جامعة سيدي محمد بن عبد الله بفاس
UNIVERSITÉ SIDI MOHAMED BEN ABDELLAH DE FES

كلية العلوم والتقنيات فاس
FACULTÉ DES SCIENCES ET TECHNIQUES DE FÈS

MOHAMMED VI
POLYTECHNIC
UNIVERSITY

Academic year 2020-2021

Master of Sciences and Technologies:

Management and Conservation of Biodiversity

A Dissertation Submitted in Partial Fulfillment for the Degree of Master Sciences and Technologies

Exploring potential effects of P Solubilizing Bacteria in improving tolerance in Wheat under Cadmium stress

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Defended on: **13/07/2021**

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Foreword

This study is part of the end-of-studies project of the Master in Science and Technology in Management and Conservation of Biodiversity **MCB** of the Faculty of Science and Technology of Fez **FSTF**, University Sidi Mohammed Ben Abdellah **USMBA,** under the supervision of

Pr. RACHIQ Saad.

The realization of this internship was granted by Mohammed VI Polytechnic University **(UM6P)** for a period of four months (*from March 1 to June 30, 2021*). The work was carried out mainly at the Agro Bio-sciences department (**AGBS)** at **UM6P-Benguerir** under the supervision of **Mr. BARGAZ Adnane** and Mr. **MAHAMMAT ISSA Ammar Ibn Yasser,** and initiated in part within the research center-Laboratory of Plant-Microbe Interactions **PMI** and in part at the Experimental Farm **(AITTC)** Agriculture Innovation and Technology Transfer.

Dedication

I dedicate this work:

To my dear parents *who have enlightened the path of my life by their exemplary devotion and the enormous sacrifices as well as and their encouragement throughout my school and university career.*

To my dearest sisters *and* ***my brother*** *for their encouragement, their help and their permanent moral support.*

To my friends *for their support and their availability and the pleasant moments we spent together.*

To all my fellow students *of the Master "****Management and Conservation of Biodiversity*** *".*

To all my teachers.

Acknowledgement

First, I thank our almighty God for giving us the chance to live and be able to study, for giving us the courage, the moral and physical strength, to complete this work.

I wish to thank the Dean of the Faculty of Sciences and Technologies, Fes (**FSTF**), for offering such rich and interesting Master program (Management and Conservation of Biodiversity) that was a starting point for us to get deep into microorganism's world. I thank as well, Mohammed VI Polytechnic University (**UM6P**) for welcoming me as well as for all the facilities they offered.

First of all, I would like to express my gratitude to my supervisor **Dr. BARGAZ Adnane** Researcher Professor at Mohammed VI Polytechnic University for granting me the permission to do this internship in the laboratory of Plant-Microbe Interactions, for offering me the opportunity to work on such an exciting and captivating research topic and for guiding and encouraging me to be professional and efficient, My thanks to **Pr. OUKKAROUM Abdellah** for his help and his great sympathy towards me and their efforts to ensure the good realization of this work, as well as Co-supervisor **Mr. MAHAMAT ISSA Ammar Ibn Yasser**, for his supervision, his help and his advice throughout the course.

I would like to thank **Pr. RACHIQ Saad** my supervisor in the Faculty of Sciences and Techniques in Fez, for having supported me by his help, his orientations and his availability as well as his encouragements. Thank you for the supervision.

I also present my thanks to **Pr. MIKOU Karima** and **Pr. BENJELLOUN Meryeme**, of the Faculty of Sciences and Techniques of Fez, who honored me by their presence at the defense of my end-of-study project and by the time they devoted to examine and judge this work.

I would also like to thank all the team of the laboratory Plants-Microbes Interaction (PMI), and the people who helped me in the realization of this work, **Mr. GHANI Rachid, ELHAISSOUFI Wissal, KHOURCHI Said, HADDINE Meryeme and NACIRI Rachida** as well as the trainees of the unit for all the great moments shared together which made this training course particularly pleasant**.**

Finally, this work would not have been completed without the concessions and encouragement of my parents to whom I simply say thank you.

A big thank you to all my family.

Project information

This study was carried out within the framework of the project **AS17** financed by **OCP** at **UM6P** under the coordination of Professor **Adnane BARGAZ**.

A B S T R A C T

Heavy metals are not degradable and can environment be including the plants, thus entering the food chain. Cadmium (Cd) contamination of soils generally impairs plant growth and affects human health. The objective of this study was to examine the effect of applying Cd-tolerant PSB to alleviate Cd stress while promoting P solubilization and improving the growth of wheat plants grown in Cd-contaminated soils by increasing their physiological parameters and reducing Cd uptake by the plant. Three isolates B8 (*Bacillus sp*.), B12 (*Bacillus sp*.) and B31 (*Rahnella sp*.) were selected for their tolerance to Cd showing MIC of 300 ppm $CdCl_2$ (Cd [300]), along with others biochemical characteristics related to promoting plant growth. To determine their effect on Wheat growth under Cd stress, seeds were inoculated with these isolates individually (B8, B12 and B31) and in consortium and grown in-vitro and in-vivo on medium containing three levels of Cd (0 ppm = Cd [0], 50 ppm = Cd [50] and 100 ppm = Cd [100]). The results indicate that among these isolates, inoculation with isolate B31 belonging to the genus *Rahnella sp*. significantly increased plant physiological parameters such as stomatal conductance (SC) by 86,66%; 50% and 40% respectively under Cd [0], Cd [50] and Cd [100], and the Chlorophyll content index (CCI) in leaves is significantly elevated with "CCI 3.9", "CCI 2.4" and "CCI 1.8" compared to control "CCI 1.8", "CCI. 1.3" and "CCI 0.7" in the presence of Cd [0], Cd [50] and Cd [100]. Also, increase significantly the morphological traits such as plant length by 66.66% and spike length by 80% at Cd [100]. This B31 isolate was found to have a relatively high Cd tolerance capacity compared to the other isolates by increasing plant dry weight (i.e. spike weight by 100%) as well as root morphological traits (such as total root length by 66.66%, root area by 95.65% under Cd [100]). On the other hand, inoculation of plants with the different isolates showed a promoting effect on P solubilization from phosphate rock. The three inoculants B12, B31 and Co showed a high solubilization of P in 75-days old roots by 80%, 78,5% and 79% respectively at Cd [100] compared to controls. In rhizosphere, isolate B31 increased the available P content by 66.66%, as well as acid and alkaline phosphatase activities in rhizosphere were increased by 83.33% and 91.66% respectively compared to controls under Cd stress [100]. Our findings suggest that isolate B31 consequently had the most pronounced effects in promoting wheat growth and decreasing Cd uptake under stress conditions. Therefore, this isolate B31 may have a positive impact on plant growth when applied in agricultural works for crop protection in fields, as an effective Cd-tolerant biofertilizer that can solubilize soil P as well as stimulate plant growth under Cd stress.

Keywords: Cadmium, wheat, rhizobacteria, phosphate, phosphate solubilizing bacteria, solubilization, tolerance.

R E S U M E

Les métaux lourds ne sont pas dégradables et peuvent se retrouver dans l'environnement, y compris les plantes, et ainsi entrer dans la chaîne alimentaire. La contamination des sols par le cadmium (Cd) nuit généralement à la croissance des plantes et affecte la santé humaine. L'objectif de cette étude était d'examiner l'effet de l'application des bactéries solubilisatrices du Phosphore tolérantes au Cd pour atténuer le stress lié au Cd tout en favorisant la solubilisation du P et en améliorant la croissance des plantes de blé cultivées dans des sols contaminés par le Cd en augmentant leurs paramètres physiologiques et en réduisant l'absorption du Cd par la plante. Trois isolats B8 (*Bacillus sp*.), B12 (*Bacillus sp*.) et B31 (*Rahnella sp*.) ont été sélectionnés pour leur tolérance au Cd avec une CMI de 300 ppm $CdCl_2$ (Cd [300]), ainsi que d'autres caractéristiques biochimiques liées à la promotion de la croissance des plantes. Pour déterminer leurs effets sur la croissance du blé sous le stress du Cd, les graines ont été inoculées avec ces isolats individuellement (B8, B12 et B31) et en consortium et cultivées in-vitro et in-vivo sur un milieu contenant trois niveaux de Cd (0 ppm = Cd [0], 50 ppm = Cd [50] et 100 ppm = Cd [100]). Les résultats indiquent l'inoculation avec l'isolat B31 *Rahnella sp.* améliore significativement les paramètres physiologiques des plantes tels que la conductance stomatique (SC) de 86.66 ; 50 et 40% respectivement sous Cd [0], Cd [50] et Cd [100]. L'indice de la teneur en Chlorophylle (CCI) dans les feuilles est élevée significativement avec "CCI 3.9", "CCI 2,4" et "CCI 1.8" par rapport au contrôle "CCI 1.8", "CCI. 1.3" et "CCI 0.7" en présence de Cd [0], Cd [50] et Cd [100]. Il augmente également de manière significative les traits morphologiques telles que la longueur de la plante de 66,66% et la longueur des épis de 80% sous Cd [100]. Cet isolat B31 s'est avéré avoir une capacité de tolérance au Cd relativement élevée par rapport aux autres isolats en améliorant le poids sec végétal (i.e. le poids des épis de 100%) ainsi que les traits morphologiques des racines telles que la longueur totale racinaire par 66,66% et la surface racinaire par 95,65% sous Cd [100]. D'autre part, l'inoculation des plantes avec les différents isolats a montré un effet promoteur sur la solubilisation du P à partir de la roche phosphatée. Les trois inoculats B12, B31 et Co ont montré une solubilisation élevée de P dans les racines âgées de 75 jours de 80, 78,5 et 79% respectivement sous Cd [100] par rapport aux contrôles. Dans la rhizosphère, l'isolat B31 a augmenté la teneur en P disponible de 66,66%, ainsi que les activités phosphatases acide et alcaline dans la rhizosphère ont augmenté de 83,33 et 91,66% respectivement par rapport aux contrôles sous Cd [100]. Nos résultats suggèrent que l'isolat B31 a donc eu, par conséquence, les effets les plus prononcés en favorisant la croissance du blé et en diminuant l'absorption de Cd dans des conditions de stress. Par conséquent, cet isolat peut avoir un impact positif sur la croissance des plantes lorsqu'il est appliqué dans les travaux agricoles pour la protection des cultures dans les champs, comme un biofertilisant efficace tolérant le Cd et solubilisant le P du sol ainsi, stimulant la croissance des plantes sous le stress du Cd.

Mots clés : Cadmium, blé, rhizobactéries, phosphate, bactéries solubilisant le phosphate, solubilisation, tolérance.

Abbreviations

ABA : Abscisic acid
ACCD : 1-aminocyclopropane-1 -carboxylate (ACC) - deaminase
AITTC : Innovation and Technology Transfer Center for Agriculture
ALPase : Alkaline phosphatase activity
APase : Acid phosphatase activity
CaCl2 : Chlorure de calcium
CCI : Chlorophyll content index
Cd : Cadmium
CHF : Chlorophyll a fluorescence
Co : Consortium
EPS : Exo-polymeric substance
HM : Heavy metals
IAA : Indole-3-acetic acid
MIC : Minimum inhibitory concentration
MT : Metallothioneins
N : Nitrogen
NaOH : Hydroxyde de sodium
NBRIP : National Botanical Research Institute Phosphate
NFB : Nitrogen fixing bacteria
OCP : Office Cherifian of Phosphates
P : Phosphorus
PC+ : Plastocyanin
PGPR : Plant Growth Promoting Rhizobacteria
PMI : Plants-Microbe Interaction
UM6P : Mohammed VI Polytechnic University
pNP : Para Nitro-phenol
pNPP : Para-4-Nitrophenylphosphate
PQ : lastoquinone
PSB : phosphate solubilizing bacteria
PSI and II : Photosysteme I and II
RP : Rock Phosphate
Sc : Stomatal conductance
TCP : Tricalcium phosphate
TSB : Tryptic Soy Broth

Table

Figure

Summary

Introduction

Introduction

Biotic and abiotic stresses cause significant yield losses in crops and can significantly affect their productivity. Abiotic stresses (salinity, drought and metal) are the most devastating factors that hamper crop growth and yield (Ahmad et al., 2014). Soil contamination with heavy metals has become a severe problem in many parts of the world (Li et al., 2014). Heavy metals are natural components of the Earth's crust; however, anthropogenic activities, like ore mining, e-waste recycling, and sewage irrigation, have greatly increased soil heavy metals concentrations (Yuan et al., 2017). They can be mobilized in the environment in a natural or anthropogenic way by forming toxic compounds. This non-biodegradable nature of heavy metals leads to a hyper accumulation of toxic compounds in different levels of the ecosystem and causes adverse effects on life forms (Mahapatra et al., 2020).

The heavy metals (HM) are deposited in soil and reach concentrations that are toxic to a variety of organisms (Ahmad et al., 2014). Among them, Cadmium (Cd) is not an essential element and does not participate in any physiological activity in plants (Rehman et al., 2019), It is relatively mobile in soils and is one of the most toxic environmental pollutants (Dell'Amico et al., 2008). In plants, Cd affects plant growth owing to its accumulation of the contaminant in the edible parts of crops. Toxicity of Cd can cause chlorosis and instability to lipid membrane, thus inhibits plant growth (Ahmad et al., 2014). It can also cause oxidative stress in plants through overproduction of reactive oxygen species, which are highly toxic causing considerable impairment to macromolecules such as proteins, RNA, DNA, lipids, pigments and cell membranes (Li et al., 2016; Sidhu et al., 2017). In addition, high Cd concentration in soils affects soil fertility, disordered physiology and metabolism of the plant that showed a limitation in plant growth, symbiosis, crop production and yield. Also, it has deleterious effects on photosynthetic process, mineral elements uptake and transport resulting massive agricultural loss (Mitra et al., 2018).

Several heavy metal extraction practices have been implemented for the removal or recovery of heavy metals from metal contaminated environments (Mitra et al., 2018). Remediation of heavy metal polluted soils can be performed using physicochemical processes such as ion exchange, precipitation, reverse osmosis, evaporation, and chemical reduction; however, the required measures are expensive (Mojiri, 2011). Phytoremediation is a viable and relatively inexpensive technology that uses plants to remove heavy metals from contaminated soil. However, among phytoremediation strategies, the uptake of heavy metals by plants can be enhanced by the use of soil and plant chemical compounds

including chemical chelates such as EDTA that have negative effects on soil fertility or structure (Mojiri, 2011) and (Jeong et al., 2012).

The application of plant growth promoting rhizobacteria (PGPR) can be considered as an important bioremediation technology for enhancing plant biomass production as well as tolerance to heavy metals (Jeong et al., 2012) . Metal resistant PGPR offer resistance to plants and have the ability to reduce their effects through several direct or indirect mechanisms (Zubair et al, 2016) including hormone synthesis, indole-3-acetic acid (IAA) production, abscisic acid (ABA) synthesis, enzyme production (Kumar et al., 2019).

In particular, phosphate (P) solubilizing bacteria (PSB) are often used to facilitate P dissolution for a better P uptake and plant growth (Jeong et al., 2012). It is worth to mention that P is one of the most important nutrients that plants need for their development. In soil, PSB could produce organic acids and phosphatase enzymes to enhance the solubilization and mineralization of insoluble P compounds (Yuan et al., 2017). They dissolve inorganic P mainly by acidification, which lowers the pH in soil (Yang et al., 2018). Moreover, PSB have a important effect on root morphological traits and their biophysical characteristics, which has a positive impact on the capacity of the roots to absorb nutrients and on the overall physiology of the crop (Elhaissoufi et al., 2020). Addition of PSB might be defined as a cost-effective and environmental-friendly technique to remediate multiple heavy metals contaminated soils (Yuan et al., 2017).

The objective of this study is to evaluate the Cd tolerance capacity in three phosphate solubilizing bacterial isolates and their Consortium, and to correlate this tolerance to P bio-solubilization. Thus, to examine the effect of these PSB on growth and development of wheat plants under cadmium stress. Two main questions can be addressed.

1) Could these PSB reduce Cd uptake and allocation to above-ground plant parts while promoting P solubilization?
2) Does the application of these PSB alleviate the effect of Cd by stimulating specific physiological parameters along with shoot and root growth of wheat plants?

Literature review

Literature review

I. Interaction of Cadmium by PGPR

The toxicity of heavy metals to plants depends largely on their mobility and bioavailability, which can be affected by many factors, including microbial activities. PGPR could reduce the bioavailability of heavy metals by immobilization, which transformed them into inactive forms to inhibit the transfer of heavy metals from soil to plants (Li et al., 2019).

The PGPR have characteristics that allow them to interact with HM in such a way that they can alter their availability in the soil environment. These characteristics include, (1) release of chelating substances, (2) acidifying the microenvironment, (3) by inducing modifications in redox potential (Zubair et al., 2016).

The diversity of micro-organisms present in the rhizosphere shows mutualistic interactions and positively regulates the growth and metabolism of the plant under normal as well as stressed conditions; they are well known to ameliorate Cd toxicities by immobilization and reducing their translocation to the plant tissues (Khanna et al., 2019). The plant growth promoting bacteria (PGPR) can bind directly with Cd in order to reduce their bioavailability and toxicity (Thiem et al., 2018). Furthermore, micro-organisms also result in the upregulation of plant defense system, heavy metal tolerant proteins (MTP family) and phytohormones under Cd stress (Khanna et al., 2019). However, Thiem et al. (2018) mentioned that high concentrations of Cd inhibit the synthesis of siderophores and accumulation of toxic ions in bacterial cells.

