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Potential of endophytic actinobacteria for biological use in remediation of soil contaminated with petroleum hydrocarbons

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Devant le jury

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“It wasn't Straight, nor Long, but

Ups and Downs were fruitful for me”

Hafida

Dedicated to

To My Beloved Parents; M'hamed and Yamina

To My Dear brothers; Farouk & Nabil

To My Husband, Youcef

Without whom none of my success would be possible

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TABLE DES MATIERES

Abbreviation list

ACC:	1-aminocyclopropane-1-carboxylate
Ant:	Anthracène
AIA:	Acide indole-3-acétique
HAM:	Hydrocarbures aromatiques monocycliques
HAP:	Hydrocarbures aromatiques polycycliques
HPT:	Hydrocarbures pétroliers totaux
PGP:	Plant growth promoting
IAA:	Indole-3-acetic acid
MAH:	Monocyclic aromatic hydrocarbons
N_2:	Nitrogen (English). Azote (Français)
PAH:	Polycyclic aromatic hydrocarbons
PGPB:	Plant growth promoting bacteria
Phe:	Phénanthrène
Pyr:	Pyrene
TCA:	Tricarboxylic acid cycle (Krebs cycle)
TPH:	Total petroleum hydrocarbon
VOC:	Volatile organic compounds

Résumé

L'objectif de notre travail est de mettre en évidence l'utilité des actinobactéries endophytes dans le processus de phytoremédiation des sols contaminés par les hydrocarbures pétroliers.

Dix-sept isolats bactériens issus des racines de plantes poussant dans les sols pollués par le pétrole brut sont testés pour leur tolérance aux hydrocarbures volatils. Parmi ces actinobactéries endophytes, six, ayant poussé en présence du pétrole sont analysées pour leur capacité à dégrader le pétrole léger algérien et à produire des métabolites favorisant la croissance des plantes (PGP). Les résultats ont montré que cinq isolats sur six peuvent dégrader le pétrole avec une efficacité atteignant jusqu'à 98% après 7 jours d'incubation. Elles présentent toutes un large éventail de produits favorisant la croissance des plantes telles que le sidérophore, la solubilisation des phosphates, la production de la 1-aminocyclopropane-1-carboxylate désaminase, la fixation de l'azote et la production d'acide indole-3-acétique, ainsi que la production de biosurfactants. Le séquençage de l'ARNr 16S de ces souches a révélé qu'elles appartiennent au genre *Streptomyces*.

Ces résultats suggèrent que la souche *Streptomyces* sp. Hlh1 peut être utile comme agent de bioremédiation en raison de son rôle important dans la dégradation des *n*-alcanes (C_6-C_{30}) et des hydrocarbures aromatiques polycycliques. La capacité de cette souche à coloniser le sol, à concurrencer la microflore native et à éliminer les hydrocarbures pétroliers sont recherchées en évaluant sa performance dans la décontamination des sols stériles et non stériles pollués artificiellement par les hydrocarbures. En plus de la production d'une biomasse importante, révélant une bonne croissance, la souche étudiée a aussi pu éliminer les hydrocarbures pétroliers totaux, les *n*-alcanes et les hydrocarbures aromatiques contenus dans les échantillons du sol.

La laitue s'est révélée un bon bioindicateur pour évaluer la toxicité des métabolites finaux de de bioremédiation. De plus, cette souche s'est avérée être un bon bioinoculant. Par ailleurs, l'efficacité de l'inoculation du maïs par *Streptomyces* sp. Hlh1 est recherchée à travers un système de phytoremédiation assistée des sols contaminés par du pétrole brut et des hydrocarbures aromatiques polycycliques. Les résultats montrent que l'inoculation des plantes avec l'endophyte testée conduit à une plus forte élimination des contaminants, améliore le développement des plantes et augmente la production des pigments photosynthétiques. L'inoculation des espèces végétales par *Streptomyces* sp. Hlh1 représente une alternative prometteuse pour éliminer et détoxifier les milieux contaminés. Par conséquent, l'interaction entre les plantes et les bactéries endophytes est un enjeu important à ne pas négliger pour améliorer les stratégies de phytoremédiation.

Mots-clés : Hydrocarbures pétroliers, actinobactéries endophytes, phytoremédiation, métabolites PGP.

Abstract

The objective of our work is to highlight the usefulness of endophytic actinobacteria in the phytoremediation of petroleum hydrocarbons contaminated soils.

