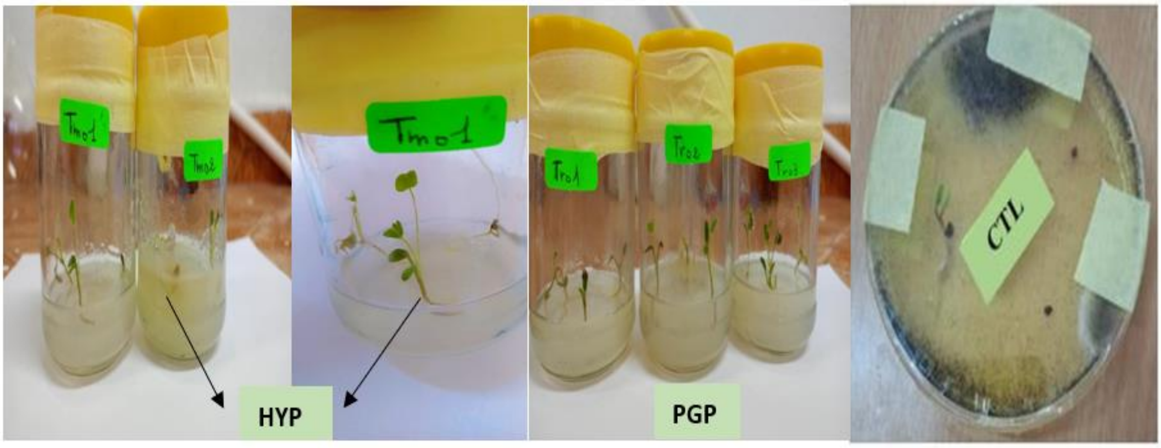

Figure 10. Germination of *Trifolium subterraneum* seeds on MS medium after different priming

Regarding the interaction between the plant roots and the bacterial consortium, the observations made could not reveal this interaction due to the limited resolution of the optical microscop.

3.2. *In planta* assay

3.2.1. Percentage of germination (%)

In this study, the germination of *Trifolium subterraneum* seeds was evaluated under three treatments: control (CTL), hydro-priming (HYD), and bacterial priming (PGP) (Fig.11 and 13). The evaluation was conducted in *in planta* conditions. The germination rates were monitored over a period of 31 days. Regarding to the germination percentage results, the analysis of variance indicated no statistically significant differences among the treatments applied (HYD, PGP, and CTL) (p = 0.848). Nonetheless, notable differences were observed in the emergence patterns. Seeds subjected to priming treatments (HYD and PGP) exhibited earlier emergence, commencing on day 11, whereas emergence in the control group (CTL) began on day 13. Although HYD and PGP treatments promoted a more rapid emergence, the overall germination trajectories remained comparable across all treatments (Fig. 12).

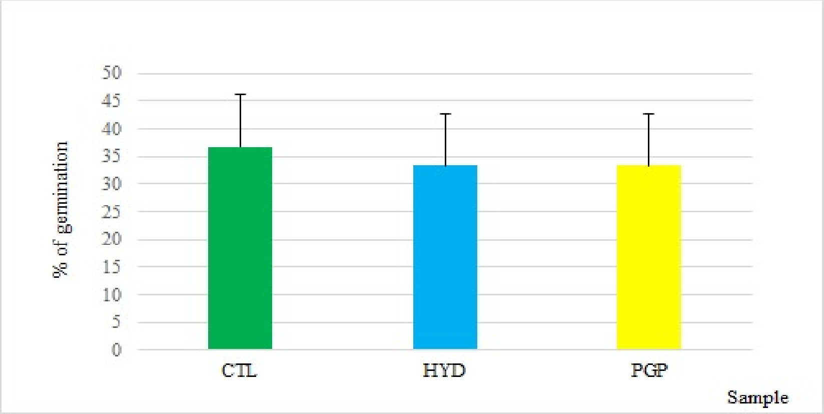

Figure 11. Germination percentage of *Trifolium subterraneum* seeds under CTL, Hydro, and PGP treatments over 31 days.