II. Uptake and transformation of Cd by PGPR

Cadmium-resistant PGPR that stimulate plant growth under metal contaminated soils play an important role in mobilization or immobilization of heavy metals (Zubair et al., 2016). They may affect the plant-metal interaction in two ways (Sharma & Archana, 2016); either by reducing the uptake and transfer of Cd into plant aboveground particularly in non-hyper-accumulator plants thus contributing to metal tolerance or plants, or by facilitating the Cd intake into plants and its accumulation in plant aboveground parts, potentiating the performance of hyper- accumulator plants. According to (Li et al., 2019), it was reported that PGPR could reduce the bioavailability of heavy metals by immobilization, transforming them into inactive forms to facilitate the development of microbes and ultimately inhibit the transfer of heavy metals from the soil to plants. They are able to enhancing plant growth through the production of plant growth-promoting substances and influence the mobility and availability of

HMs in the soil by acidification, redox changes, secretion of chelating agents and mobilization of metal phosphates (Zubair et al., 2016) and (Manoj et al., 2020).

Phosphorus (P) plays important roles in reducing Cd uptake and translocation via the processes involved in bonding Cd^{2+} to the cell wall fraction and forming Cd-phosphate complexes. Amendment of phosphate rock to soils resulted in immobilization of Cd^{2+} in the soil with a decrease in Cd uptake (Sharma & Archana, 2016). Another study by (Arshad et al., 2016) also reported that P application decreased uptake of Cd by plants and enriches mineral nutrients, chlorophyll contents, antioxidants, gas exchange attributes in wheat under Cd stress.

phosphate-solubilizing bacteria (PSB), which can be benefit for the growth of host plants by secreting organic acids to dissolve insoluble phosphorus and mineral elements in soil, can promote the mobility and bioavailability of insoluble heavy metals in soil during the process of dissolving phosphorus (Gao et al., 2019). However, (Ahmad et al., 2014) reported that the application of Cd-tolerant bacteria can improve the tolerance of wheat to Cd stress. They increase plant growth, biomass and relative water content and decrease oxidative stress in Cd-stressed wheat seedlings (Rizwan et al., 2016).

III. Effects of Phosphate-solubilizing bacteria on Cd mobility in contaminated soil

Soil contaminated by heavy metals (such as Pb, Cd, Zn) pose additional threats to natural ecosystems and human health (Li et al., 2019). In agriculture, Soil contaminated by Cd is a serious problem because of the severe negative effect on plant growth, productivity and the quality of crops. thereby reducing the exchangeable Cd fraction and its accumulation in crops using the potential solubilization effect of bacteria is a main challenge for researches (Gao et al., 2019).

Foremost, PSB facilitate P dissolution through numerous P solubilization mechanisms for plant growth from organic and inorganic forms of total soil P by P hydrolyzing enzymes and organic acids production (yang 18). The PSB produce a wide variety of organic acids, including acetic, lactic, citric, malic, tartaric, gluconic and 2-ketogluconic acids... (Rodríguez & Fraga, 1999). These organic acids improve the P solubility by ionizing protons to decrease the soil pH and to combine PO_4^{3-} to form HPO_4^{2-} or $H_2PO_4^-$. The organic acid anions can also form a complex with metal cations and consequently, release PO_4^{3-} (Yang et al., 2018) and (Gao et al., 2019).

Yuan (Yuan et al., 2017) showed that PSB are able to enhance Cd immobilization in contaminated soil by significantly increasing the nutritive value of solubilized phosphate for plants exposed to Cd stress

from the insoluble phosphate compound. Therefore, the ability to solubilize P could perform better promote plant growth under Cd stress(Mitra et al., 2018).

IV. PSB traits involved in reducing Cd accumulation in plants

Species belonging to PSB group may have multiple PGP traits (more than localized rhizosphere P solubilization) reverberating positively on growth and development of crops (Elhaissoufi et al., 2020).The PSB have the capacity to render soluble P more accessible for uptake by plants. They can also promote growth and development of plants through various mechanisms including N2 fixation, synthesis of phytohormones such as indoleacetic acid, synthesis of siderophores, reduction of ethylene toxicity through 1 aminocyclopropane1carboxylate (ACC) deaminase and by solubilizing or reducing the toxicity of metals (M. S. Khan et al., 2009).

Bioavailability of heavy metals in soil causes toxicity and negatively affects plant growth. PSB strains have the ability to improve plant growth by suppressing heavy metal-induced toxicity (Singh et al., 2019). Ahmad (Ahmad et al., 2014) showed that certain rhizosphere bacterial strains including PSB are able to tolerate high levels of Cd and can potentially be applied to reduce Cd accumulation in plants.

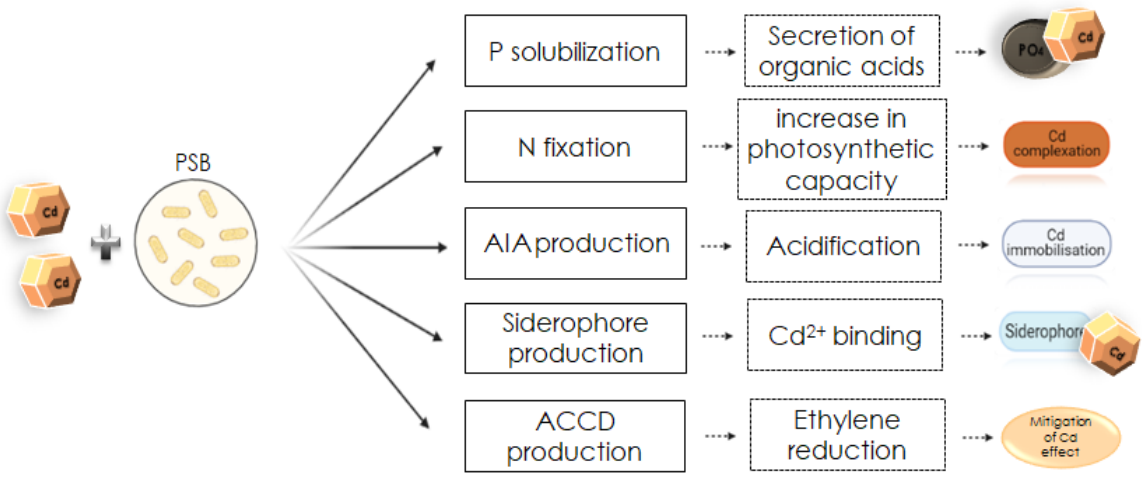


Figure 1 : *Illustration of various traits of PSB involved in Cd reduction.*

1. Phosphate solubilization

Phosphorus (P) is the second important macronutrient that plants need at an adequate rate in the early stages of their development. Similarly, at the cellular level, P is of vital importance, due to its role in cell division, the growth of new tissues and in the biosynthesis of the main cellular constituents, i.e. nucleic acids, enzymes, phospholipids, ATP and nucleotides, as well as in the control of vital processes such as photosynthesis, respiration and energy production (Elhaissoufi et al., 2020) (Janati et al., 2021).

In general, phosphate (P) existed in an insoluble form, plants unable to utilize directly from the soil. However, some soil bacteria play an important role in P solubilization, through the secretion of enzymes and organic acids, which convert insoluble form of P into a soluble form (Manoj et al., 2020).

(Teng et al., 2019) showed that PSB enhanced the concentration of soluble P and that acidification might be the main strategy for the solubilizing phosphate from inorganic phosphate, and P solubilization is associated with pH, acid phosphatase activity and the production of multiple organic acids. (Sharma & Archana, 2016) showed also that the application of these PSB rapidly enhances Cd2+ immobilization by solubilization of P and subsequent formation of insoluble Cd phosphate precipitates. Immobilization of Cd in soils by P-containing compounds decreases Cd toxicity. This is done by phosphate-induced adsorption of the metal including: increase in pH values, co-adsorption of phosphate and Cd as an ion pair, and formation of a Cd surface complex on phosphate compounds (Rahman et al., 2020).

2. Nitrogen fixation

Nitrogen (N) is an essential element involved in the composition of urea and amino acids (proteins), nucleic acids (DNA and RNA), adenosine triphosphate (ATP), nicotinamide adenine dinucleotide (NAD) in all living cells as well as chlorophyll. It is the most necessary pigment for photosynthesis, and plays an essential role in plant growth and production (Lindström & Mousavi, 2020). Nitrogen is present as an inert gas N_2 in the atmosphere. However, only the oxidized or reduced forms of nitrogen can be assimilated by plants (Burén and Rubio, 2017).

Biological N-fixation is a symbiotic process by which N_2 gas is converted to ammonia by photosynthetic rhizosphere N-fixing bacteria (NFB), which naturally provide nitrogen for crop and native plant growth (Welz et al., 2018). Cd toxicity may cause essential nutrient deficiency and changes in the concentration of basic nutrients such as N and P in plant tissues. N supplementation leads to improved plant tolerance to Cd by increasing photosynthetic capacity (Jalloh et al., 2009), which allows for greater root exudation and consequently Cd complexation (Walker et al., 2003). Many bacteria have the ability to fix atmospheric N_2 and increase N supply to plants and decrease Cd content in grain. Bacterial N fixation improves plant Cd tolerance by increasing root-associated Cd and reducing Cd levels in aerial parts (Sharma & Archana, 2016).

In contrast, P is required for growth and development of plants and promotes N_2 fixation (M. S. Khan et al., 2009). P deficiency strongly limits the activity of nitrogen fixing bacteria. The application of PSB facilitate plant growth by stimulating the efficiency of N_2 fixation and consequently, the process

of N-fixation correlates with the availability of P and therefore with the availability of PSB (Janati et al., 2021). Therefore, based on available knowledge, it can be possible that improved N_2 fixation by PSB leads to a better tolerance of plants to Cd and therefore a better plant growth in Cd-contaminated soils.

3. Production of indole-3-acetic acid (IAA)

Phosphate solubilizing bacteria are able to assimilate inorganic P from insoluble compounds through solubilization and mineralization. According to (Jeong et al., 2013), these PSB activities strongly affect metal speciation in soil, It showed that the pH of the Cd-contaminated soil inoculated with PSB decreased and this was due to the secretion of organic acid IAA and increase in its concentration that led to the acidification of the soil or could have directly solubilized the Cd from the soil. Mitra et al. (2018) has mentioned that the production of IAA by Cd resistant rhizobacteria could be lowering the stress ethylene level resulted in enhancement of plant tolerance under Cd stress.

4. Siderophore production

Soil microorganisms may release chelating substances, which may acidify the soil by releasing protons and thus alter metal availability in soil solution (Ahmad et al., 2014). The siderophores are low molecular weight compounds secreted by many plants and microorganisms with a strong affinity to Fe^{3+} to recover this ion for metabolic needs (Ma et al., 2016). Thus, siderophores act as iron solubilizing agents from minerals or organic compounds under iron-limiting conditions. In addition to iron, siderophores bind Cd ions and can also form stable complexes with Cd (Rajkumar et al., 2010). These authors also noted that Cd induced siderophore production in some PSB strains resistant to high levels of Cd.

5. ACC deaminase production

Cadmium (Cd) is known to be one of the most stressful heavy metals to plants. An excessive amount of Cd affects the physiological functions and morphological features of most crop species as well as photosynthetic efficiency. In addition to, negatively root growth, elongation and architecture (diameter, volume, surface…) (Chtouki et al., 2021).

According to (Pramanik et al., 2018) Cd-resistant PSB include *Bacillus, Enterobacter, klebsiella, Ochrobactrum, Pseudomonas…* have been reported to improve Cd immobilization and P bioavailability, as well,they could alleviate the effect of Cd toxicity by reducing ethylene stress through the production of ACC deaminase, which hydrolases ACC, the immediate precursor of ethylene. Thus, bacterial strains which have ACCD activity can partially mitigate Cd-induced

increased stress ethylene content in plants that stimulates length of plant roots and shoots, and in biomass caused by high ethylene levels under Cd stress (Dell'Amico et al., 2008).

V. Effects of PSB on growth and development of Cd-stressed Wheat

(Verma et al., 2015) Showed that some Cd-tolerant strains of genus pseudomonas sp. positively improves the growth and germination of wheat in Cd-containing soil by solubilizing P and producing NH_3 and IAA. This group of bacteria show a significant increase in shoot and root length and play an important role in improving plant growth and remediating metal-contaminated soils. In fact, findings by Malekzadeh et al. (2012) revealed that the presence of Cd in soils decreased dry weight of shoots and roots of maize plants. However several PSB strains significantly increased plant biomass in Cd polluted soil, This is most likely through the assimilation of P and other nutritional elements, as well as the production of the enzyme ACC deaminase which is known to reduce ACC levels in plant roots, reducing ethylene and increasing root length and plant growth under Cd-polluted conditions (Malekzadeh et al., 2012). All together, results from these studies highlight the capacity of PSB in helping the plants withstand environmental constraints such like heavy metals contamination.

VI. Tolerance mechanisms PSB against Cd

In response to Cd contamination, many PSB strains can cause Cd mobilization or immobilization through multiple mechanisms related to detoxification and resistance via biosorption, bioaccumulation, complexation, precipitation and metal efflux (Singh et al., 2019).

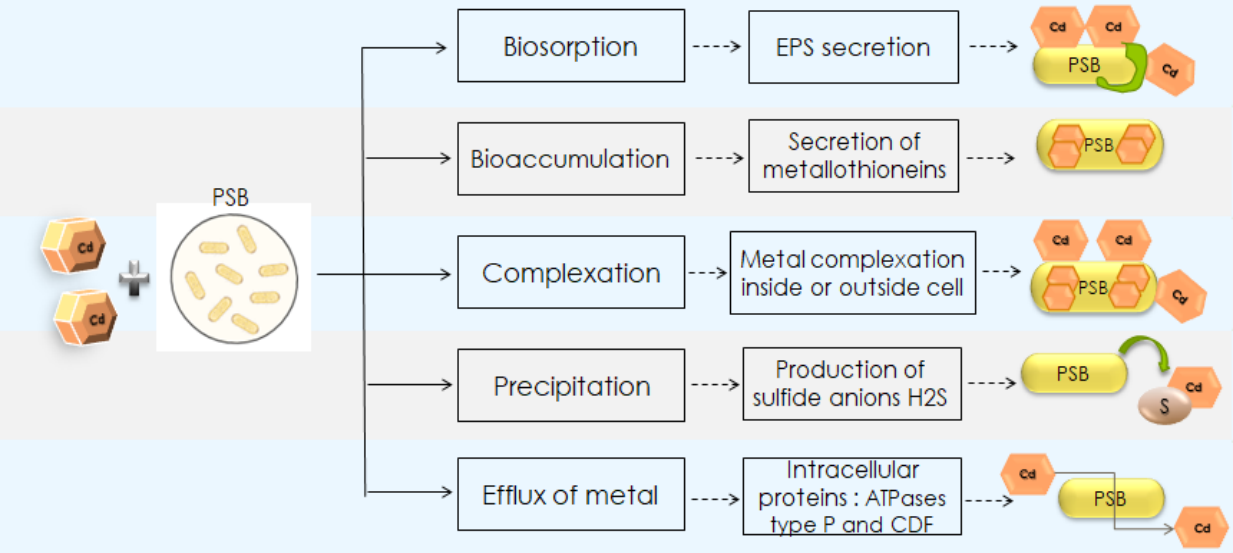


Figure 2 : *Illustration of different tolerance mechanisms by Cd tolerant phosphate solubilizing bacteria.*

1. Bioaccumulation and biosorption

According to Mishra et al. (2017), bioaccumulation/biosorption of heavy metals by bacterial cells removes heavy metal ions. Bioaccumulation is responsible for the uptake and detoxification of heavy metals. It consists of two processes, passive uptake or biosorption which is the metabolism-independent accumulation of metals, while active uptake or bioaccumulation occurs only in living cells, requires metabolism and energy for transport and bioaccumulation of metals.

a. Biosorption

Bacterial cell surface anionic functional groups (sulfhydryl, sulfonate, carboxyl, hydoxyl, amine and amide groups), extracellular polymeric substances (EPS) and extracellular capsules are playing an important role in heavy metal adsorption (Sessitsch et al., 2013), as shown by (Guiné et al., 2006) in the (Fig. 3)These compounds could easily entrap the cationic metal ions, which leads to reducing the availability and mobility of heavy metals in the rhizosphere (Manoj et al., 2020).

Singh et al. (2019) noted that some PSB strains of *Pseudomonas species* secreted EPS such as exopolysaccharides, lipopolysaccharides, proteins, etc. with anionic functional group, which help mobilize or immobilize Cd by binding with the cationic ions of the metal and removing it by the biosorption process. According to (Mahapatra et al., 2020) the EPS produced from bacteria is having higher stability to environmental stresses and higher heavy metal removal ability than the EPS secreting biomass. EPS or bacterial cell-mediated biosorption involves the interaction between positively charged metal ions and negatively charged EPS and/or bacterial cell wall or surfaces (Fig. 4). The production of EPS by metal-resistant bacteria induced the formation of biofilms that improve the tolerance of bacterial cells by forming a protective sheath and transforms toxic metal ions into non-toxic forms after adsorption (Mishra et al., 2017). Another study by (Ma et al. 2016) mentioned that the mechanisms involved in metal biosorption onto to EPS include metal ion exchange, complexation with negatively charged functional groups, adsorption and precipitation.