Seventeen isolates obtained from the roots of plants grown in polluted soils with crude petroleum are tested for their tolerance to volatile hydrocarbons. Among those endophytic actinobacteria, six, grown in the presence of petroleum were analyzed for their capacity to degrade of Algerian light petroleum and to produce plant growth promoting metabolites (PGP). The results have shown that five out of six isolates could degrade petroleum with efficiency achieved up to 98% after 7 days of incubation. They showed a wide range of plant growth promoting features such as siderophores, phosphate solubilization, the production of 1-aminocyclopropane-1-carboxylate deaminase, nitrogen fixation and indole-3-acetic acid, as well as biosurfactant production. The sequence of the 16S rRNA of these strains revealed that all the strains belong to the genus *Streptomyces*.

These results suggest that the strain *Streptomyces* sp. Hlh1 may be useful as a bioremediation agent due to its important role in the degradation of *n*-alkanes (C_6-C_{30}), and polycyclic aromatic hydrocarbons. The ability of this strain to colonize the soil, to compete with native microbiota and to remove petroleum hydrocarbons are conducted by evaluating its performance in the decontamination of artificially polluted sterilized and non-sterilized soils. In addition to the production of important biomass.

Lettuce was found to be good bioindicator to assess the toxicity of the final metabolites of bioremediation. Furthermore, this strain was found to be good bioinoculant. Otherwise, the effectiveness of the inoculation of maize with *Streptomyces* sp. Hlh1 was carried out through a system of assisted-phytoremediation of soils contaminated with crude petroleum and polycyclic aromatic hydrocarbons. The results showed that the inoculation of plant with the tested endophyte lead to a strong hydrocarbons removal, enhancing plant development and increasing the photosynthetic pigments production. The inoculation of plant species by *Streptomyces* sp. Hlh1 would represent a promising alternative to remove and detoxify contaminated environments. Therefore, the interaction between plants and endophytic bacteria is an important issue not to be neglected for improving phytoremediation strategies.

Key-words: Petroleum hydrocarbons, endophytic actinobactéries, phytoremediation, PGP metabolites.

ملخص

الهدف من دراستنا هو إثبات فائدة الأكتينوبكتيريا المتواجدة داخل النبات في عملية المعالجة النباتية للتربة الملوثة بالهيدروكربونات البترولية.

سبعة عشر سلالة بكتيرية تم عزلها من جذور النباتات التي تنمو في التربة الملوثة بالنفط الخام تم اختبارهاعلى تحمل الهيدروكربونات المتبخرة. من بين هذه الأكتينوبكتيريا المتعايشة داخل النباتات، ستة سلالات قادرة على التكاثر في وجود البترول تم تحليل قدرتها على تفكيك البترول الجزائري الخفيف وإنتاج نواتج الأيض التي تعزز نمو النبات (PGP). أظهرت النتائج أن خمسة سلالات من بين ستة تمكنت من تفكيك البترول بفعالية تصل إلى 98٪ بعد 7 أيام من التحضين. تتميز جميعها بمجموعة واسعة من نواتج الأيض التي تعزز نمو النبات مثل، لاقطات الحديد، إذابة الفوسفات، إنتاج 1- أمينوسيكلوبروبان-1- كربوكسيلات ديميناز، تثبيت النيتروجين، إنتاج حمض 3-الخليك الإندول وأيضا إنتاج جزيئات السطح الحيوية. التحليل الجيني 16S rRNA لهذه السلالات بين أنها تنتمي إلى الجنس *Streptomyces* .

تشير هاته النتائج إلى أن السلالة *Streptomyces* sp. Hlh1 يمكن أن تكون مفيدة كعامل معالجة بيولوجية بسبب دورها المهم في تفكيك *n*-الألكانات (C_6-C_{30}) والهيدروكربونات العطرية متعددة الحلقات. البحث عن قدرة هذه السلالة على استعمار التربة، التنافس مع البكتيريا الأصلية وإزالة الهيدروكربونات البترولية بتقدير أدائها في إزالة تلوث التربة المعقمة وغير المعقمة والملوثة صناعياً بالهيدروكربونات. بالإضافة إلى إنتاج كتلة حيوية معتبرة مما يكشف عن نمو جيد، السلالة المدروسة قادرة أيضًا على القضاء على الهيدروكربونات البترولية الكلية، الألكانات والهيدروكربونات العطرية الموجودة في عينات التربة.