Figure 12. Germination kinetics of *Trifolium subterraneum* seeds under CTL, HYD, and PGP treatments over 31 days.

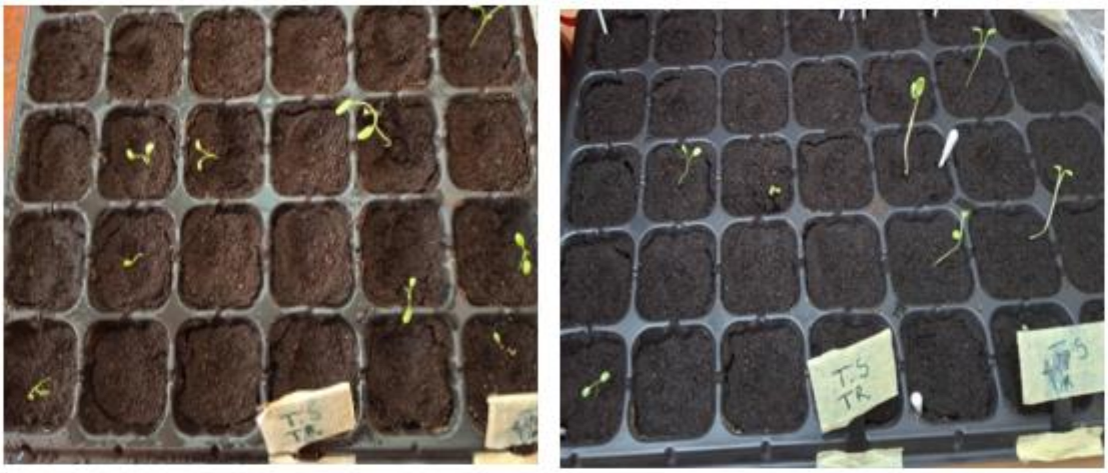

Figure 13. Germination *In planta* of primed *Trifolium subterraneum* seeds.

3.2.2. Shoot and root lengths

As illustrated in Figure 14, bacterial priming (PGP) appeared to enhance root elongation, with an average root length of 26.00 mm, compared to 14.96 mm in hydro-primed seeds. Conversely, shoot length was slightly reduced in the PGP treatment (39.13 mm) relative to hydropriming (45.68 mm). These trends suggest a potential stimulatory effect of bacterial priming on root development. Nonetheless, one-way analysis of variance (ANOVA) indicated that the differences among treatments were not statistically significant for shoot length ($p = 0.434$), root length ($p = 0.109$), or the root-to-shoot length ratio ($p = 0.262$).