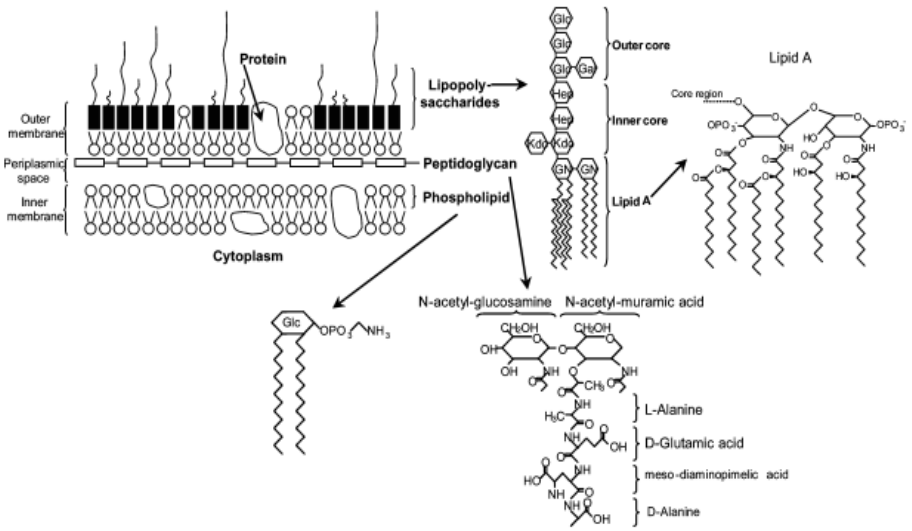


Figure 3 : *Schematic representation of the major reactive components of E. Coli cell wall (Guiné et al., 2006).*

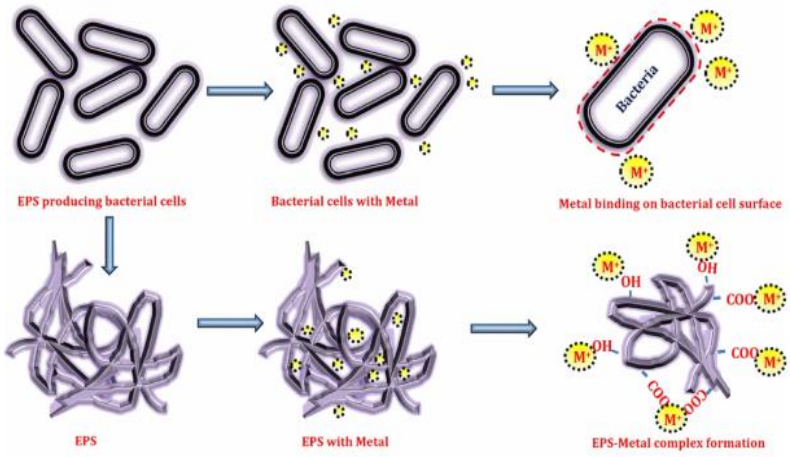


Figure 4 : *Illustration of EPS production, bacterial cell wall and mechanism of metal-EPS interaction (Mahapatra et al., 2020).*

b. Bioaccumulation

The secretion of metallothioneins (MT) and glutathione-derived peptides by many PSB in the presence of heavy metals, may allow the metal to be trapped and transported inside the microbial cells. These proteins are negatively charged and allow the sequestration of the metal in a non-toxic form for the bacteria (Singh et al., 2019). These (MT) are cysteine-rich intracellular proteins with high affinity for Cd.

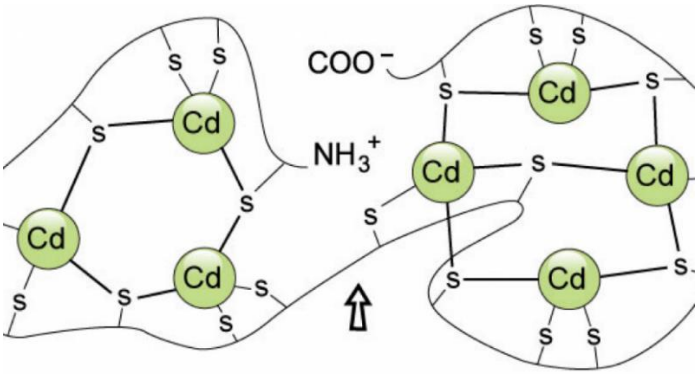


Figure 5 : *Schematic representation of the form of metallothioneins.*

2. Complexation

Complexation of Cd with polyphosphate granules and intracellular Cd-binding proteins has been reported in some strains of PSB. They can also take up soluble $Cd^{2+}$to complex the metal outside the cell and reduce its phyto-absorptive capacity (Sharma & Archana, 2016).

3. Precipitation

The mobility and toxicity of a metal can be reduced by the reduction of that metal to a redox state. This process can accompany other reduction mechanisms such as bacterial sulfate-reducing systems (Gadd, 2004). Sharma & Archana (2016) mentioned the process of precipitation of Cd^{2+} with produced anions such as sulfides and phosphates also reduces its bioavailability. Some sulfate-reducing bacteria produce more H_2S under metal stress conditions. This H_2S reacts with available Cd^{2+} and precipitates extracellularly as CdS, according to this reaction:

$$H_2S + Cd = CdS + H_2$$

4. Efflux of metal

The Cd^{2+} resistance in soil bacteria is primarily based on active efflux of metal ions by P-type ATPases, cation diffusion facilitator (CDF) transporters and chemiosmotic transporters (Sharma & Archana, 2016) :

* P-type ATPases constitute a super-family of transporter proteins that transport ions against the concentration gradient using energy provided by ATP hydrolysis.

* CDF comprises of a group of transporters which can catalyze either influx or efflux of heavy metals. They provide very low level of resistance and their main role is to function as a kind of heavy metal buffer for the cell at low cytoplasmic metal concentrations.

* Bacteria bind heavy metals to intracellular proteins as a defense system to make them non-bioavailable. This biding mechanism reduces Cd^{2+} availability. Bacterial metallothioneins are small cystein-rich proteins capable of binding Cd^{2+}.

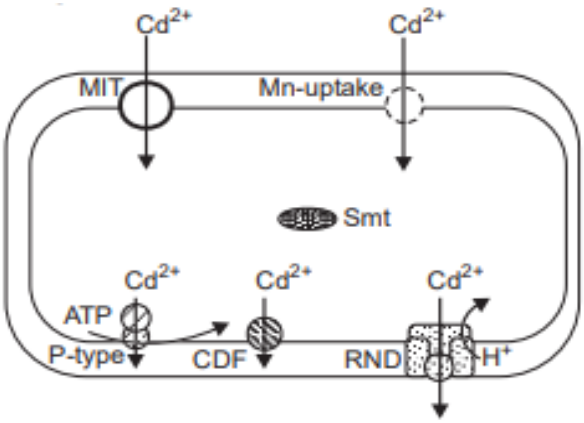


Figure 6 : *Schematic representation of Cd transport inside the bacterial cell and metal efflux by different transporters (Nies, 1999).*

Magnesium (MIT) and/or manganese Mn uptake systems are responsible for Cd^{2+} uptake. In gram-positive bacteria, metal efflux is mediated by P-type ATPases and in gram-negative bacteria, it takes the form of RND-driven trans-envelope transport, and may also be mediated by CDF transporters (Nies, 1999).

Material and Methods

Material and Methods

I. Isolation of Phosphate-solubilizing bacteria and determination of PGP traits

In this study, three PSB were obtained from the rhizosphere of several crops grown in Moroccan soils (wheat, intercropping wheat/faba beans and alfalfa); Isolates B8 (*Bacillus sp.*), B12 (*Bacillus sp.*) and B31 (*Rahnella sp.*) were selected first for their tolerance to Cadmium ($CdCl_2$) at concentrations ranging from 20 ppm to 500 ppm.

Work mentioning that before screening for Cd [20], these PSB were biochemically analyzed for their tolerance (by Elhaissoufi et al., 2020 – UM6P Benguerir) such as P solubilization, ammoniac production, production of phytohormones (indole-3-acetic acid (IAA)), ACC deaminase production and siderophore production. Then, analyzed for their tolerance to Cd following the description provided below.

II. Cadmium tolerance test of isolated PSB isolates

1. Determination of minimum inhibitory concentration MIC under $CdCl_2$ - Cadmium chloride

Before selecting the above three PSB isolates as Cd-tolerant, all PSB isolates belonging to the Plant-Microbe Interaction Lab at UM6P *(PMI-UM6P)* were screened for their tolerance to a starting Cd concentration of Cd [20] on TSB growth medium (*Appendix 1*). Afterward, only bacteria that were developed were selected to undergo another tolerance test at five $CdCl_2$ concentrations (Cd [0]: 0 ppm, Cd [50]: 50 ppm, Cd [100]: 100 ppm, Cd [200]: 200 ppm, Cd [300]: 300 ppm and Cd [400]: 400 ppm of $CdCl_2$) to determine their MIC. Three Cd-tolerant bacterial isolated with MIC greater than or equal to Cd [300] were retained in this study to carry out further analyses (Ahmad et al., 2014).

III. Effect of $CdCl_2$ on P solubilization in NBRIP liquid medium

The three selected PSB (DO:1 = 10^8UFC/ml) as well as the consortium (DO: 0,5 = 10^4UFC/ml of each liquid bacterial culture) were tested on *National Botanical Research Institute's Phosphate* (NBRIP) liquid medium (*Appendix 1*) at five concentrations of Cd (Cd [0], Cd [50], Cd [100], Cd [200] and Cd [300]) in the presence of a P source (5 g/L TCP or RP), in order to accurately quantify the available (measure the amount of P solubilized). Other parameters were measured such as activities of acid phosphatase (APase) and alkaline phosphatase (ALPase), which will help indicate a possible link between P solubilization and tolerance to Cd.

The NBRIP medium was inoculated with 1 ml of a liquid bacterial culture (10^8 UFC/ml), and incubated at 150 rpm for seven days at 28°C. The supernatant of each PSB suspension was obtained by centrifugation (12000 rpm for 10min).

The available P fraction was estimated by spectrophotometry using the acid ascorbic method (*Appendix 2*) against P standards using KH_2PO_4. Optical density of both P from standards and samples were read at 880 nm and converted to P concentrations expressed in mg. L^{-1} (Fernandez et al., 2007).

The enzymatic activities of Phosphatase (APase and ALPase) were estimated by spectrophotometry using the Tabatabai method (*Appendix 2*), or the standard curve was traced using spectrophotometer at 405 nm using *p*NP 10 mM (*para* Nitrophenol). The optical density was read using the standard curve using the same wavelength and converted to APase and ALPase activities (nmol of *p*NP produced. $min^{-1}.ml^{-1}$) (Tabatabai and Bremner, 1969).

IV. In vitro seeds experiment germination inoculated by PSB and Co under Cd stress (10-day old seedlings)

1. Preparation of liquid bacterial inoculum

The medium used for inoculum preparation is TSB (30g/L). Under sterile conditions, the bacterial (B8, B12 and B31) solutions added were 1ml in each 3 flakes (*Appendix 3*), while for Consortium (Co) preparation, 0.5ml of each bacterial solution was added at a concentration of (10^8 UFC/ml). This was followed by incubation for 48h at 28°C under agitation (150rpm) in the dark. After 48h of incubation, the inoculum is collected in tubes and centrifuged at 12000rpm for 7min. The pellet is recovered and suspended with 30ml of sterile distilled water under gentle agitation and set at 4°C.

2. Sterilization of wheat seeds

Durum wheat (*Triticum durum*) seeds of KARIM variety were sterilized as follows:

- Sterilization in Bleach 6% for 2min;
- Washing with sterile distilled water for 5min;
- A second sterilization in 6% bleach for 2min;
- A second washing (3 times) with sterile distilled water for 5min.

3. Inoculation and germination experiment

Under sterile conditions, the sterile seeds were placed in 4 different sterile tubes that will be added with the 4 bacterial treatments (3 induvial and 1 consortium) followed by a gentle agitation at 150 mg. L^{-1} during 15 to 20min. Then, the inoculated seeds were placed on germination paper while adding 1ml of bacterial inoculum and 2ml of Cd-solution with a different concentration corresponding to each

treatment and Control (without bacterial treatment) (*Appendix 3*). Irrigation with sterile distilled water is necessary for the seed's germination.

V. Post-harvest analyses of 10-day old seedlings

1. Analyses of morphological traits

Morphological root trait analysis was done using LA2400 scanner and the WinRHIZO software (Regent Instruments Inc. Canada) (*Appendix 3*), which is a professional system of analysis of the roots of the plants allowing to scan to obtain images of which it can analyze the morphology (length, surface, volume...), the topology, the architecture and the color of roots. It consists of computer software and image acquisition components that can be combined to meet different needs. In this work, WinRHIZO was used to scan the roots of young seedlings (10-day trial) inoculated with the different bacterial isolates with different Cd concentrations.

2. Microscopic observation of roots

The Nikon SMZ25 Research Stereomicroscope (*Appendix 3*) combines macro and micro imaging in a single instrument to facilitate visualization and manipulation of single cells or whole organisms. The SMZ25 allows for the observation of roots with different magnification scales and illumination options that increase the versatility of the system.

I, this work, the Nikon SMZ25 was used to observe and differentiate the root architecture of young seedling (10-day old seedlings), according to the bacterial treatments (B8, B12 and B31) and at different $CdCl_2$ concentration (Cd [0], Cd [50] and Cd [100]). The parameters fixed are: Camera type Nikon DS-Fi3, Gain 1,0x, Magnification 1X, Zoom 4 and exposure time 8ms.

3. Determination of tissue P content and phosphatase activity in roots

To evaluate the effect of PSB isolates on P uptake by young plants (10-old day) and to get an idea of the P solubilization mechanism during the Cd stress test, the P contents of root tissues were determined by the ascorbic acid colorimetric method (*Appendix 2*) (Fernandez et al., 2007) and an assay of Phosphatase activity was carried out by the Tabatabai method using pNP substrate (*Appendix 2*) (Tabatabai and Bremner, 1969).

Aliquot of 200mg sample of fresh roots was ground (Retsch mill) with 2ml of acetate buffer (mixture of solutions A and B) for the P and APase assay and modified universal buffer for ALPase. The whole set was centrifuged for 10min at 13000rpm and then transferred to eppendorf tubes.

For the P assay, 1ml of supernatant was mixed with 160µl of reagent. After 30min of incubation, the DO was read at 880nm. P levels were determined by reference to a standard range established by KH_2PO_4.

For APase and ALPase activities, 125µl of supernatant (enzyme extract) was mixed with 125µl of pNPP (para-4-Nitrophenylphosphate) buffer. This was incubated for 30min at 30°C (For controls, the reaction was blocked directly by adding 500µl of NaOH and 500µl of $CaCl_2$). After incubation, the reaction was stopped by adding 500µl of NaOH and 500µl of $CaCl_2$. The DO reading was taken at 405nm. Phosphatase activity was determined by reference to a standard range established with pNP (para-Nitrophenyl) (nanomoles of pNP/hour/g fresh root).

VI. 75-day old plants growth experiment under PSB inoculation and Cd stress

The three bacterial isolates and their consortium were used in an experimental greenhouse test at the Experimental Farm in the Innovation and Technology Transfer Center for Agriculture (**AITTC**) to further evaluate their effects for their potential to promote plant growth. An agricultural soil (rich in nutrients), deficient in P (in the order of 5ppm) was sampled and autoclaved as well as the bags. OCP soil Rock Phosphate (containing total P: 30.65% (including 2 ppm of P available)) was prepared. Cadmium solution levels (Cd [50] and Cd [100]) were prepared and autoclaved. Then the surface disinfected seeds were inoculated with the different bacterial inoculum already prepared. For the non-inoculated control treatment, the disinfected seeds contained no bacterial culture.

The disinfected seeds were sown in bags containing autoclaved agricultural soil (1. 5kg/bag), the RP was added in the bags using 0.5g/kg equivalent to 0.75g/bag and then the soil was pre-irrigated with 20ml of sterile distilled water to moisten it, then the bacterial inoculum was added at a rate of 5ml/bag according to each treatment and 20ml of Cd solution at different concentrations (Cd [50] and Cd [100]) according to the treatments, for the control without Cd (Cd [0]), it was irrigated only by sterile distilled water. In total, three selected isolates (B8, B12 and B31) and their Consortium (Co) as well as the non-inoculated control and three levels of Cd were allocated under normal conditions and each treatment was replicated six times. 30 days later, a soil booster with bacterial inoculum corresponding to each

treatment was made with an amount of 2 ml/bag and the cultures were harvested after 75 days (*Figure 7*).

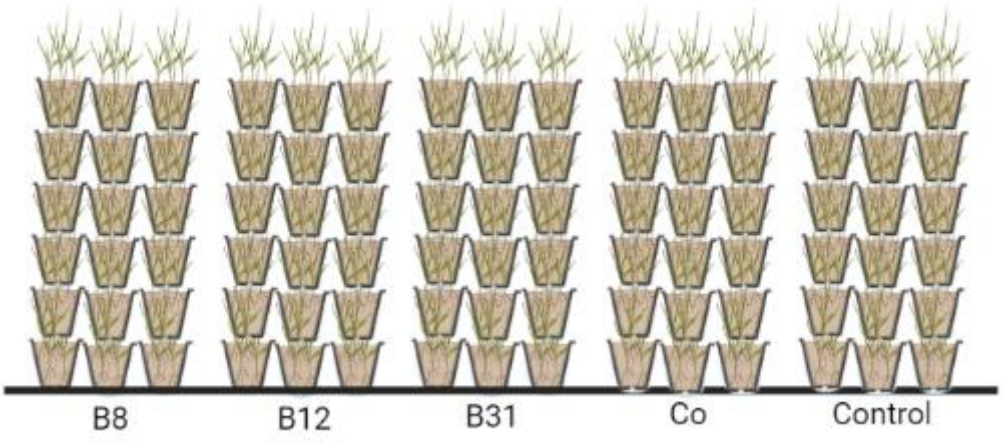


Figure 7 : *Experimental design of the trial of plant growth plants fertilized with natural P (RP) and inoculated with B8, B12, B31 and Co under three levels of Cd concentrations (Cd [0], Cd [50] and Cd [100]) is represented by 6 replicates distributed under normal greenhouse conditions. The control is the one without any bacterial culture.*

VII. In-situ analyses in 75-day old plants

1. Stomatal conductance

This parameter is measured by the SC-1 Leaf Porometer (*Figure 8*) which consists of a portable box with a cable connected to a leaf clip sensor. The final reading appears on the screen in terms of conductance or resistance; this device has the advantage of a non-destructive measurement. In this work, the SC-1 leaf porometer is used to measure the stomatal conductance (mmol/m^2.s) of leaves under PSB inoculation and Cd stress.

Figure 8 : *Stomatal conductance measurement in Wheat leaves inoculated with B8, B12, B31 and Co by the leaf porometer under three Cd-levels (Cd [0], Cd [50] and Cd [100]).*

2. Chlorophyll content index

Chlorophyll plays a crucial role in plant photosynthesis. Its content is an important indicator of plant nutritional stress.