لقد ثبت أن الخس هو مؤشر بيولوجي جيد لتقييم سمية النواتج النهائية للمعالجة البيولوجية. بالإضافة إلى ذلك، يبدو أن هذه السلالة هي عامل لقاح بيولوجي جيد. علاوة على ذلك، تم البحث عن كفاءة تلقيح الذرة بـ *Streptomyes* sp. Hlh1 من خلال نظام المعالجة النباتية للتربة الملوثة بالنفط الخام والهيدروكربونات العطرية متعددة الحلقات. أظهرت النتائج أن تلقيح النباتات بالبكتيريا المتواجدة داخل النبات يؤدي إلى إزالة أكبر للملوثات، يحسن نمو النبات ويزيد من إنتاج أصباغ التمثيل الضوئي. تلقيح الأصناف النباتية بـ *Streptomyces* sp. Hlh1 يعد بديلاً واعدًا للتخلص وإزالة السموم من الأوساط الملوثة. وبالتالي، فإن التفاعل بين النباتات وبكتيريا النبات الداخلية هو قضية مهمة لا ينبغي إغفالها من أجل تحسين استراتيجيات المعالجة النباتية.

الكلمات المفتاحية: الهيدروكربونات البترولية، الأكتينوبكتيريا المتواجدة داخل النبات، المعالجة النباتية، نواتج الأيض PGP.

I. Aperçu général

Une énorme quantité d'hydrocarbures pétroliers pénètre dans l'environnement, que ce soit accidentellement, ou en raison des activités humaines. Leur toxicité et leurs propriétés destructrices des écosystèmes naturels sont à l'origine de problèmes environnementaux majeurs (Moliterni *et al*., 2012). L'agence Américaine de Protection de l'Environnement (*US Environmental Protection Agency*), (1986) a classé ces composés comme des polluants environnementaux prioritaires. Par conséquent, la pollution des sols par le pétrole brut est un problème environnemental mondial nécessitant une intervention immédiate (Koshlaf *et al*., 2016).

L'Algérie compte parmi les plus grands producteurs de pétrole. C'est à Ouargla que se situent les deux plus importants sites d'exploitation (Hassi Messaoud et Haoud Berkaoui). C'est donc l'une des régions les plus exposées à la contamination des sols par les produits pétroliers.

Au cours des deux dernières décennies, une attention particulière est accordée à la phytoremédiation comme alternative aux méthodes physico-chimiques, reconnues coûteuses et destructrices de l'environnement (Li *et al*., 2012). A l'opposé, la phytoremediation est une approche efficace, respectueuse de l'environnement et rentable (Bisht *et al*., 2014). Elle est utilisée dans le traitement des sols contaminés par divers polluants tels que les hydrocarbures pétroliers, les herbicides, les explosifs et les métaux lourds (Li *et al*., 2012).

Certaines espèces végétales ont montré leur capacité à faire face à des concentrations relativement élevées de produits chimiques et organiques, qui peuvent être absorbés, transférés, métabolisés et/ou volatilisés. De plus, ces plantes peuvent stimuler la dégradation des xénobiotiques dans la rhizosphère (la rhizodégradation) libérant des exsudats racinaires (Alvarez *et al*., 2012; Álvarez *et al*., 2015).

D'autre part, les bactéries endophytes colonisant les tissus internes des plantes sans causer de dommages, établissent une relation harmonieuse avec la plante hôte (Tan et Zou, 2001; Kidd *et al*., 2017). La phytoremediation peut donc être améliorée par l'utilisation des endophytes pour éliminer les contaminants organiques et inorganiques du sol et de l'eau (Pilon-Smits, 2005).

Les efforts actuels sont essentiellement concentrés sur les bactéries endophytes capables de dégrader les contaminants organiques et/ou d'améliorer la croissance des plantes (Khan *et al.,* 2013). Plusieurs études ont rapporté que de nombreuses bactéries endophytes présentent un rôle important dans la dégradation de différents hydrocarbures. Ces micro-organismes comprennent des bactéries Gram négatif telles que *Pseudomonas* et *Brevundimonas* (Phillips *et al*., 2008 ; Peng *et al*., 2013;

Zhang *et al*., 2014) et des bactéries Gram positif, à savoir les actinobactéries (Kukla *et al.*, 2014; Singh et Sedhuraman, 2015).