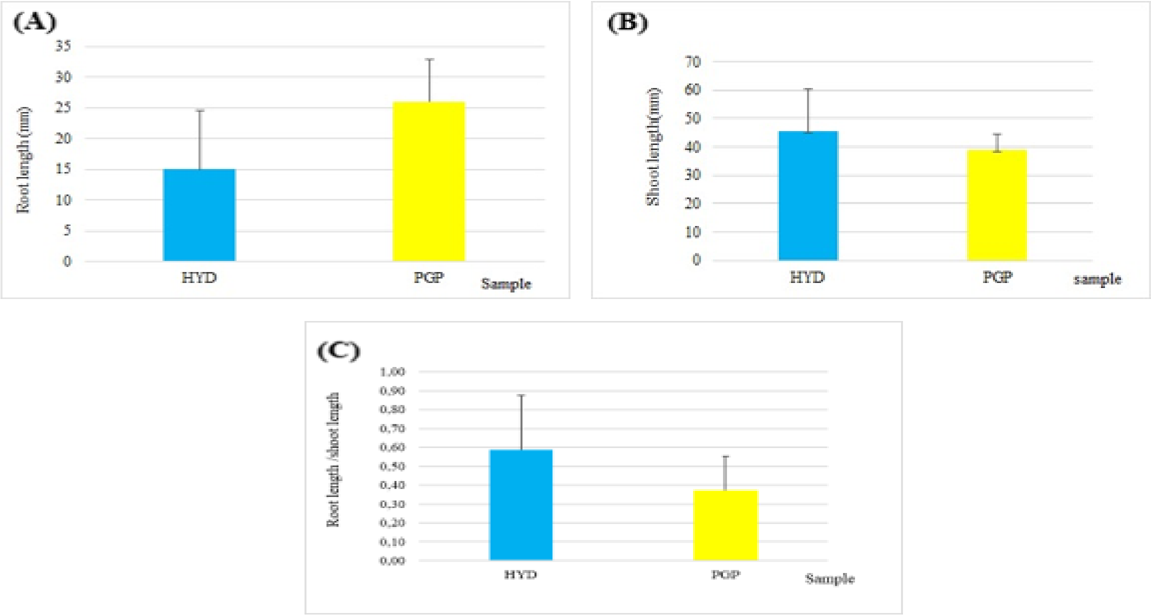

Figure 14. Root length **(A)**, Shoot length **(B),** and root length/shoot length ratio **(C)** of *Trifolium subterraneum* plants grown with hydropriming and bacterial priming (PGP) treatments.

3.2.3. Shoot and root weight

Analysis of variance revealed no significant differences in shoot fresh weight between treatments (p = 0.434). In contrast, root fresh weight was significantly higher in the bacterial priming (PGP) treatment (1.50 mg) compared to hydro-priming (0.418 mg), indicating a strong positive effect of PGP on root biomass accumulation (p = 0.008). Furthermore, the root to shoot fresh weight ratio was markedly higher under PGP treatment (0.056) than under hydro-priming (0.012), suggesting a shift in biomass allocation favoring root development (Fig.15). This difference was also statistically significant (p = 0.017). These findings highlight the potential of bacterial priming to enhance root growth and modify biomass partitioning in favor of the underground part of the plant.