The impact of cadmium on the photosynthetic efficiency of inoculated and non-inoculated plants was evaluated by measuring the chlorophyll content index (CCI) using a Chlorophyll meter (CL-O1, Hansatech instruments) (*Figure 9*) in the middle of leaves after 75days of treatment.

Figure 9 : *Chlorophyll content index measurement in Wheat leaves inoculated with B8, B12, B31 and Co by the Chlorophyll meter under three Cd-levels (Cd [0], Cd [50] and Cd [100]).*

3. Chlorophyll a fluorescence of leaves

Wheat leaves from the different treatments were kept in the dark for 15min before the chlorophyll fluorescence (CHF) measurements were started by the Handy PEA^+ fluorometer (Handy PEA^+, Hansatech instruments) (*Figure 10*). Then measurements were taken from a single strong 650nm light pulse of 3000 $\mu mol.m^{-2}.s^{-1}$ (which is an excitation intensity to ensure closure of all PSII reaction centers) for 1s, on the middle of each leaf already darkened.

Figure 10 : *Measurement of Chlorophyll a fluorescence in wheat leaves inoculated with B8, B12, B31 and Co by fluorometer under three Cd-levels (Cd [0], Cd [50] and Cd [100]).*

4. Phenotypic characteristics of plants

Phenotypic characteristics are descriptive tools that allow to differentiate the morphology of different treatments. This study is conducted to evaluate the morphological characteristics that are based on a set of parameters related to the morphology and agronomic characteristics of the plant, such as plant height, number of leaves, spike length and number of spikes for each treatment after 75day.

VIII. Post-harvest analyses in 75-day old plants

1. Plants dry weight

All treated and untreated plants were dried in an oven at 70 °C for 48h to determine root, shoot and spikes dry weights.

2. Morphological traits roots

After root harvesting, the plants were firstly washed with distilled water and root parts were immediately dipped to analyze the root architecture using the LA2400 scanner and WinRHIZO software (Regent Instruments Inc. Canada). Root morphology measurement focused mainly on total root length, average root diameter, and total volume roots.

3. Determination of P in rhizosphere and roots

To evaluate the effect of PSB isolates on rhizosphere P availability in 75-day old roots P uptake under Cd stress. Rhizosphere and root P levels were determined using the ascorbic acid colorimetric method (*Appendix 2*) (Fernandez et al., 2007).

For P content in rhizosphere, 20ml of 0.5M sodium bicarbonate was added to 1g of dry soil. After 20min of agitation, the extracts were filtered. Then, 1ml of the filtrate was added to 160µl of reagent. For the root tissue part, a 200mg sample of fresh material was ground (Retsch mill) with 2ml of acetate buffer (mixture of solutions A and B). The whole set was centrifuged for 10min at 13000rpm and then transferred to an Eppendorf tube. 1ml of supernatant was mixed with 160µl of reagent. After 30min of incubation, the DO was read at 880nm and P content were determined by reference to a standard range established by KH_2PO_4.

4. Determination of enzymatic activity in rhizosphere and roots

To evaluate APase and ALPase activity of PSB and Co in soil, 500µl of buffer and 125µl of 10mM pNPP (A were added to 0.125g of soil and vortexed and incubated for 20min at 37°C. After incubation, 125µl of $CaCl_2$ (0.5M) and 500µl of NaOH (0.5M) were added successively to stop any enzymatic reaction. Then the whole set was centrifuged at 15000rpm for 5 min, 150 µl of supernatant was transferred to the cuvettes and the assay reading was taken at 405nm.

Aliquot 200 mg sample of the fresh roots was ground (Retsch grinder) with 2ml of acetate buffer (mixture of solution A and B) (*Appendix 2*). The whole set was centrifuged for 10 min at 13000rpm and then transferred to an eppendorf tube. 125µl of supernatant (enzyme extract) was mixed with 125µl of *pNPP* buffer (para-4-Nitrophenylphosphate). The whole was incubated for 30 min at 30°C (For controls, the reaction was blocked directly by adding 500µl of NaOH and 500µl of $CaCl_2$). After incubation, the reaction was stopped by adding 500µl of NaOH and 500µl of $CaCl_2$. The DO reading was taken at 405 nm. Phosphatase activity was determined by reference to a standard range established with pNP (para-Nitrophenyl).

5. Statistical analysis

This experiment was designed in a completely randomized factorial arrangement. It was performed with two factors: 1) three bacterial isolates, a consortium as well as an uninoculated treatment (B8, B12, B31, Co and Control), and, 2) factor is the concentration of Cd (Cd [0], Cd [50] and Cd [100]) (5 treatments ×3 Cd-levels with 6 replicates). Analysis of variance (ANOVA) was performed to analyze the data using SPSS software ver. 20 (IBM-SPSS Inc. Chicago, IL). The means of the treatments were compared using the HSD Tukey post hoc test at a 5% level of significance and the graphs were used by the GraphPad Prism 8 software.

Results and Interpretations

Results and interpretations

I. PGPR traits and minimum inhibitory concentration (MIC) determination

The Cd-tolerant isolates were identified and subjected to functional analysis with respect to plant growth promotion characteristics. Their biochemical characteristics (quantitative and/or qualitative) were determined (*Tab. 1*) such as P solubilization, N fixation, IAA production, CCA production and siderophore production.

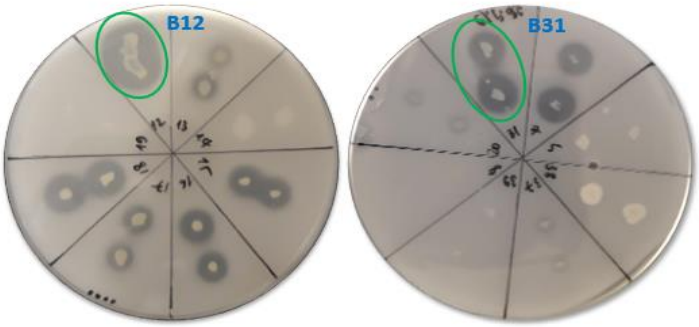


Figure 11 : *Halos indication of P solubilization of two isolates B12 and B31 in NBRIB solid medium in vitro.*

Based on the qualitative P solubilization test (*Fig. 11*), the figure shows the bacteria placed in a solid nutrient medium (NBRIP) to evaluate their P solubilization halos in order to determine the most important isolates capable of solubilizing P. The presence of P solubilization halos are observed in both isolates B12 and B31. In order to evaluate P solubilization in the isolates, a quantitative P solubilization assay was initiated to quantify PSB. Three bacteria (B8, B12 and B31) that show a better P solubilization capacity were selected and then the biochemical characteristics of these isolates are presented in the following table (*Tab. 1*). Therefore, they show significant P solubilization in vitro and can be considered as PSB.

Table 1 : *Plant growth-promoting traits of three PSB isolates and their MIC under three Cd-concentrations* (Cd [0], Cd [50] and Cd [100]). *Different letters indicate values that are statistically different at p < 0.05.*

Bacteria	Solubilization Index	N_2 Fixation	AIA (µg/ml)		ACC	Siderophore		Selection test Cd 20ppm	MIC determination				
			Tryp +	**Tryp -**	**Qualitatif**	**P.C**	**%**		**Cd50**	**Cd100**	**Cd200**	**Cd300**	**Cd400**
B12	4.55	-	1.14b	0	+++	2ab	44.84a	++++	++++	++++	+++	+	-
B31	0.39	+	31.99a	20.13a	+++	2.83a	44.35ab	++++	++++	++++	+++	++	-
B8	0	++	0	0.22b	-	1.5b	19.91b	++++	++++	++++	+	-	-

- : Negative development, **+:** Positive development, **P.C:** Production capacity,

All isolates were evaluated for their potential to promote plant growth (*Tab. 1*). The solubilization index of P was observed with 'IS 4.55' and 'IS 0.39' respectively in the two isolates B12 and B31 that developed solubilization halos (*Fig. 11*). The same isolates B12 and B31 were able to produce IAA although in different amounts in the presence of L-tryptophan with 1.14 and 31.99µg/ml respectively along with the ability to produce ACCD. While in the absence of L-tryptophan the two isolates B8 and B31 produced IAA with amounts of 0.22 and 20.13µg/ml respectively as well as the ability to fix N. All three isolates (B8, B12 and B31) have the capacity to produce siderophores with a significant value of 44.84% in B12, 44.35% in B13 and a low value of 19.91% in B8. While only isolates B8 and B31 showed nitrogen fixation. And isolates B12 and B31 showed ACC deaminase activity.

A first selection of these isolates was made on a solid medium with Cd [20], and they all developed Cd tolerance. In addition, a second selection was made on solid medium in the presence of different Cd concentrations to determine their MIC. The three isolates (B8, B12 and B31) were selected as highly Cd-tolerant bacteria, showing a minimum inhibitory concentration less than or equal to Cd [300]. Isolate B31 showed the highest positive development compared to the other two isolates under Cd stress.

II. Cadmium tolerance test of isolated PSB isolates

1. P solubilization and Phosphatase activity by PSB from TCP and RP

To evaluate the ability of different isolates and their Consortium to solubilize P, the P and Phosphatase activity were determined from two P sources (TCP and RP) and in the presence of different Cd concentrations (Cd [0], Cd [50], Cd [100], Cd [200] and Cd [300]).

Figure 12 shows the solubilization of P (µg/ml) by the three isolates selected from two forms of P: TCP (*Fig. 12A*) and RP (*Fig. 12B*), in a growth medium with five levels of Cd. And the tables (*2 and 3*) show the production of acid and alkaline phosphatases (µmol/ml/min) from the forms of P (TCP or RP) in the same five levels of Cd concentrations, which help bacteria to solubilize P.

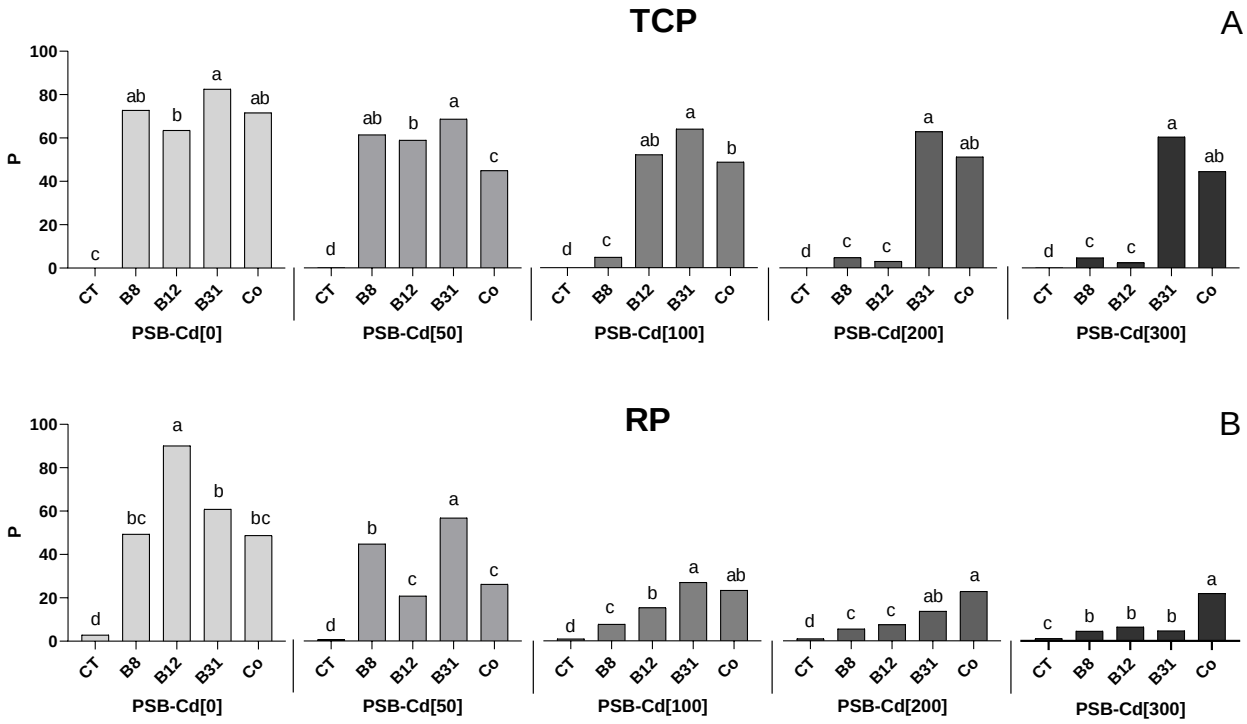


Figure 12 : *Determination of P content (µg/ml) of the three isolates selected (B8; B12 and B31) and their Consortium (Co) under five Cd-concentrations* (Cd [0], Cd [50], Cd [100], Cd [200] and Cd [300]) *in presence of TCP(A) or RP(B). Different letters indicate values that are statistically different at $p < 0.05$.*

The solubilization of P differs from isolate to isolate and from concentration to concentration. But all isolates can solubilize P from both forms (Fig. 12) and their growth is not inhibited by increasing Cd stress on the bacteria. In contrast, this P solubilizing activity is weak or absent in the non-inoculated treatment at all Cd levels. At Cd [0], the inoculated treatments show a high P solubilization activity especially in isolate B31 from the TCP form and isolate B12 from the RP form. This activity remains important compared to the control by increasing the concentration of Cd [50] and Cd [100], it is significantly high especially in isolate B31 in case of TCP and RP, and it decreased especially in isolate B8. Under Cd stress (Cd [200] and Cd [300]), solubilization decreased strongly in both isolates B8 and B12, and was significantly elevated in isolate B31 for both concentrations from TCP form and in isolate B12 for both concentrations from RP form.

Table 2 : *The APase (A) and ALPase (B) levels in the presence of TCP produced by PSB and Co isolates under three Cd concentration levels (Cd [0], Cd [50] and Cd [100]). Different letters indicate values that are statistically different at p < 0.05.*

	Acid Phosphatase-TCP (µmol/ml/min) **A**				
PSB	**Cd [0]**	**Cd [50]**	**Cd [100]**	**Cd [200]**	**Cd [300]**
B12	65.35^{a}	28.45^{ab}	39.4^{ab}	47.3^{a}	57.65^{a}
B31	32.87^{b}	16.3^{b}	15.3^{c}	8.9^{c}	31.55^{b}
B8	52.55^{ab}	29.95^{ab}	28.15^{b}	25.9^{b}	24.4^{c}
Co	60.5^{a}	37.25^{a}	48.35^{a}	35.55^{ab}	35.25^{b}

	Alkaline Phosphatase-TCP (µmol/ml/min) **B**				
PSB	**Cd [0]**	**Cd [50]**	**Cd [100]**	**Cd [200]**	**Cd [300]**
B12	80.95^{a}	76.75^{a}	102.3^{a}	104.3^{a}	215.8^{a}
B31	60.1^{ab}	67.65^{ab}	96.8^{a}	50.3^{c}	20.35^{c}
B8	47.45^{b}	49.55^{b}	54.55^{b}	84.75^{b}	98.7^{b}
Co	74.8^{ab}	59.5^{b}	64.4^{ab}	$66.9b^{c}$	81.8^{bc}

Table 3 : *The APase (A) and ALPase (B) levels in the presence of RP produced by PSB and Co isolates under three Cd concentration levels (Cd [0], Cd [50] and Cd [100]). Different letters indicate values that are statistically different at p < 0.05.*

	Acid Phosphatase-RP (µmol/ml/min) **A**				
PSB	**Cd [0]**	**Cd [50]**	**Cd [100]**	**Cd [200]**	**Cd [300]**
B12	50.1^{b}	13.9^{b}	17.8^{b}	27.9^{b}	47.8^{ab}
B31	124.1^{a}	12.7^{b}	16.5^{b}	37^{ab}	47.5^{ab}
B8	52.8^{b}	15^{b}	21.1^{b}	37.5^{ab}	55.1^{a}
Co	124.55^{a}	85.7^{a}	64.05^{a}	58.55^{a}	47.95^{ab}

	Alkaline Phosphatase-RP (µmol/ml/min) **B**				
PSB	**Cd [0]**	**Cd [50]**	**Cd [100]**	**Cd [200]**	**Cd [300]**
B12	53.3^{c}	42.7^{ab}	111.65^{a}	148.05^{a}	194.85^{a}
B31	166.35^{b}	54.35^{a}	57.65^{b}	80.6^{b}	75.5^{b}
B8	188.55^{a}	41.85^{ab}	50.65^{b}	75.65^{b}	98.75^{b}
Co	71.8^{bc}	15^{c}	10.3^{c}	30.7^{c}	67.1^{c}

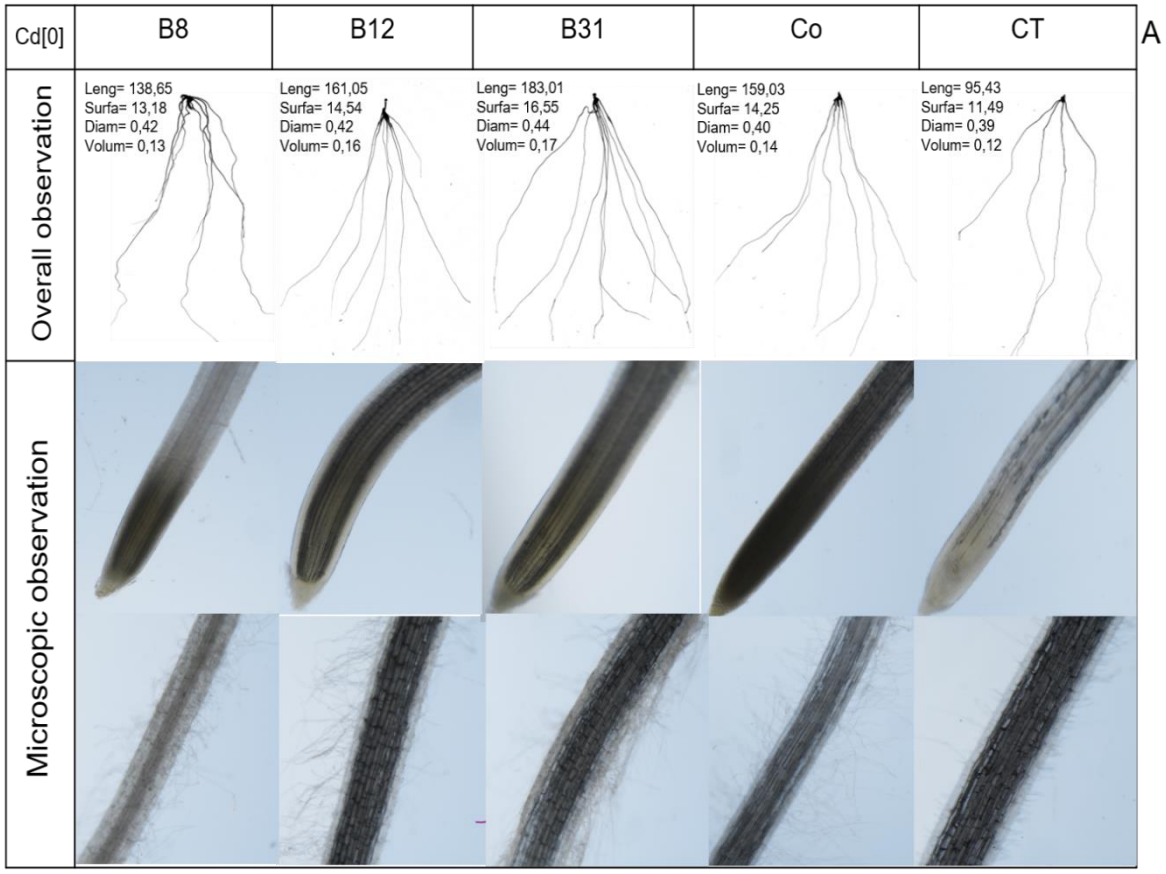
The results obtained in *Table 2* show that acid phosphatase activity is almost in excess in the presence of TCP at Cd [0] with a concentration of 65.35(µmol/ml/min) in isolate B12 and 60.5 in Co. The activity is significantly elevated at Cd [50] in Co with 37.25(µmol/ml/min) and 48.35(µmol/ml/min) at Cd [100]. For the two concentrations Cd [200] and Cd [300] the B12 isolate presents 47.3 and 57.65(µmol/ml/min) respectively. It should be noted that the activity of alkaline phosphatases from TCP is significantly in excess at all Cd concentrations in isolate B12.