Les actinobactéries sont un groupe de bactéries qui joue un rôle important dans le métabolisme de la matière organique complexe dans la nature, éliminant ainsi efficacement les composés xénobiotiques tels que les hydrocarbures pétroliers (Barabás *et al*., 2001). De plus, les actinobactéries endophytes isolées à partir de tissus végétaux sains dont la surface est désinfectée, jouent un rôle important dans la métabolisation des polymères complexes tels que les composés toxiques organiques et inorganiques, en des nutriments plus facilement assimilables (Doumbou e*t al*., 2005). Elles sont également bien connues comme des productrices d'un large éventail de métabolites promoteurs de la croissance des plantes (*PGP*) (Subramaniam *et al*., 2016). Ces caractères les ont rendus des candidats prometteurs pour améliorer la phytoremédiation des sols pollués. Les bactéries du phylum Actinobacteria semblent avoir le potentiel d'utiliser différentes voies métaboliques (Polti *et al*., 2011).

De plus, le genre *Streptomyces* est connu pour la production de plus de 60% de métabolites bioactifs, qui sont généralement explorés pour leurs activités antimicrobiennes (Goldman et Green, 2009). Quelques études indiquent que certaines espèces du genre *Streptomyces* isolées du sol ont la capacité de dégrader les hydrocarbures pétroliers (Ferradji *et al*., 2014). A ce jour, aucune information n'est disponible sur l'action des *Streptomyces* endophytes dans la phytoremédiation des sols contaminés par les contaminants pétroliers.

En raison du rôle potentiel des actinobactéries dans la métabolisation de différents composés complexes, l'étude des actinobactéries hydrocarbonoclastes endophytes, associées aux plantes poussant dans les sols contaminés, peut fournir des informations précieuses sur les potentiels avantages économiques et environnementaux de l'utilisation des actinobactéries endophytes dans la phytoremédiation.

II. Structure de la thèse

Le manuscrit de la thèse est composé de trois chapitres. Il est ainsi structuré :

Introduction générale ; qui consiste en une brève revue de la littérature sur les hydrocarbures pétroliers et leur toxicité, les technologies de bioremédiation et de phytoremédiation ainsi que le rôle du complexe endophytes-plantes dans la phytoremédiation. Les objectifs et les contributions originales de la thèse sont aussi précisés dans cette introduction.

Plan expérimental ; qui représente les différents dispositifs expérimentaux adoptés lors de la réalisation des différentes parties de notre travail.

Chapitre I ; dans lequel sont présentés les résultats et les discussions des travaux de recherche sur les isolats d'actinobactéries endophytes tolérant les hydrocarbures pétroliers volatils et leur pouvoir de biodégradation du pétrole brut Algérien et de production de PGP.

Chapitre II ; consacré à l'évaluation de la capacité de *Streptomyces* sp. Hlh1 à remédier des sols contaminés avec différentes concentrations de pétrole brut Algérien. La toxicité des métabolites finaux est également évaluée dans ce chapitre.

Chapitre III ; rapporte les résultats et les discussions de l'efficacité du processus de remédiation de l'ensemble plante-*Streptomyces* sp. Hlh1 dans des sols contaminés artificiellement par du pétrole brut ou des hydrocarbures aromatiques polycycliques (HAP) dans des conditions de laboratoire.

Conclusion ; qui vient clôturer le travail réalisé et annoncer les perspectives envisagées à l'échelle du laboratoire et à l'échelle du terrain pour une meilleure compréhension de la thématique entreprise.

Il est à noter que la présente thèse est sous forme de thèse-Articles. Elle commence par une introduction générale rédigée en Français et traduite en Anglais. Chaque chapitre est représenté par un article scientifique paru dans une revue de classe A, précédé d'un résumé détaillé rédigée en langue Française. Une conclusion générale des travaux réalisés et publiés est également rédigée en Français et traduite en Anglais.

Introduction générale

I. Introduction générale

Le pétrole, du latin « huile de roche » est un liquide sombre, collant et visqueux (Varjani, 2017). Il est composé de carbone, d'hydrogène, et peut également contenir de l'azote, du soufre et de l'oxygène (Balachandran *et al*., 2012). Il s'appelle pétrole brut, une fois extrait du sous-sol et transporté vers des raffineries, où il est transformé en d'autres produits (Ugochukwu et Fialips, 2017). L'emplacement du champ de pétrole et l'âge et la profondeur du puit déterminent la composition du pétrole brut (Varjani 2017). Il peut être classé comme léger, moyen ou lourd, en fonction des proportions de composants de poids moléculaire élevé (Xu et Lu, 2010).