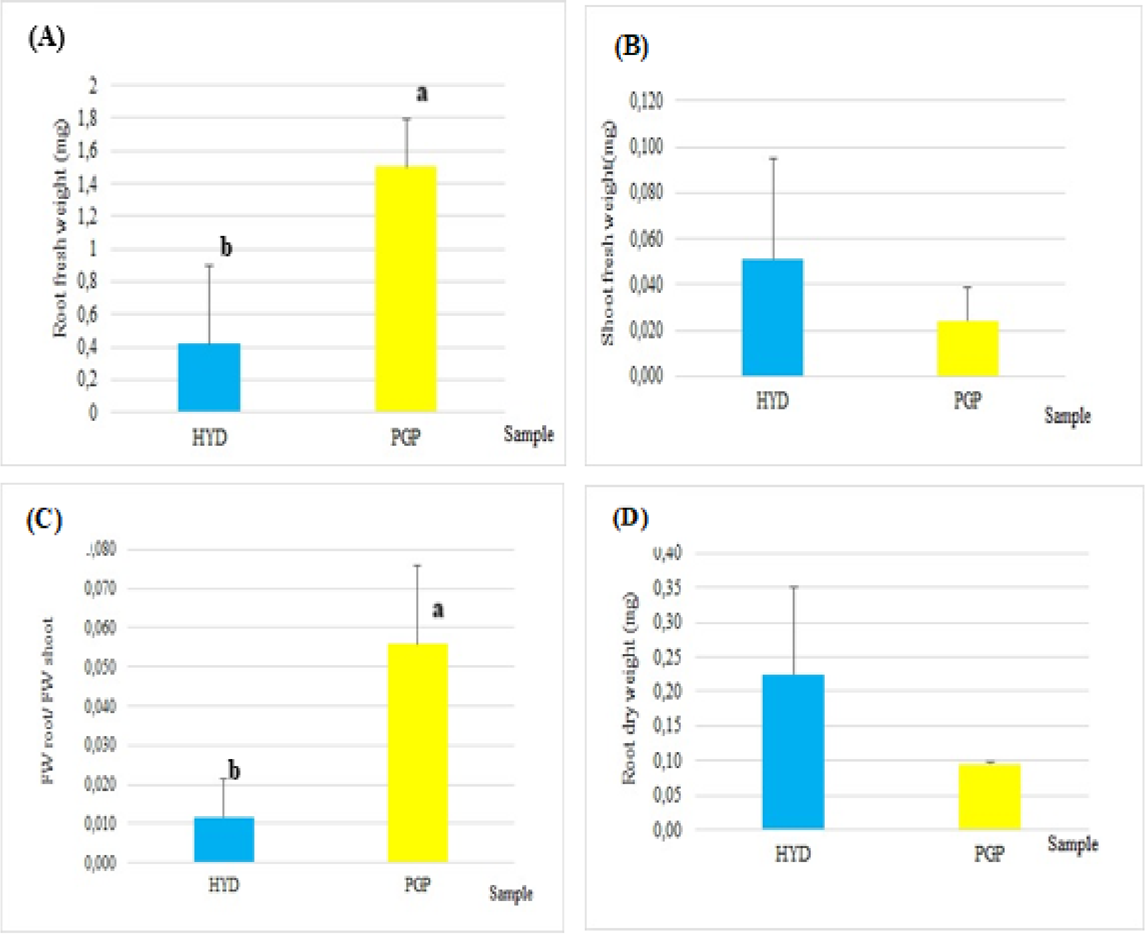

Figure 15. Shoot fresh weight **(A)**, Root fresh weight **(B)**, FW root/FW shoot ratio **(C)**, and Root dry weight **(D)** of *Trifolium subterraneum* plants grown with hydropriming and bacterial priming (PGP) treatments.

3.2.4. Photosynthetic pigment content

Analysis of variance (ANOVA) revealed significant differences between hydropriming (HYD) and bacterial priming (PGP) treatments in terms of chlorophyll a, b, and total chlorophyll content in *Trifolium subterraneum* (Fig.16).Chlorophyll a content was significantly higher in the Hydro treatment (3.08 mg100g FW^{-1}) compared to the PGP treatment (2.10 mg100g FW^{-1}) ($p < 0.05$).Conversely, chlorophyll b content was significantly greater in the PGP treatment (3.52 mg100g FW^{-1}) than in the Hydro treatment (1.06 mg100g FW^{-1}) ($p < 0.001$).Similarly, total chlorophyll content was higher under PGP (5.63 mg100g FW^{-1}) compared to Hydro (4.15 mg100g FW^{-1}) ($p < 0.0001$).Tukey's post-hoc test confirmed that bacterial priming significantly increased chlorophyll b and total chlorophyll contents, while hydropriming favored chlorophyll accumulation. These results suggest that the two priming methods differently influence photosynthetic metabolism by specifically modulating the accumulation of distinct chlorophyll types.

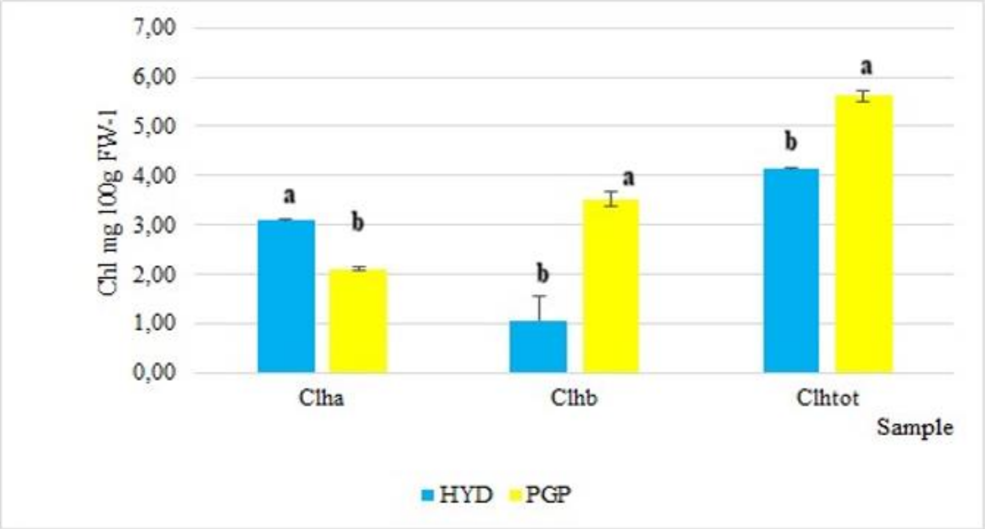

Figure 16. Chlorophyll content in *Trifolium subterraneum* seeds under hydro-priming (HYD) and bacterial priming (PGP) Chlorophyll a (Clha), Chlorophyll b (Clhb), and Total Chlorophyll (Clhtot).