Thus, APase is in excess in the presence of RP (*Tab. 3A*) in isolates B31 and Co has Cd [0], and in significant excess in Co with 85.7; 64.05; 58.55(µmol/ml/min) at concentrations Cd [50], Cd [100] and Cd [200] respectively. While ALPase production is very significant in B31 in the presence of RP (*Tab. 2B*) and in all three isolates and Co by values of 111.65; 148.05 and 194.85(µmol/ml/min) at Cd [100], Cd [200] and Cd [300] concentrations, respectively.

III. Post-harvest analyses in 10-day old seedling

1. Observation of roots morphology

Root observation was done with a stereomicroscope (Nikon SMZ25) to observe the piliphere zone and root caps of PSB-treated and untreated roots as well as root morphology observation of different treatments was done by WinRHIZO (LA2400 scanner) at different Cd concentrations (Cd[0], Cd[50] and Cd[100]).



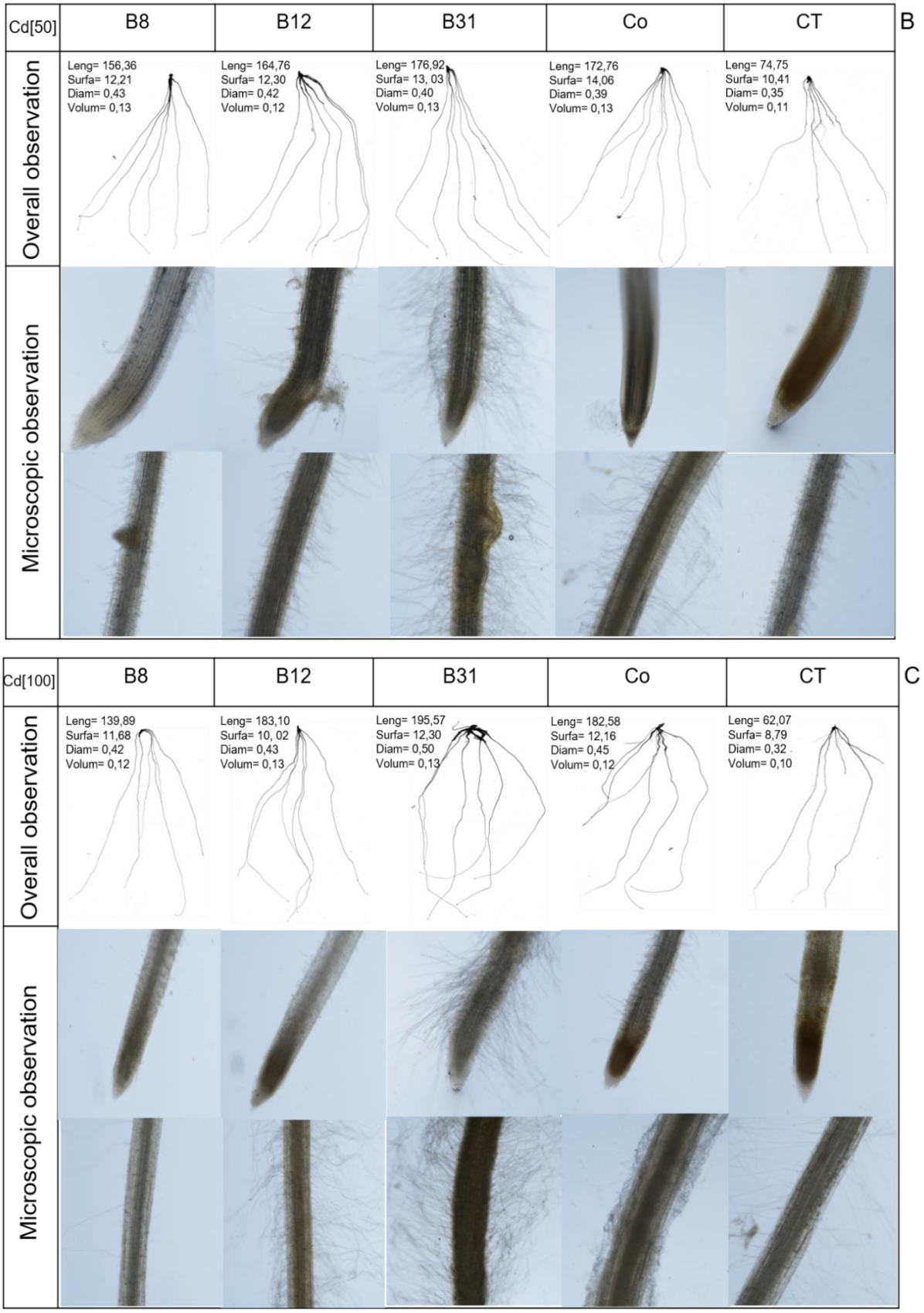


Figure 13 : *Morphological traits illustration of root observation by WinRHIZO scanner and Stereomicroscope (cap and piliferous zone) of plants inoculated with PSB and Co and non-inoculated plants at three levels of Cd concentrations (Cd [0] (A), Cd [50] (B) and Cd [100] (C)). Accompanied with morphological parameters such as root length (cm), area (cm^2), diameter (mm) and volume(cm^3).*

At the level of a global and microscopic observation of the roots at Cd [0], we notice that all isolates have well developed roots compared to the Control. (*Fig. 13A*). While at Cd [50] (Fig. 13B) and at a global observation, the development of the treated roots was improved compared to the control. And at the level of observation by stereomicroscope, the treated roots with B31 and Co showed absorbing hairs development. Roots of 10-day-old seedlings treated with Cd [100] concentration showed an improvement in length and volume development as well as well-developed absorptive hairs especially for isolate B31 and Co (*Fig. 13C*).

2. Determination of P and Phosphatase activity in roots

The results obtained (Figure 14) show that the P and Pase content in roots of plants inoculated with **BSP** isolates and **Co** is significantly higher than that of the non-inoculated controls.

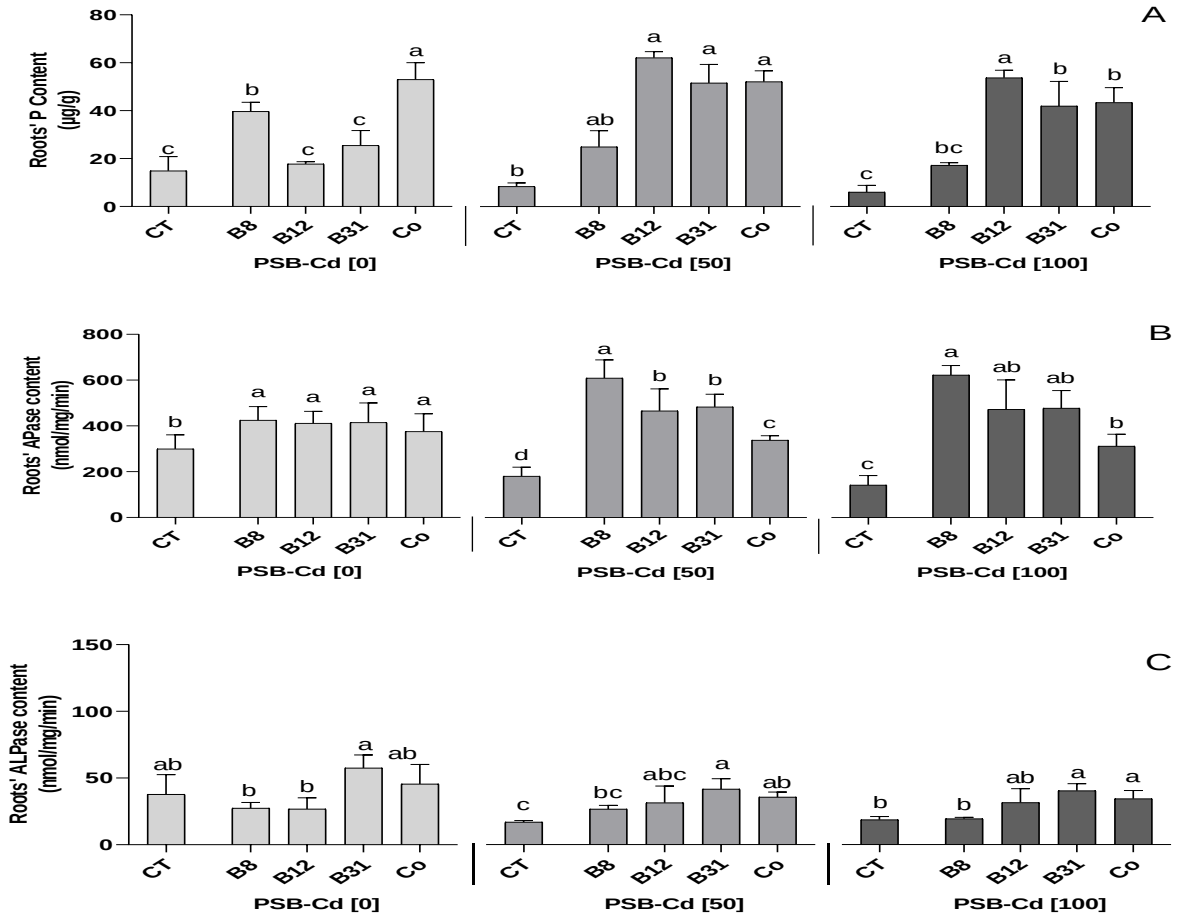


Figure 14 : *Phosphorus (µg/g) (A) Acid phosphatase (nmol/mg/min) (B), Alkaline phosphatase (nmol/mg/min) (C) content in the roots of germination test (10 days) with four treatments (B8, B12, B31 and Co) and control; the absence and presence of Cadmium (Cd [0]), (Cd [50]) and (Cd [100]). Different letters indicate values that are statistically different at $p < 0.05$.*

Figure (*Fig. 14A*) shows the P content in treated and untreated plants by the different isolates. Under normal conditions (Cd [0]), with the exception of the two treatments B8 and Co which have a significantly high P content compared to the other treatments and the control. The highest content is noted without the roots inoculated with Co with 54 µg/g (P). In the presence of Cd, the three inoculants B12, B31 and Co have a significant increase in root P content compared to B8 and the control and the highest value is noted in isolate B12 with 65µg/g and 55µg/g P under both Cd [50] and Cd [100] concentrations respectively.

The four inoculations (B8, B12, B31 and Co) showed significantly higher APase levels than the non-inoculated controls at the different Cd levels (*Fig. 14.B*). The results obtained (*Fig. 14.C*) show that the ALPase content in roots of plants inoculated with isolate B31 is significantly higher than in the other treatments and presents a content of 60 nmol/mg/min in the absence of Cd. In the presence of Cd (Cd [50] and Cd [100]), the highest levels of ALPase were found in both inoculants B31 and Co.

IV. Pre-harvest analyses in 75-day old plants

1. Stomatal conductance

The *figure 15* shows the effect of Cd stress on the stomatal conductance of different treated and untreated plants.

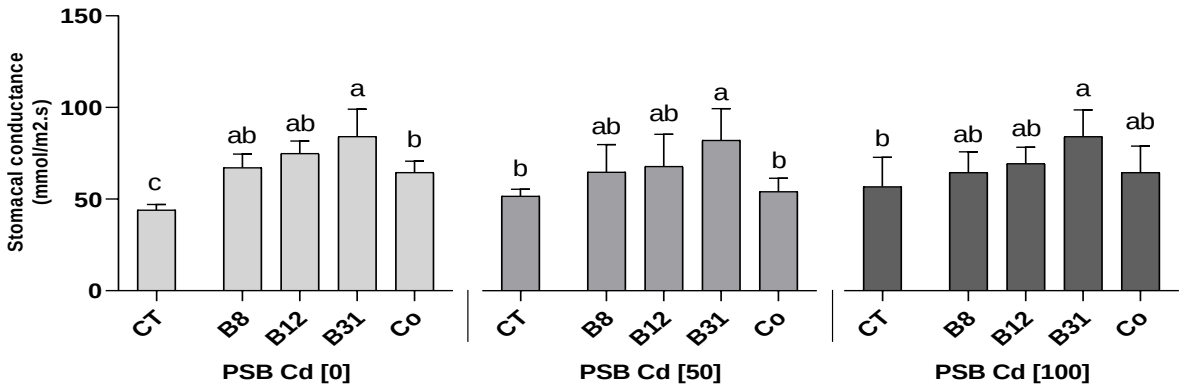


***Figure 15** : Leaf stomatal conductance of Wheat leaves under cadmium stress (Cd [0]), (Cd [50]) and (Cd [100]) after 75 days. Different letters indicate values that are statistically different at $p < 0.05$.*

The results obtained (*Fig. 15*) show that the conductance of the stomata in the leaves of plants inoculated with the different isolates and Co at a concentration of 0, are significantly higher than that of the non-inoculated controls. The highest value was recorded in plants treated with isolate B31 (84mmol/m^2.s) compared to the control (45mmol/m^2.s). In the presence of Cd, isolate B31 showed a significant increase with a content of (75mmol/m^2.s) at both Cd [50] and Cd [100] concentrations compared to the control (5o and 55 mmol/m^2.s) at Cd [50] and Cd [100] respectively.

2. Chlorophyll content index

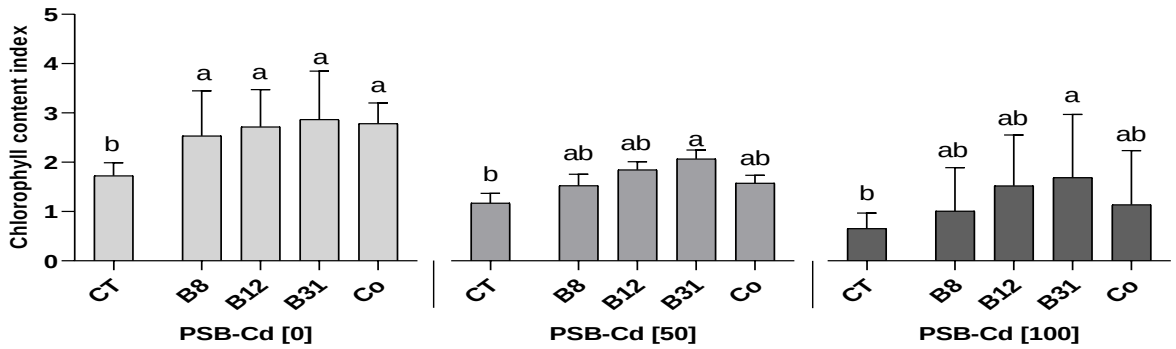


***Figure 16** : Chlorophyll content index of Wheat leaves inoculated with PSB (B8, B12, B31 and Co) and control under Cadmium (Cd [0]), (Cd [50]) and (Cd [100]). Different letters indicate values that are statistically different at $p < 0.05$.*

For chlorophyll content in leaves (*Fig. 16*) and under normal Cd [0] conditions, all inoculated treatments had significantly higher chlorophyll content than the untreated treatment. In the presence of Cd [50] and Cd [100], activity was significantly elevated in leaves inoculated with isolate B31 "CCI 2.4" compared to the control "CCI 1.3" with a concentration of Cd [50] and "CCI 1.8" compared to the control "CCI 0.7" with a concentration of Cd [100].

3. Chlorophyll a fluorescence intensity

The plants grown in culture medium with different isolates and Co showed a typical Chlorophyll a polyphasic fluorescence rise OJIP transient (*Fig. 17*) exhibited by Cd stressed Wheat plants. The transients are plotted on a logarithmic time scale. The first O–J phase represents the reduction of the acceptor side of PSII and J–I phase represents the reduction/oxidation of the plastoquinone (PQ) and the last phase I–P represents the re-reduction of plastocyanin (PC)+ and P700+ in PSI (Naciri et al., 2021).