L'appellation hydrocarbures pétroliers totaux (HPT) est un terme utilisé pour décrire une grande famille de composés hétérogènes que l'on trouve dans le pétrole brut et dont les principaux constituants chimiques sont les atomes de carbone et d'hydrogène. Les HPT peuvent être divisés en quatre groupes (fractions) d'hydrocarbures pétroliers; aliphatiques, aromatiques (contient un ou plusieurs cycles), résines et asphaltènes (Bojes *et* Pope, 2007; Abdel-Shafy et Mansour, 2016).

Les hydrocarbures aliphatiques représentent la fraction prédominante du pétrole brut. Ils sont saturés ou insaturés, linéaire ou ramifiée à chaîne ouverte tels que les alcanes, les iso-alcanes, les cycloalcanes (naphtènes), les terpènes et les stéranes (Zhang *et al*., 2011). En fonction de leur poids moléculaire, ils sont classés en quatre classes : les alcanes gazeux, les aliphatiques de poids moléculaire faible (C_8-C_{16}), les aliphatiques de poids moléculaire moyen (C_{17}-C_{28}) et les aliphatiques de poids moléculaire élevé (> C_{28}) (Varjani, 2017).

Les hydrocarbures aromatiques contiennent un ou plusieurs cycles et classés en deux classes, à savoir ; les hydrocarbures aromatiques monocycliques (HAM) qui ne contiennent qu'un seul cycle et les hydrocarbures aromatiques polycycliques (HAP) contenant généralement plus d'un cycle benzénique. Les HAP constitués de deux ou trois cycles sont connus sous le nom de HAP légers, tandis que ceux constitués de quatre cycles et plus, sont appelés HAP lourds (Abdel-Shafy et Mansour, 2016). Les résines et les aspahltènes sont des composés polaires, avec de l'azote, du soufre, des atomes d'oxygène et des traces des métaux (Varjani, 2017). Ils peuvent contenir des groupes alkyles qui participent à leur résistance à la biodégradation (Zhu *et al*., 2010).

I.1. Source de pollution pétrolière et son impact sur l'environnement

Le terme contamination est attribué à la présence d'une substance dont la concentration ne doit pas être égale ou supérieure au niveau de la concentration référence. En d'autre terme, un contaminant est un matériau

indésirable bien qu'il ne doit pas nécessairement être nocif. Tandis que, la pollution est référée à la présence d'un contaminant nocif pour les organismes ou l'infrastructure (Chapman, 2007).

La pollution pétrolière dans l'environnement peut résulter des déversements et des fuites de ces produits des réservoirs souterrains, des bateaux à vapeur, du débranchement des puits de pétrole, des sites de raffinerie de pétrole abandonnés, dans, les sols, les eaux souterraines et les océans (Varjani, 2017).

Les hydrocarbures sont des polluants persistants, récalcitrants et hautement toxiques, car ils causent des dommages permanents à l'environnement (Das et Chandran, 2011). Ce sont des composés hémotoxiques, cancérigènes et tératogènes. Les composés organiques volatils (COV) sont extrêmement toxiques pour l'Homme (Ghosal *et al*., 2016). Les hydrocarbures aromatiques polycycliques (HAP) posent un problème international en raison de leurs fortes propriétés écotoxiques (Chen *et al*., 2016).

L'Algérie est l'un des trois premiers producteurs de pétrole en Afrique. Elle est considérée comme le pays le plus exportateur de pétrole vers l'Europe et l'Eurasie (US Energy Information Administration, 2019). Elle possède trois principaux terminaux d'exportation et cinq raffineries, trois situées à Skikda, Alger et Arzew et deux dans les villes sahariennes de Hassi Messouad et Adrar.