3.2.5. Evaluation of antioxidant activity

The antioxidant activity of *Trifolium subterraneum* seeds was evaluated using the DPPH assay following hydro-priming (HYD) and bacterial priming (PGP) treatments (Fig. 17). Analysis of variance revealed a highly significant difference between the two treatments ($p < 0.001$), as confirmed by Tukey's post-hoc test. Hydro-primed seeds showed a higher IC_{50} value (57.80 µg-mL^{-1}) compared to those treated with plant growth-promoting (PGP) bacteria (48.55 µg-mL^{-1}), indicating lower antioxidant efficiency. However, bacterial priming resulted in a significant reduction in IC_{50}, suggesting enhanced DPPH radical scavenging activity and superior antioxidant potential.

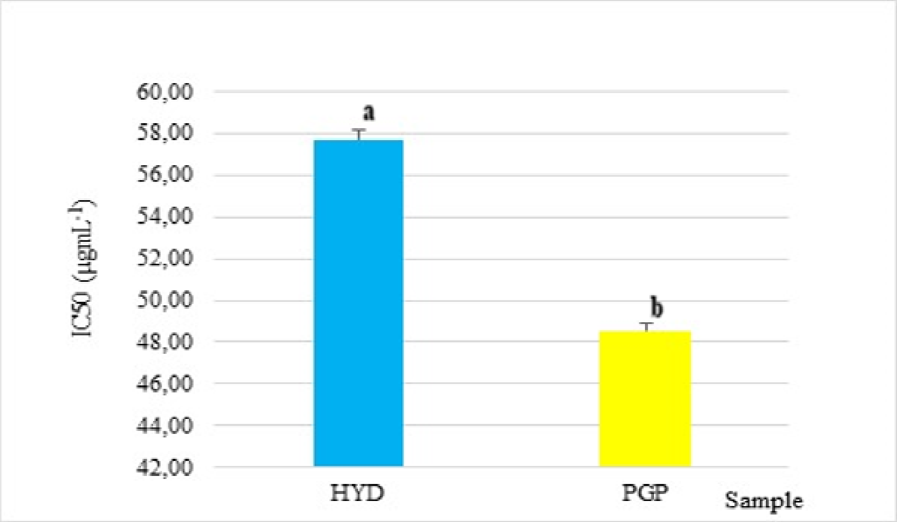

Figure 17. IC50 values of hydro-primed (HYD) and bacterial primed (PGP) *Trifolium subterraneum* seeds.

4. Discussion

In this study, we evaluated the effect of two priming methods (hydro-priming and bacterial priming) on the germination and growth of *Trifolium subterraneum* under controlled (*In vitro*) and natural (*In planta*) conditions. The results obtained from both tests revealed similar, non-significant germination rates between the two pre-treatment methods. However, both PGP and hydropriming influenced the germination rate, with faster germination observed compared to the control seeds. Indeed, numerous studies have demonstrated the positive effects of hydropriming and biopriming during the early stages of growth, resulting in uniform and rapid germination (Pagano *et al*., 2023 ; Tarchoun *et al*., 2024). Similarly, Catiempo *et al*., (2021) and Goufa *et al*., (2025) reported that although seed priming treatments are effective in accelerating seedling emergence and enhancing early growth under stress conditions, they do not always lead to a significant improvement in germination percentage. Regarding plant growth, the results of the present study revealed a significant increase in root fresh weight, root length, and the root-to-shoot fresh weight ratio. Similar findings were reported by Goufa *et al*., (2025) in their study on grass pea varieties, where biopriming with *Bacillus subtilis* led increase in root fresh weight and a notable enhancement in root length under saline stress conditions. According to Singh *et al*., (2016), bio-priming of pea seedlings with *Trichoderma asperellum* resulted in a significant increase in both root length and root fresh weight. In soybean, Miljaković *et al*., (2022), in their sand pot experiment, showed that treatment with *Bacillus megaterium* significantly improved shoot length, root length, dry root weight and seedling robustness index, compared with the control.