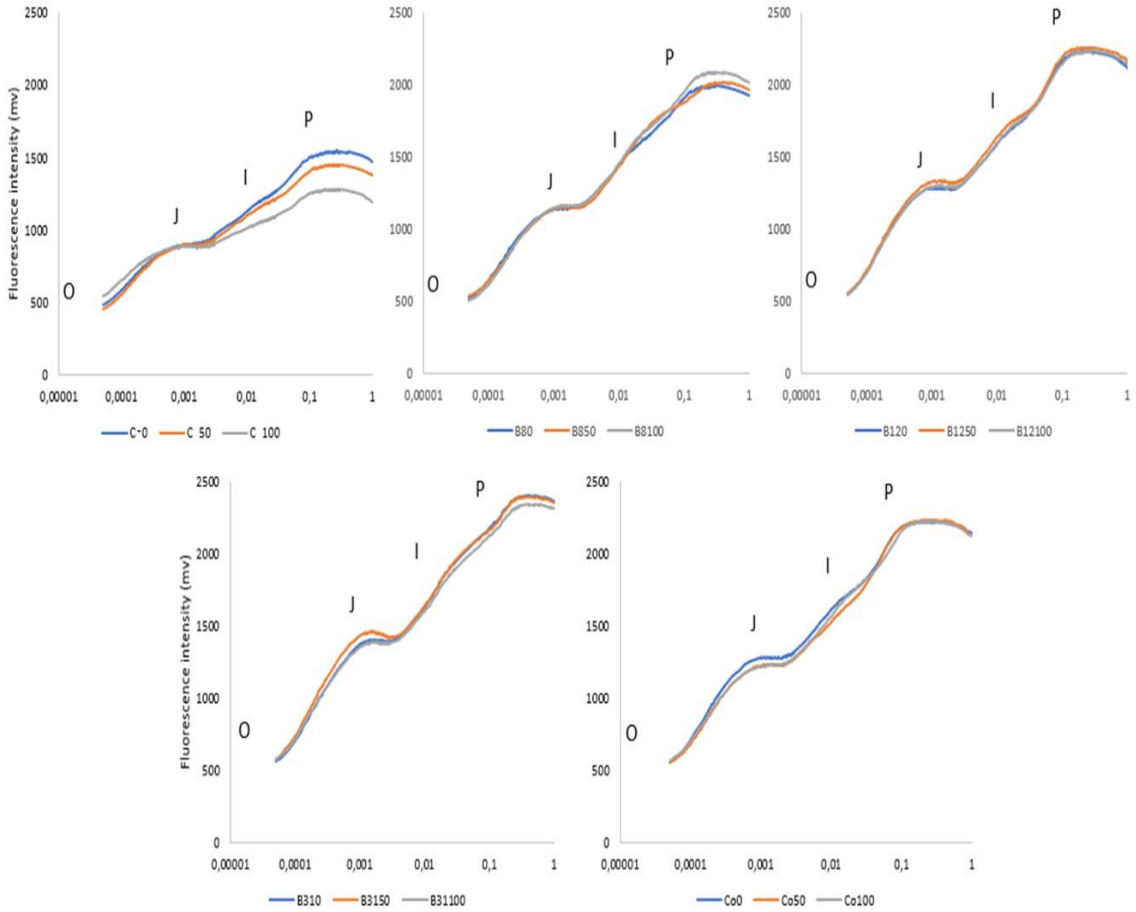


Figure 17 *: Effects of three Cadmium concentrations (Cd [0]), (Cd [50]) and (Cd [100]) on Chlorophyll a fluorescence transient curve of plants inoculated with PSB (B8, B12, B31 and Co) and control. The different letters represent the phases of chlorophyll fluorescence transition. The first O–J phase represents the reduction of the acceptor side of PSII and J–I phase represents the reduction/oxidation of the plastoquinone (PQ) and the last phase I–P represents the re-reduction of plastocyanin (PC)+ and P700+ in PSI.*

We noticed here (*Fig. 17*) that OJIP fluorescence transients were affected under two concentrations of Cd [50] and Cd [100], compared to Cd [0] concentration in non-inoculated plants. This explains that the reduction of the PQ pool and the electron transport chain through the PSI were altered by the Cd treatment. In the case of inoculated plants, the typical ChlF OJIP transient was increased in all inoculated treatments and under different Cd levels; this shows the ability of the selected isolates to increase and protect the photosynthetic processes in plants under Cd stress. Indeed, we noticed that plants treated with B31 showed a better observable increase in OJIP phases (FI 2400) both in the presence and absence of Cd compared to the control (FI 1550; FI 1450 and FI 1350 respectively under the three Cd levels Cd [0], [50] and Cd [100].

4. Phenotypic characters

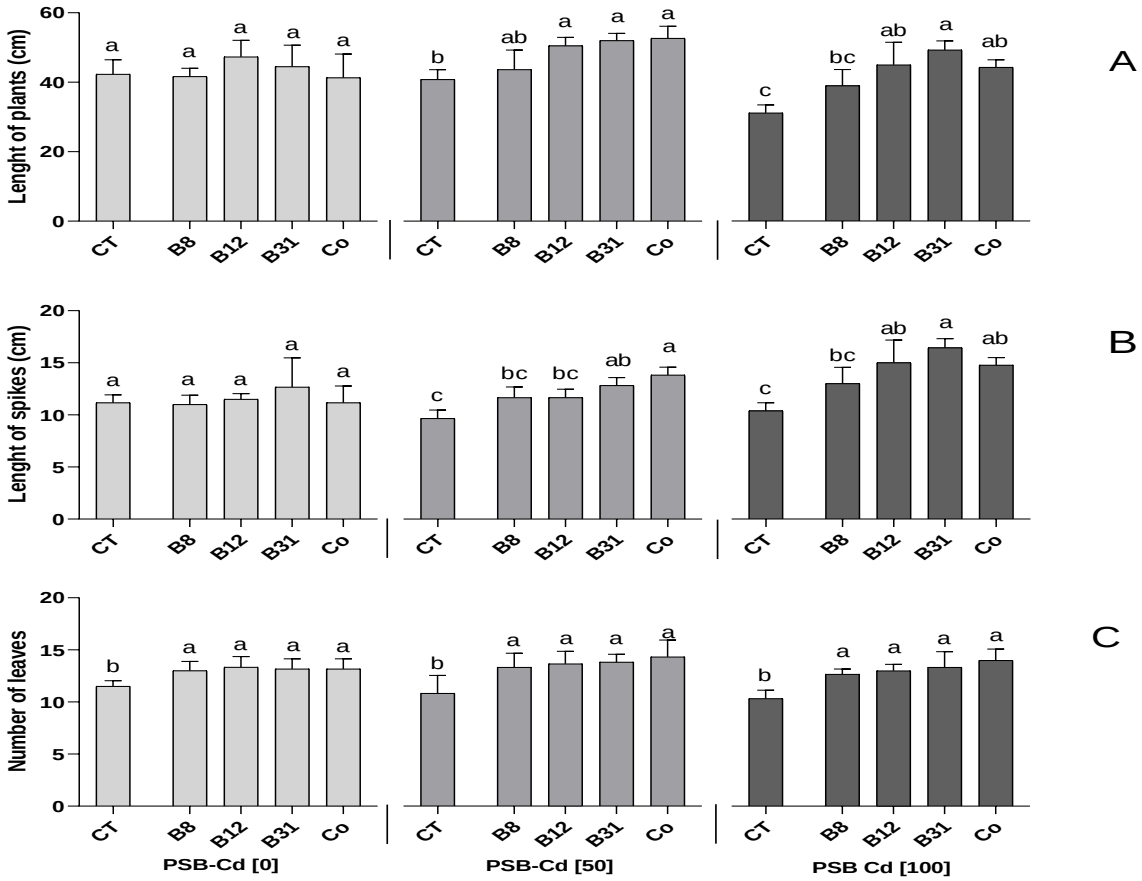


Figure 18 : *Measurement of plants length (A), spikes length (B) and number of leaves (C) on plants inoculated with different treatment (B8, B12, B31, Co and Control) at three Cd-concentrations (Cd 0, 50 and 100 ppm) after 75 days. Different letters indicate values that are statistically different at $p < 0.05$.*

The results obtained by measuring phenotypic traits (*Fig. 18*) showed a significant improvement in the length of plants (*Fig. 18A*) inoculated with B12, B31 and Co under both Cd [50] and Cd [100], with a maximum value noted in plants treated with isolate B31 (50cm) compared to the control (30cm) at Cd [100].A significant increase in spike length (*Fig. 18B*) of plants inoculated with B31 and Co in the presence of two Cd concentrations with a higher value recorded in plants treated with Co (15 cm) compared to the control (9 cm) at concentration Cd [50] and in plants treated with B31 (18 cm) compared to the control (10 cm) at concentration Cd [100]. All plants treated with different inoculants showed a significant increase in the number of ears (*Fig. 18C*) compared to the untreated controls in presence and in absence of different Cd concentrations.

V. Post-harvest destructive analyses for inoculation plants test

1. Determination of shoot and root dry weight

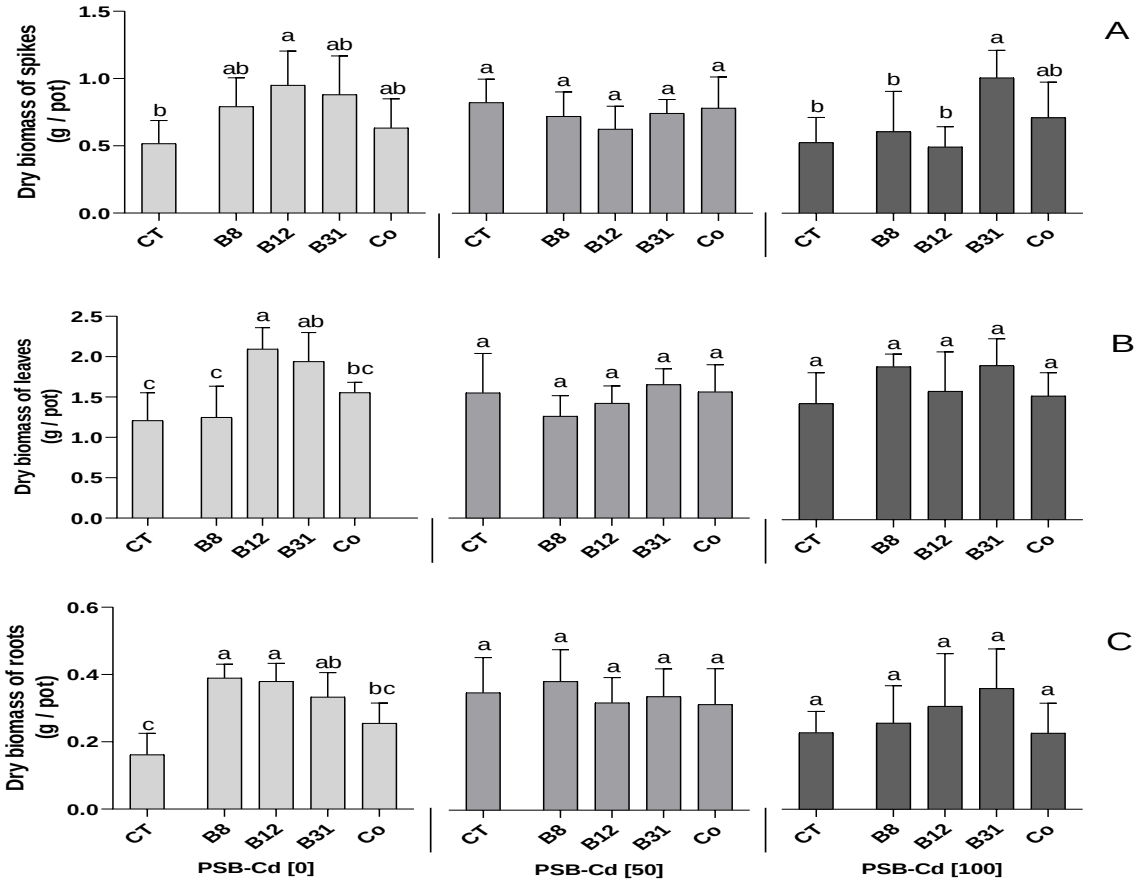


Figure 19 : *Dry weights of spikes (A), leaves (B) and roots (C) on wheat plants inoculated with PSB (B8, B12 , B31 and Co) and control at three Cd-concentrations (Cd [0]), (Cd [50]) and (Cd [100]) in 75-day old plants. Different letters indicate values that are statistically different at $p < 0.05$.*

Under normal conditions Cd [0], dry weight of ears (A), leaves (B) and roots (C) were significantly higher in treatments inoculated with (B12 "1g/pot"), (B12 "2.1g/pot" and B31 "2g/pot"), and (B8 "0.4g/pot", B12 "0.38g/pot", and B31 "0.3g/pot") respectively, compared to control plants "0.6g/pot" "1.3g/pot" and "0.18g/pot". Under Cd stress [100], only the treatment inoculated with B31 significantly increased the dry weight of ears (A) with a value of "1g/pot" compared to the control "0.5g/pot" and the other treatments.

2. Determination of roots morphological parameters

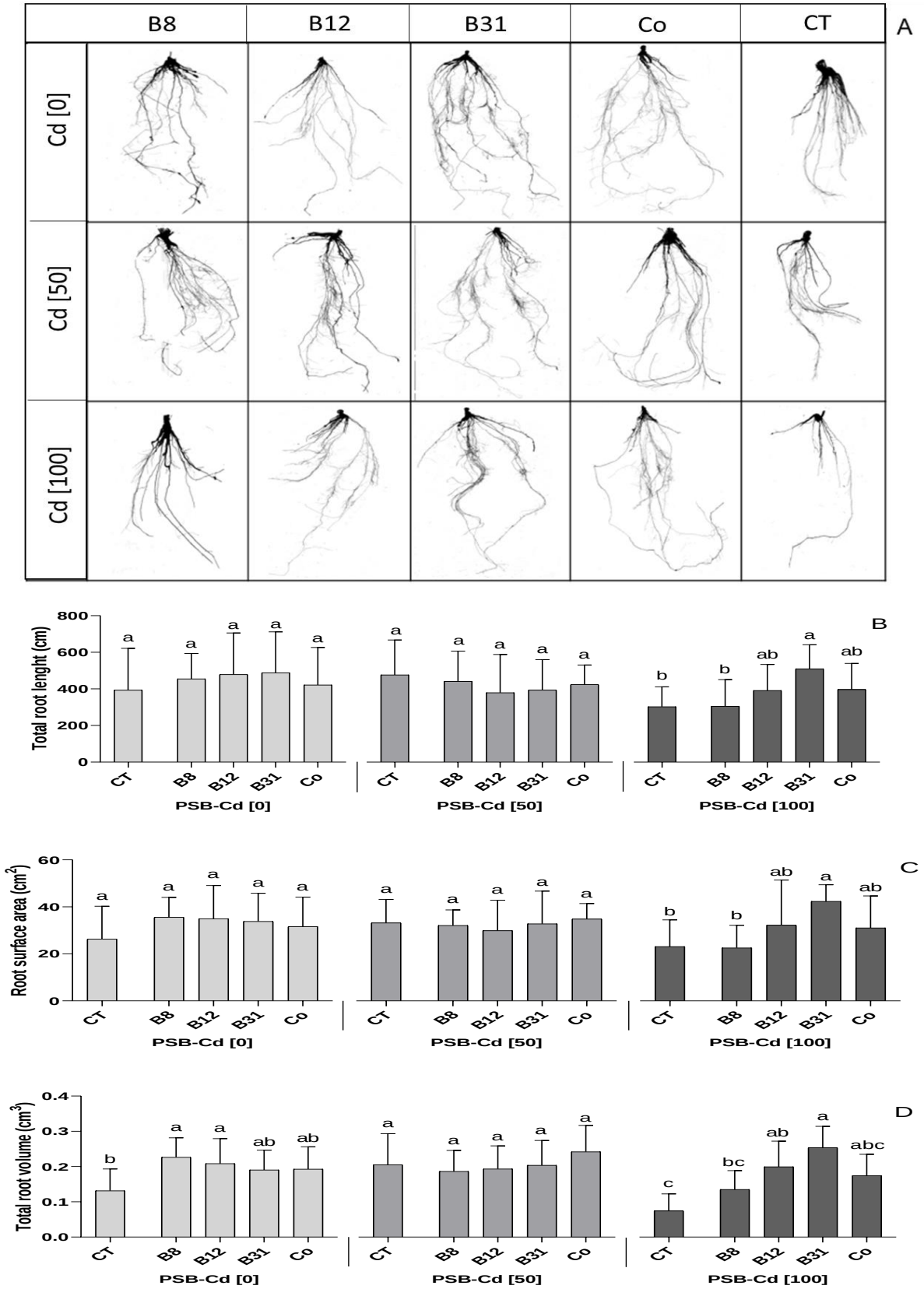


Figure 20 : *Root morphological traits (A) accompanied with their parameters in response to different treatments (B8, B12, B31, Co and control): (B) total root length (cm), (C) root surface area (cm^2) and (D) total root volume (cm^3). Different letters indicate values that are statistically different at $p < 0.05$.*

The data collected by WinRHIZO and based on the global observation of the roots (*Fig.20A*) showed that Cd exposure affects the normal growth of the roots at different Cd concentrations and a remarkable observation especially in the roots treated with B31. From the analysis of morphological traits, the inoculation of plants with B31 showed a promoting effect on the total length of roots (B) with a value of almost "500 cm" compared to the control "300 cm", as well as the surface of roots (C) with a value of "45 cm^2" compared to the control "23 cm^2" at high concentration of Cd [100]. Thus, both isolates B12 and B31 also significantly increased the root volume (D) at Cd [100]. The highest value was recorded in the roots inoculated with B31 "0.27 cm^2" when compared with the control "0.08 cm^2".

3. Determination of P and Phosphatase activity in rhizospheric soil

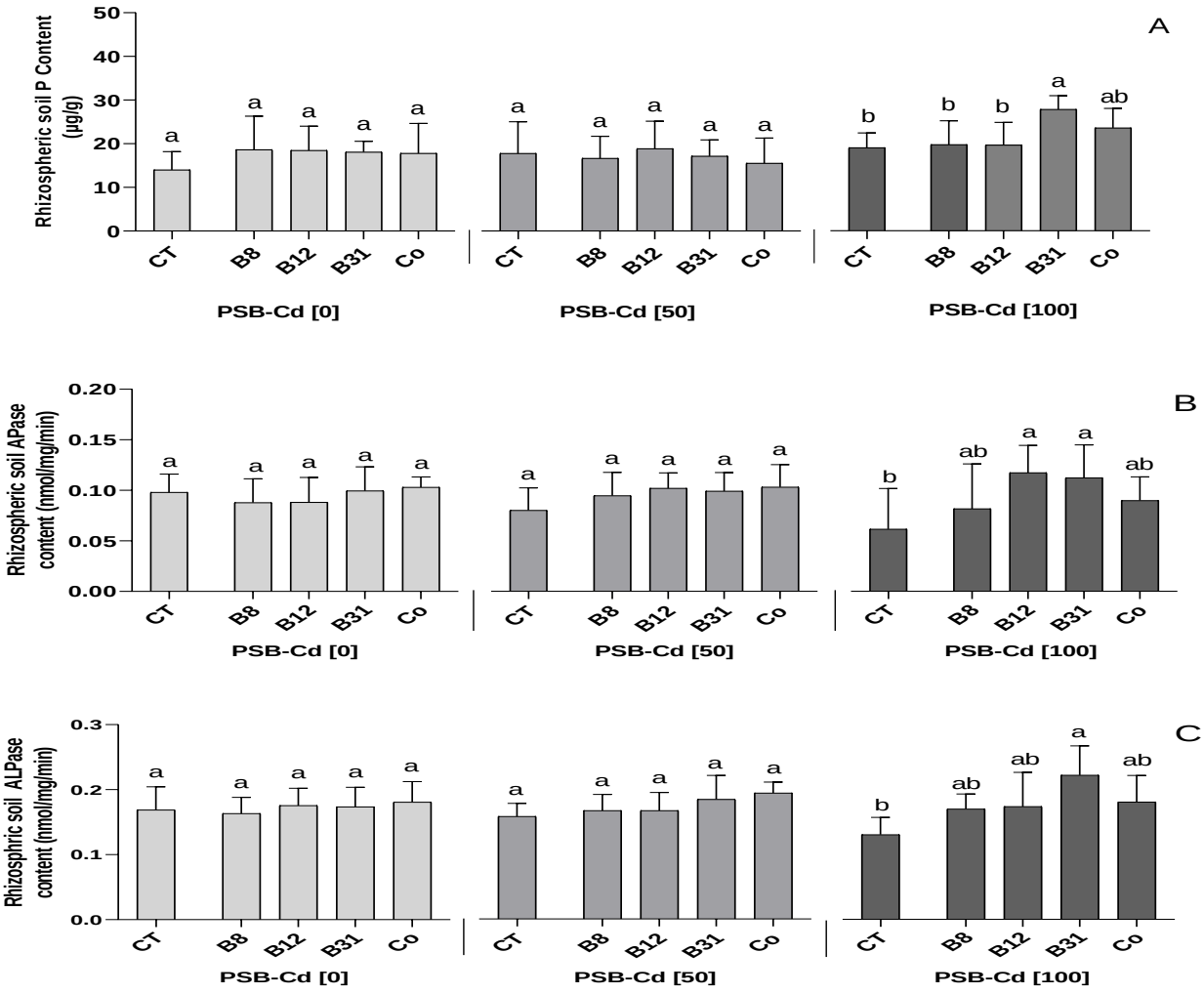


Figure 21 : *The P content (µg/g) (A) APase content (nmol/mg/min) (B) and ALPase content (nmol/mg/min) (C) in rhizospheric soil of inoculated plants with four treatments (B8, B12, B31 and Co) and control; the absence and presence of Cadmium (Cd [0]), (Cd [50]) and (Cd [100]). Different letters indicate values that are statistically different at $p < 0.05$.*

Available P content was measured in rhizospheric soils of Wheat crops to determine the effect of isolates and Co on P availability. Figure 21 (*Fig. 21A*) shows that the available P content in rhizospheric

soil of seedlings inoculated with isolate B31 (30µg/g) at a concentration of Cd [100] is high compared to that recorded in the control soil (18µg/g).

For the activity of APase in soil (*Fig. 21B*), it is noted that both isolates B12 and B31 have a significant positive effect on the content of APase in rhizospheric soils inoculated with these isolates in a concentration of Cd [100], with values of 0.13 and 0.11 (nmol/mg/min) respectively compared to the control (0.06 (nmol/mg/min). The highest ALPase content (*Fig. 21C*) was recorded in the soils inoculated with isolate B31 with a content of 0.23 (nmol/mg/min) of soil compared to control (0.12 nmol/mg/min).

4. Determination of P and Phosphatase activity in roots

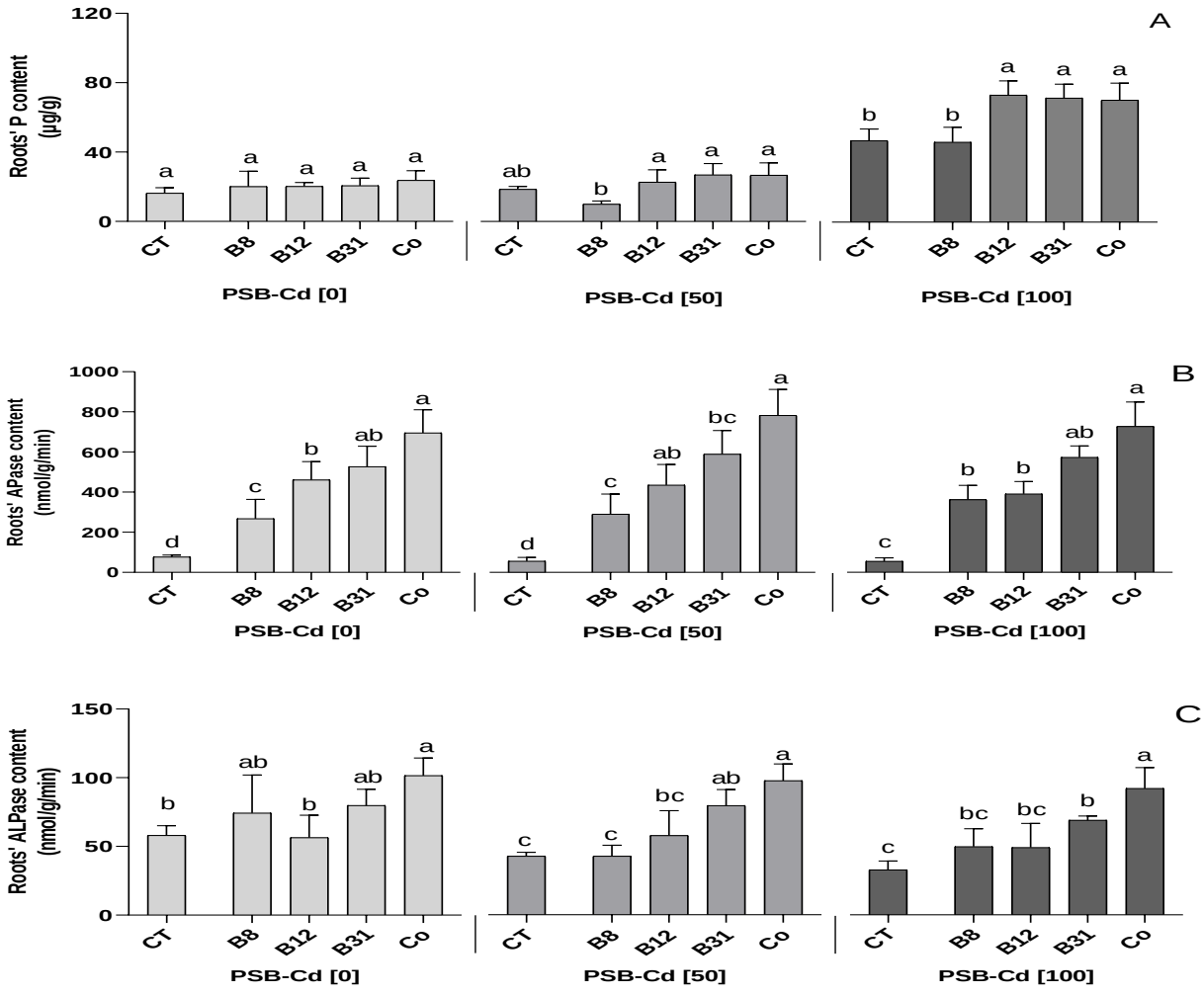


Figure 22 : *The P content (µg/g)(A) APase content (nmol/mg/min) (B) and ALPase content (nmol/mg/min) (C) in roots of inoculated plants with four treatments (B8, B12, B31 and Co) and control; the absence and presence of Cadmium (Cd [0]), (Cd [50]) and (Cd [100]). Different letters indicate values that are statistically different at $p < 0.05$.*

The results obtained (*Figure 22.A*) show that the P contents in the root part of plants inoculated with isolates B12, B31 and Co are significantly higher than those of the non-inoculated controls at the level

of two Cd concentrations [50] and Cd [100]. In the absence of Cd, no effect was observed between the different treatments.

Figure 22.B shows APase activity quantified at the root level. All plants inoculated with the different isolates B8, B12, B31 and Co have a significantly positive effect compared to the control. The highest content is noted in the seedlings inoculated with Co with contents of 700, 800 and 750 (nmol/mg/min), compared to the control 90, 50 and 50 (nmol/mg/min) respectively at the three levels of Cd [0], Cd [50] and Cd [100].

Regarding ALPase activity (*Fig. 22C*), the figure shows that the ALPase content in Co-inoculated roots (100 nmol/mg/min) is significantly higher than that of uninoculated control (60 nmol/mg/min) in Cd [0]. In the presence of Cd, the highest value is noted in plants inoculated with Co (95 and 90 nmol/mg/min), followed by plants inoculated with B31 (80 and 70 nmol/mg/min) compared to the control (45 and 30 nmol/mg/min) under two concentrations Cd [50] and Cd [100] respectively.

Discussion

Discussion

In the present study, we are interested in PSB that are able to tolerate high concentrations of Cd that can potentially be used to reduce Cd accumulation in plants. Three isolates were selected as Cd tolerant PSB. These isolates were identified and functional analysis in regard to plant growth promotion traits. Their biochemical characteristics were determined (*Tab. 1*) such as P-solubilization, N-fixation, AIA-production, ACC-production and Siderophore-production.

Given the importance of Auxin for plant growth and knowing that bacteria can synthesize auxin in the rhizosphere from its precursor tryptophan, which is responsible for enhancing plant root growth (Ahmad et al., 2014). In our experiments, we showed that the **three isolates** tested are able to produce IAA (Tryp + or Tryp -) (*Tab. 1*). In addition, **B8** and **B31** were able to fix nitrogen, whereas, for ACC-production, it was present only for **B12** and **B31** isolates. As well, when it comes to Cd tolerance, the three isolates **(B8**, **B12** and **B31)** which were selected as highly Cd-tolerant bacteria, showed a minimum inhibitory concentration (MIC) less or equal to Cd [300]. However, Similar results were reported in a previous study by (Ahmad et al., 2014), who showed that bacterial isolates had in vitro ability to produce IAA, ACC deaminase and EPS. Also, all the bacterial isolates have shown in vitro tolerance to Cd concentrations with an important MIC. Similarly, (Pramanik et al., 2018) have successfully isolated a potent multi-heavy metal resistant PGPR isolates including Cd, in a bacterium that shows several important PGP traits like IAA production, nitrogen fixation, phosphate solubilization, ACC deaminase activity even under high Cd stress.

Regarding the solubilization of P from two forms: TCP (*Fig. 12A*) and RP (*Fig. 12B*), the three isolates that were selected, in a culture medium in the absence and presence of 4 Cd concentrations (Cd [50], Cd [100], Cd [200], and Cd [300]), showed different potential in solubilizing P, and it depends essentially on the isolates and also the Cd concentration. Consequently, all isolates can solubilize P with its two forms, and their growth is not inhibited by increasing Cd stress. This is in concordance with previous evidence that have demonstrated that PSB could solubilize P from TCP and from RP in culture medium (Karunai Selvi et al. (2011). In fact, it is well known that the mechanism of mineral phosphate solubilization by the PSB strain is associated with the production of low molecular weight organic acids, which through their hydroxyl and carboxyl groups chelate the cation bound to phosphate, the latter being converted to soluble forms (Sagoe et al., 1998). Furthermore, the three isolates tested in this work can also produce Apase and ALPase from TCP (*Tab. 2*) and RP (*Tab. 3*). However, taken together, P solubilization from TCP and RP and the production of APase and ALPase

in **B8, B12** and **B31** and their **Co** in vitro at different Cd concentrations do support our claim regarding the three isolates being considered as Cd resistant PSB.

In this study we aimed to take advantage of the Cd resistance characteristic of the selected PSB and then evaluate their traits activity on the growth and development of wheat plants under Cd stress conditions. Seeds were inoculated with these isolates individually (**B8**, **B12** and **B31**) and their Consortium (**Co**) and grown in soil contaminated with three Cd levels (Cd [0], Cd [50], and Cd [100]).

Based on previous literature, the use of PSB as inoculants simultaneously increases P uptake by the plant and improve crop yield (Elhaissoufi et al., 2020b; Khan et al., 2009; Jeong et al., 2013 and Chen et al., 2006). According to Rodríguez & Fraga, (1999) , the main mechanism of mineral P solubilization is the production of organic acids, and acid phosphatases, this process seems to play a major role in the mineralization of organic P in soil, enhancing thereby the activity of PSB and improving plant growth. Moreover, PSB promoted the P-availability to plants exposed to heavy metal stress and the solubilized phosphate helped in immobilization of heavy metals (Park et al., 2011). In addition, the P solubilization ability of PSB could better promote plant growth under Cd-stress. Several Cd-tolerant plant growths promoting strains having P solubilization ability was reported previously. For instance, Guo and Chi (2014) have identified the (Bradyrhizobium sp. YL-6). Also, many Cd resistant Enterobacter strains have showed interesting results in enhancing plant growth under Cd stress condition (Chen et al., 2010; Płociniczak et al., 2013; Ahmad et al., 2016). However, based on our results (Figures 14 and 22), the P content in roots has increased in the treated plants (especially those inoculated with **B12**, **B31** isolates and **Co**) which point to a potential effect of these isolates in promoting plant growth under Cd-stress (Cd [100]). Whereas, the isolate **B8** had a low level of P contents compared to other treatments. Moreover, the analysis of APase activity in roots showed a significant effect of all treatments compared with the control, which indicates that all the isolates and their Consortium (**Co**) improve the P solubilization in roots by enhancing the activity of APase. On the other hand, only the plants treated with **B31** and **Co** increase have shown an increase in the ALPase content in roots, which may improve the intracellular P in roots.

Furthermore, based on our results, the highest available P content in soil was observed with **B31** in presence of a maximum Cd concentration (Cd [100]) compared to other treatment and the control (*Fig. 21A*). Similarly, for the acid and alkaline phosphatase activity, (*Fig. 21B and 21C*) the same isolate (**B31**) had the highest APase and ALPase content is soil at the high Cd concentration. Interestingly, (Yuan et al., 2017) reported that the application of (HM)-tolerant PSB significantly increased available phosphate content in soil by enhancing the immobilization rate of Pb and Cd present in soil. Further,

This is in agreement with the study of (Teng et al., 2019) showed that all the selected (HM)-tolerant PSB strains exhibited P solubilizing capacity under stressful conditions and this P solubilizing capacity of those PSB was related to the concentrations of organic acids, acid phosphatase activity and pH. Indeed , In soil, the PSB could produce organic acids and phosphate enzymes to enhance the solubilization of insoluble phosphate compounds (Chen et al. 2006), and hence, PSB have been widely used as inoculants to increase soil available P contents and crop yield (Rodríguez & Fraga, 1999).

Previous studies had already demonstrated the ability of PSB strains and isolates to produce organic acids and promote the release of phosphate into soil. The content of acid phosphatase activities was approximately the same in the different strains of PSB, all of which have the ability to solubilize P under stressed conditions (Teng et al., 2019).Thus, the tested PSB could have an interesting capacity in increasing Rock P dissolution under stressful conditions which can enhance plant growth by providing nutrients. Among these PSB, the **B31** and **Co** have already demonstrated a high P solubilizing activity. The effect of Cd on cellular functioning is well established and it has been shown that Cd stress induces an imbalance in cell redox homeostasis leading to oxidative damage in plants. The reduction of growth traits in presence of Cd (CCI, dry weights, root diameters and root surface area) may be due to the results of alteration of photosynthesis activity, and imbalance in nutrients uptake (i.e. K, P …) (Naciri et al., 2021).

The effect of different treatments on the physiological characteristics of plant growth, notably stomatal conductance (*Fig. 15*), chlorophyll content (*Fig. 16*), chlorophyll fluorescence intensity *(Fig. 17*), and phenotypic measurements of plants (*Fig. 18*) were monitored and analysed. Indeed, the stomatal conductance of the leaves and the chlorophyll content of the plants treated with **B31** showed a significant and positive effect compared to the control in the presence of Cd (Cd [50] and Cd [100]). Also, chlorophyll fluorescence intensity (*Fig. 17*) was significantly elevated in all treated plants and especially higher in plants inoculated with **B31** compared to the control at a concentration of Cd [100]. While for plant length (*Fig. 18A*) and spike length (*Fig. 18B*) an observable increase was recorded compared to control in plants treated with **B12**, **B31** and **Co** in the presence of Cd. Likewise, a high leaf number was observed (*Fig. 18C*) in all treatments inoculated with PSB and Co.