For the chlorophyll content, The results of the present study indicate that, while hydropriming primarily promotes chlorophyll a, bacterial priming resulted in a significant increase in chlorophyll b and total chlorophyll, which is consistent with the studies by Purwanto *et al.*, (2021) and (Rotaru *et al.*, 2015), who observed similar effects on other species such as rice and soybean. These observations suggest that the differential effects of the treatments on chlorophyll types may be attributed to their specific mechanisms, with hydropriming enhancing photosynthetic efficiency, while bacterial priming may modulate nitrogen uptake, a key component of chlorophyll. The work of Mathivanan *et al*., (2017) also highlights that PGPRs can play a crucial role in improving photosynthesis, particularly under environmental stress, by enhancing plant growth and increasing their resistance to adverse conditions.

Thus, Basu *et al*., (2023) confirm the importance of PGPRs in enhancing photosynthesis and plant growth, particularly under stress conditions, offering prospects for sustainable and effective agricultural applications. The antioxidant activity of *Trifolium subterraneum* seeds was assessed using the DPPH test, revealing that bacterial priming resulted in a more pronounced reduction in IC_{50}, indicating enhanced antioxidant efficiency and superior protection against oxidation. These results are consistent with those reported by Yilmaz (2025), who observed that Bacillus subtilis increased antioxidant activity in *Vicia faba* in correlation with a higher phenolic content. Similarly, Chiappero *et al.*, (2019) noted that the application of *Bacillus amyloliquefaciens* and *Pseudomonas fluorescens* enhanced the antioxidant activity of Mentha piperita under drought conditions, associated with a greater accumulation of phenols and a reduction in lipid peroxidation. The IC_{50} values highlighted a more robust antioxidant defense and improved management of free radicals.

Conclusion

This study evaluates the effect of bio-priming with plant growth-promoting microorganisms (PGP priming) and hydro-priming on germination and growth of *Trifolium subterraneum* L. The PGP priming was carried out using a bacterial consortium consisting of three actinobacterial strains (C1A, C1B, G1B) and a strain of *Bacillus* (B3), while hydro-priming involved soaking the seeds in sterile warm water. An *In planta* trial was conducted under ambient conditions to assess the effects of these treatments on plant growth. In parallel, an in vitro test was performed to estimate germination rates and observe root interactions with the bacterial consortium. The main findings of this study are as follows : For the *In vitro* test, *T. subterraneum* seeds showed a positive response to both priming methods, with comparable results between the two treatments. This lack of significant difference was confirmed by variance analysis. Similar to the *In vitro* test, the in planta assay showed that PGP priming stimulated seed germination of *T. subterraneum*. However, no significant difference in germination behavior was observed between PGP priming and hydropriming. Although germination rates were comparable to, or slightly lower than, those of the control seeds, both priming methods resulted in a faster germination process. Furthermore, both *In vitro* and *In planta* tests revealed comparable germination rates between the two priming methods ; however, overall germination was lower under in planta conditions. Regarding growth parameters, PGP priming significantly promoted greater root growth in the seedlings and had a positive effect on the production of photosynthetic pigments, notably chlorophyll b and total chlorophyll levels, suggesting a distinct impact on photosynthetic metabolism. Results related to antioxidant activity showed that hydro-priming exhibited higher overall antioxidant capacity. However, PGP priming led to a more pronounced reduction in IC_{50} values, suggesting more effective antioxidant protection against oxidative stress. The promising results of this study highlight the pivotal role of plant growth-promoting rhizobacteria (PGPR) in enhancing seed germination, vegetative growth, and photosynthetic activity of *Trifolium subterraneum*. However, to better exploit and preserve this valuable genetic resource in Algeria from a sustainable development perspective, it is essential to broaden the range of plant material evaluated.