In fact, the increased tolerance of plants to Cd could be linked to the increased availability of P to the plants following PSB inoculation, which further enhances plant growth parameters such as chlorophyll content and photosynthetic efficiency. Thus, demonstrating an indirect effect of Cd-stress alleviation mediated by the tested PSB (Chtouki et al., 2021). This result is following that of (Manikandan et al., 2016) who reported that supplementation P enhanced chlorophyll content of plants grown in Cd stress

conditions. Likewise, (Chtouki et al., 2021) have shown the same results concerning the analysis of chlorophyll fluorescence intensity, they demonstrated that P plays an important role in protecting electron transfer from PSII to PSI in presence of Cd. Additionally**,** similar research has found that the Cd stress in the root growing medium reduced significantly the photosynthetic rate, stomatal conductance, transpiration rate, internal CO_2 concentration, water use efficiency and relative sub-stomatal CO_2 concentration. Whereas, P application enhanced these gas exchange attributes by the reduction of the Cd concentration in wheat shoots reduced Cd translocation from the roots to shoots, and a dilution effect due to the increase in biomass after P application in the rooting media. (Arshad et al., 2016).

Overall, Improvement of physiological parameter in plants treated with the selected PSB may help in understanding how the availability of P solubilized by PSB is directly involved to increase the physiological parameters of plants and consequently the morphological growth (biomass) of plants as shown in *Figure 18 (A, B and C*). Interestingly, in our study, among different PSB treatments, the isolate **B31** has shown the greatest increase in several physiological parameters of plant growth. But concerning the phenotypic measurements, all the plants treated by the PSB and their Co showed an observable positive effect against the control (without PSB).

For the root system, the importance of the below-ground part of the plant in increasing nutrients uptake, and the overall plant growth have been historically evidenced (Elhaissoufi et al., 2020). In line with this, results from our experiments (*Fig. 19*) showed that the treatments with the isolate **B12** increased the dry weight of spikes, leaves, and roots in the absence of Cd. Only the **B31** isolate increases spikes dry weight at a high concentration of Cd (Cd [100]). Thus, the following, the root morphological parameters including, root length (*Fig. 20B*), root surface (*Fig. 20C*) and root volume (*Fig. 20D*), the same isolate (**B31**) that presents a desired impact on root growth at a Cd [100] and the effect this isolate is observed in the root morphological traits obtained by WinRHIZO and Stereomicroscope in 10-day old seedling (*Fig. 13*) and by WinRHIZO in 75-day old seedling (*Fig. 20A*) which subsequently led to an increased plant growth.(Elhaissoufi et al., 2020) show that all PSB isolates increased the root traits of inoculated plants Wheat, and they had a significant positive effect on root diameter, as well as the length, surface area, root volume and dry weight of the roots increased significantly in the inoculated treatments compared to non-inoculated treatments. Indeed, This is in accordance with the study conducted by (Mitra et al., 2018), showing a significant enhancement in root-shoot length, fresh weight and dry weight was observed after inoculation of PSB-strain under Cd-treated condition and the negative impacts of Cd on the rooting characters were Alleviated by inoculation of PSB-strain and alleviated Cd-toxicity on seedling growth. Likewise, (Pramanik et al., 2018) also reported similar

result showing that PSB could significantly ameliorate the above-mentioned growth indices such us root length, shoot length, root fresh weight and root dry weight under Cd stress (Cd [100]). Furthermore, the root-shoot length and biomasses were directly related to plant growth which was reported to increase after inoculation with PGPR under Cd stress (Kamran et al., 2015). Moreover, the increased biomass under Cd stress depicted the bioremediation ability of the selected strain (Lin et al., 2016). Interestingly, a similar increase of the root-shoot length and biomasses under Cd stress also found after isolate inoculation of **B31**.

Although above discussion highlights the potential of the three Cd-tolerant isolates tested to improving P availability under Cd stress, as well as physiological parameters and plant growth. The results obtained from in-vitro and in-vivo study are interesting, but we couldn't expect what would happen when these isolates will be used on agricultural soils contaminated by Cadmium under several other factors such as their application on other crops, the presence of other microorganisms in the soil, other stress or other contaminants that may influence the results obtained in this study. Therefore, we believe that the present study may be extended further with large-scale experiment in the field with replicated field trials to confirm the results and for a better understanding of the potential impact of our isolates on plant growth in normal environmental conditions.

Conclusion and perspective

Conclusion and perspectives

Our results conclude that PSB can contribute to alleviate the effects of Cd through different mechanisms such as:

- The three selected PSB isolates and their consortium positively affected the growth and development of wheat plants. These 4 inoculants have the ability to make P more accessible for uptake by plants in the presence of Cd, making them tolerant to Cd. They can also promote plant growth and development through various traits, including IAA synthesis, siderophore synthesis and reduction of ethylene toxicity through ACC deaminase production by reducing the negative effect of Cd and increasing plant leaf and root biomass. Physiological parameters such as SC, CCI, ChlF and dry weight... were influenced in the presence of Cd and were increased in the presence of PSB compared to the control under Cd stress.

- Isolate B31 was able to reduce Cd accumulation in wheat plants grown in Cd-containing soil, which conferred Cd tolerance on the plants. Thus, the presence of the set of plant growth-promoting traits in isolate B31 resulted in various morphological improvements in wheat plants. The application of B31 increased the majority of activities involved in plant growth, which triggered better growth enhancement of wheat plants under Cd stress. Therefore, this isolate could be exploited for bioremediation in Cd-contaminated agricultural fields for a better crop growth and yield.

Our results partially answered the questions posed about reducing Cd uptake and allocation to shoots while promoting P solubilization by the different isolates. And we completely answered the questions raised about the alleviation of the effect of Cd by stimulating specific physiological parameters and the growth of leaves and roots of plants while comparing the treated plants with untreated plants with different Cd-concentrations. It can be concluded that Cd-tolerant PSB have positive effects on the growth and development of plants under Cd-stress while promoting P solubilization and growth especially in isolate B31 and Co, which show the best results compared to the other isolates.

Therefore, and in this context, (1) the optimization of the effects of these isolates can be confirmed by measuring the Cd content in the soil and in the different parts of the plant; (2) Thus, to study the mechanisms of Cd attenuation by these isolates in order to understand their behavior in front of Cd stress in agriculture soils; (3) Molecular analyses may be necessary to target the genes responsible for Cd resistance in bacteria and; (4) Or, analyses of the oxidative enzymes SuperOxide Dismutase (SOD), peroxidase, proline, Manolaldehyde... which can confirm these promoting effects of these isolates.

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Appendix

Appendix 1

➡ Composition of NBRIB medium (g/l, pH7)

P solubilisation was confirmed in a medium *National Botanical Research Institute's Phosphate* NBRIP without P. The basal medium without P was used to examine the P solubilisation capacity of the isolated microorganisms and this medium contains glucose (10g), $Ca_3(PO_4)_2$ (5g), $MgCl_2$-$6H_2O$ (5g), KCl (0.2g), $MgSO_4$-$7H_2O$ (0.25g), $(NH_4)2SO_4$ (0.1g), 1000ml distilled water and Agar (15.0g) for a solid medium to get an idea of the presence or absence of P solubilisation capacity and without Agar for a liquid NBRIP medium to quantify this capacity.

➡ Composition of NFB medium

The fixation of free N_2 was confirmed in a medium NFB Nitrogen Free Base (Zhou et al., 2013) without N. The nitrogen-free base medium (NFB) was used to examine the nitrogen fixation capacity of isolated microorganisms and contained 1.20g of KH_2PO_4, 0.80g of K_2HPO_4, 5.0g of glucose, 0.20g of $MgSO_4$ - $7H_2O$, 0.20g of NaCl, 0.02g of $CaCl_2$ - $2H_2O$, 0.002g of $FeSO_4$ - $7H_2O$ and 2.0ml of metal solution in 1.0L of distilled water (pH 7.0). The metal solution contained 0.40g $CaCl_2$ - $2H_2O$, 0.30g H_3BO_3, 0.04g $CuSO_4$ - $5H_2O$, 0.10g KI, 0.20g $FeSO_4$ - $7H_2O$, 0.40g $MnSO_4$ - $7H_2O$, 0.20g $NaMoO_4$ - $2H_2O$ and 10.0mL HCl concentrated in 1.0L distilled water. The positive witness was NFB containing ammonium sulphate as the only source of nitrogen. After 7 days of incubation at 28°C, PSB isolates developed in NFB environment were considered free N-fixer isolates.

➡ TSB medium (BioKar Diagnostics)

For 1 litre of medium:

- Tryptone ..... 15,0 g
- Soybean papain peptone ..... 5,0 g
- Sodium chloride ..... 5,0 g

- Bacteriological agar ..... 15.0 g pH of the ready-to-use medium at 25 °C: 7.3 ± 0.2.

➡ **Salkowsky reagent**

Mix:

- 2ml 0.5M $FeCl_3$ (Dissolve 1.35g in 10ml H_2O),

- 49ml H_2O

- 49ml of 70% perchloric acid.

➡ **Protocol of Siderophore production** (rapport Ammar)

1st step: Blue dye

- Solution 1: Dissolve 0.06g CAS (Fluka Chemicals) in 50ml H_2O.

- Solution 2: Dissolve 0.0027g of $FeCl_3$-6 H_2O in 10ml of 10mM HCl.

- Solution 3: Dissolve 0.073g of HDTMA in 40ml of H_2O.

Mix solution 1 with 9ml of solution 2. Then mix with solution 3. The solution should be blue in colour. Autoclave and store in a plastic container/bottle.

2 nd step: Mixing solution

- Salt solution Minimum medium 9 (MM9): Dissolve 15g KH_2PO_4, 25g NaCl and 50g NH_4Cl in 500 ml H_2O.

- 20% glucose stock: Dissolve 20g glucose in 100ml H_2O.

- NaOH Stock: Dissolve 25g NaOH in 150ml H_2O; pH should be about 12.

- Casamino acid solution:

1. Dissolve 3g of Casamino acid in 27 ml of H_2O.

2. Extract with 3% 8-hydroxyquinoline in chloroform to remove all traces of iron.

- Preparation of CAS agar

1. Add 100ml of MM9 saline to 750ml of H_2O.

2. Dissolve 32.24g of piperazine-N, N-bis (2-ethanesulfonic acid) PIPES. PIPES will not dissolve below pH 5. Bring the pH to 6 and slowly add the PIPES with stirring. The pH will decrease as the PIPES dissolve. While stirring, slowly bring the pH to 6.8. Do not exceed 6.8 as this will turn the solution green.

3. Add 15g of Bacteriological Agar.

4. Autoclave and cool to 50°C.

5. Add 30ml of sterile Casamino acid solution and 10ml of sterile 20% glucose solution to the MM9 / PIPES mixture.

6. Slowly add 100ml of blue dye solution along the glass wall with sufficient agitation to mix well.

7. Aseptically pour the dishes.

8. Inoculate the bacterial isolates onto the medium and incubate at 28°C for one week.

Appendix 2

➡ Ascorbic acid method

❖ Acetate buffer

Take 164.8 ml of solution B and adjust the pH with solution A to PH ⩽ 6.5.

- Solution A: (Acetic acid 0.2M): 11.42ml of pure CH_3COOH (17.5 M (100%, d=1.05)) in 1 litre;
- Solution B: (Sodium acetate 0.2M): 16.4g CH_3COONa or 27.2g CH_3COONa, $3H_2O$ in 1 litre.

❖ Reagent

For 100ml, combine:

- 50ml of 5N sulphuric acid (dilute 70ml of the concentrated sulphuric acid in 500ml),
- 5ml Potassium antimonyl tartarate (Dissolve 1.3715g in 400ml H_2O, dilute in 500ml),
- 15ml Ammonium Molybdate 4% (Dissolve 20g in 500ml) and
- 30ml Ascorbic Acid (Dissolve 1.76g in 100ml H_2O).

➡ Dosage de Phosphatase Method Tabatabai

❖ Acetate (for pH below 6.5)

- Place 164.8 mL of 0.2 M sodium acetate in a beaker.
- Using a pH meter, adjust the pH with the 0.2 M acetic acid solution.
- Transfer to a 200 mL volumetric flask and make up to the mark with with demineralised water.
- Shake and store in the refrigerator

❖ Modified universal buffer (for pH 11)

Universal buffer modified at pH 11 is used for alkaline phosphatase.

Preparation of the stock solution

In 500ml of 1M NaOH dissolve successively:

- 12.1g of tris (hydroxy methyl) aminomethane

- 11.6g of maleic acid

- 14g of citric acid monohydrate

- 6.3g boric acid

Make up to 1000ml with demineralised water and store at +4°C.

- **Buffer at pH 11**

Collect 200ml of the stock solution + 500ml of demineralised water, adjust the pH to 11 with NaOH and make up to 1L.

❖ **Preparing solutions**

- **pNPP 10 mM**

M (pNPP) = 371.12 g.mol-1

For 20mL of solution, in a volumetric flask dissolve 0.0742g of pNPP in 20mL of deionised water demineralised water.

Shake and store in the refrigerator for a maximum of 2 days.

- **10 mM pNP**

M (pNP) = 139.11 g.mol-1

For 50mL of solution, in a volumetric flask dissolve 69.55mg +/- 0.2mg of pNP in 50ml of demineralised water.

Shake and store in the refrigerator.

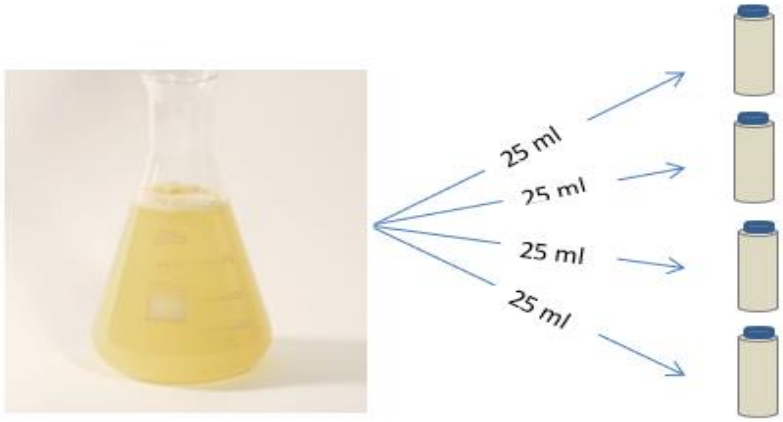
NB: It is also possible to use a commercial solution.

- **0.5 M NaOH**

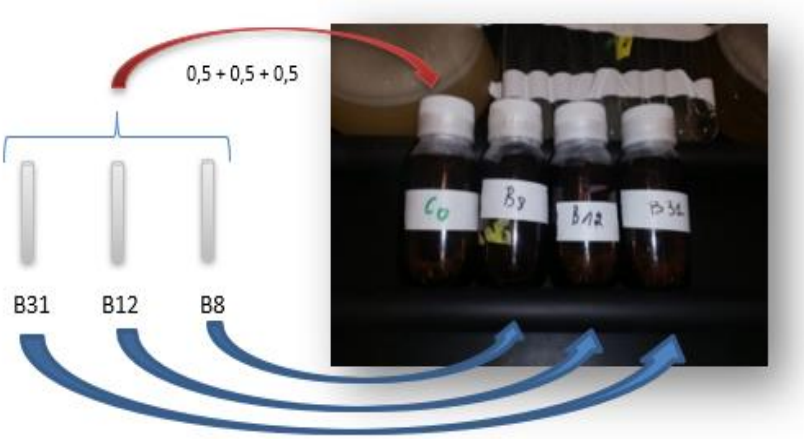
We have M (NaOH) = 39.997g.mol-1.

For 0.5L of solution, in a volumetric flask, dissolve 8.4992g +/- 0.5mg of NaOH in 500ml of demineralised water.

Appendix 3

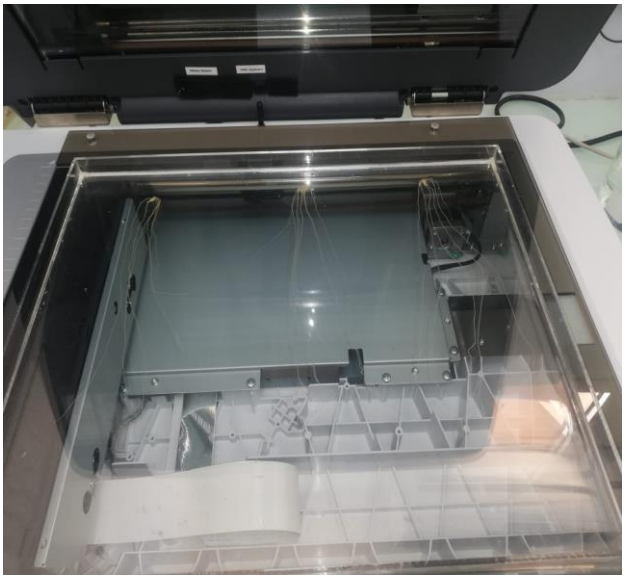


Preparation of culture medium TSB

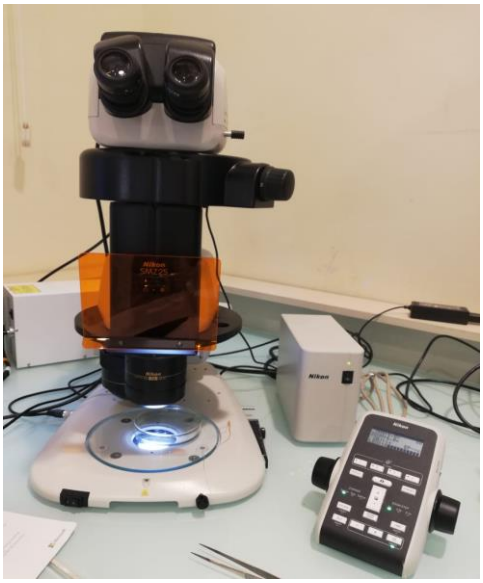


Preparation of liquid bacterial inoculum

In vitro seeds germination experiment inoculated by three isolates and Co under three Cd



Microscopic observation of root by WinRHIZO



Overall observation of roots by Stereomicroscope