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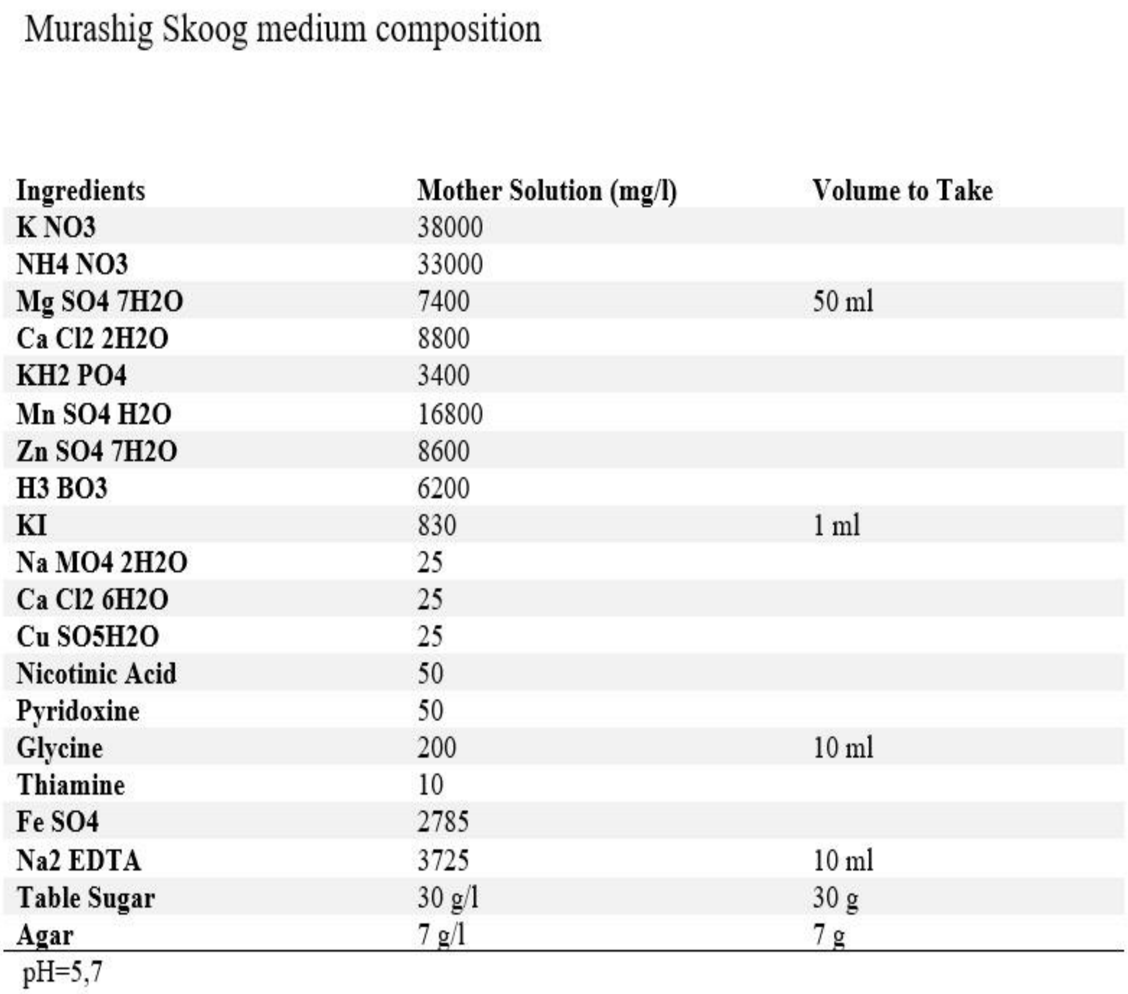
Appendix 1



Murashig Skoog medium composition

Ingredients	Mother Solution (mg/l)	Volume to Take
K NO3	38000	
NH4 NO3	33000	
Mg SO4 7H2O	7400	50 ml
Ca Cl2 2H2O	8800	
KH2 PO4	3400	
Mn SO4 H2O	16800	
Zn SO4 7H2O	8600	
H3 BO3	6200	
KI	830	1 ml
Na MO4 2H2O	25	
Ca Cl2 6H2O	25	
Cu SO5H2O	25	
Nicotinic Acid	50	
Pyridoxine	50	
Glycine	200	10 ml
Thiamine	10	
Fe SO4	2785	
Na2 EDTA	3725	10 ml
Table Sugar	30 g/l	30 g
Agar	7 g/l	7 g

pH=5,7

Appendix 2

Statistical Analysis

- Percentage of germination « *In vitro* test »

Source	DL	Sum of Squares	Mean Square	F	p-value
Betwen group	1	0,001633	0,001633	0,04	0,837
Within group	8	0,290833	0,036354		
Total	9	0,292467			

- Percentage of germination « *In planta* test »

Source	DL	Sum of Squares	Mean Square	F	p-value
Betwen group	2	0,01135	0,005675	0,45	0,655
Within group	6	0,0749	0,012484		
Total	8	0,08625			

- Shoot lenght

Source	DL	Sum of Squares	Mean Square	F	P- value
Betwen group	1	85,76	85,76	0,7	0,434
Within group	6	732,75	122,13		
Total	7	818,51			

- Root lenght

Source	DL	Sum of Squares	Mean Square	F	p-value
Betwen group	1	243,8	243,76	3,54	0,109
Within group	6	412,8	68,81		
Total	7	656,6			

- Shoot fresh weight

Source	DL	Sum of Squares	Mean Square	F	p- value
Betwen group	1	0,001463	0,001463	1,35	0,289
Within group	6	0,006504	0,001084		
Total	7	0,007967			

- Root fresh weight

Source	DL	Sum of Squares	Mean Square	F	p- value
Betwen group	1	2,3436	2,3436	14,84	0,008**
Erreur	6	0,9477	0,1579		
Total	7	3,2913			

- Root dry weight

Source	DL	Sum of Squares	Mean Square	F	p- value
Betwen group	1	1,051	1,0513	1,13	0,329
Within group	6	5,598	0,9329		
Total	7	6,649			

- Root lenght/Shoot lenght

Source	DL	Sum of Squares	Mean Square	F	p-value
Betwen group	1	0,09007	0,09007	1,53	0,262
Within group	6	0,35311	0,05885		
Total	7	0,44318			

- Root fresh weight/Shoot fresh weight

Source	DL	Sum of Squares	Mean Square	F	p-value
Betwen group	1	0,003917	0,003917	10,68	0,017
Within group	6	0,002201	0,000367		
Total	7	0,006118			

Homogenes group of tukey

Trait	N	Mean	Group	
PGP	4	0,0559	A	
HYD	4	0,0117		B

- a Chlorphyll

Source	DL	Sum of Squares	Mean Square	F	p-value
Betwen group	1	1,43797	1,43797	1275,34	0,000
Within group	4	0,00451	0,00113		
Total	5	1,44248			

Tukey homgene group

Trait	Mean	Group	
HYD	3,08	A	
PGP	2,10		B

- b Chlorphyll

Source	DL	Sum of Squares	Mean Square	F	p-value
Betwen group	1	9,07189	9,07189	566,37	0,000
Within group	4	0,06407	0,01602		
Total	5	9,13596			

Tukey homgene group

Trait	Mean	Group	
PGP	3,52	A	
HYD	1,06		B

- Total chlorophyll

Source	DL	Sum of Squares	Mean Square	F	p-value
Betwen group	1	3,28627	3,28627	317,32	0,000
Within group	4	0,04143	0,01036		
Total	5	3,32769			

Tukey homgene group

Trait	Mean	Group	
PGP	5,63	A	
HYD	4,15		B

- IC50

Source	DL	Sum of Squares	Mean Square	F	p-value
Betwen group	1	125,652	125,652	686,47	0,000
Within group	4	0,732	0,183		
Total	5	126,384			

Tukey homgene group

Trait	Mean	Group	
HYD	57,70	A	
PGP	48,55		B