La principale source de pollution pétrolière dans les eaux algériennes est les effluents des raffineries à terre et les différents rejets et ruissellements des terminaux d'exportation de pétrole (Carpent et Kostianoy, 2019), alors que la plupart des sources de pollution des sols en Algérie résultent des déversements et des fuites des réservoirs souterrains et du débranchement des puits de pétrole **(Figure 1).**

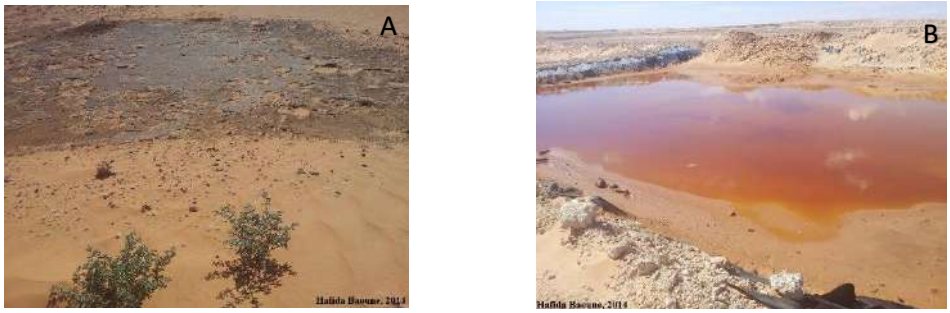

Figure 1. Exemples d'une source de pollution pétrolière ancienne (A) et récente (B) à Haoud Berkaoui (prises en 2014) (Baoune, 2014).

Les hydrocarbures peuvent avoir divers effets sur les humains et les organismes vivants. Selon le temps d'exposition, les effets sur la santé peuvent être aigus après une exposition unique, ou chroniques après exposition répétée (Abdel-Shafy et Mansour, 2016). De plus, les concentrations des contaminants, la toxicité spécifique de certains HAP, la voie d'exposition et les caractéristiques des individus exposés tels que le sexe et l'âge, pourraient influencer de manière significative l'effet indésirable global (Rengarajan *et al*., 2015).

I.2. Elimination des polluants hydrocarbonés

Plusieurs méthodes de décontamination basées sur des processus physico-chimiques ont été appliquées pour éliminer les hydrocarbures pétroliers polluant l'environnement. Le coût élevé, l'efficacité limitée, les dommages environnementaux causés par ces traitements ont conduit à la recherche d'autres alternatives, plus efficaces, respectueuses de l'environnement, polyvalentes et moins couteuses. L'utilisation des plantes et des micro-organismes dans la décontamination des milieux pollués (Bioremediation) (Fingas, 2011 ; Varjani, 2017).

Les HAP ont une forte adsorption sur les particules du sol, ce qui entraîne une diminution de leur accessibilité et de leur disponibilité pour les micro-organismes, en particulier dans les sols pollués âgés (Chen *et al*., 2016). De gros efforts ont été consacrés à la recherche de stratégies efficaces et fiables pour remédier les sols contaminés par les HAP.

I.2.1. Biodégradation microbienne

La biodégradation microbienne ou bioremédiation consiste à utiliser des micro-organismes pour dégrader les polluants ou les transformer en composés moins ou non toxiques (Varjani, 2017).

Des microbes dégradant les hydrocarbures ont été trouvés dans des habitats allant des sols polaires aux environnements marins (McGenity *et al*., 2012). Dans les environnements naturels, de nombreuses espèces microbiennes sont reconnues comme des candidats prometteurs avec une activité de dégradation des HAPs souhaitée (Chen *et al*., 2016).

La diversité phylogénétique des bactéries hydrocarbonoclastes est énorme ; plusieurs groupes récurrents se retrouvent dans la plupart des études de phytoremédiation, tels que *Pseudomonas, Burkholderia, Arthrobacter, Flavobacterium, Sphingomonas, Sphingobium, Rhodococcus, Mycobacterium, Acinetobacter, Alcaligenes* (Popp *et al.,* 2006; Chi *et al.,* 2018).

La biodégradation des hydrocarbures commence par les composés simples et se termine par les composés complexes dans l'ordre suivant : les alcanes linéaires, les alcanes ramifiés, les alkyles

aromatiques de poids moléculaire faible, les monoaromatiques, les alcanes cycliques, les polyaromatiques puis les asphaltènes (Varjani, 2017). Un ensemble de différentes enzymes intervient dans la biodégradation des polluants. Autrement dit, l'attaque des hydrocarbures par les bactéries pourrait commencer par la fixation des cellules microbiennes au substrat et/ou la production de biosurfactants ou de bioémulsifiants, permettant la libération de ces hydrocarbures adsorbés aux particules de sol (Karigar et Rao, 2011). Les communautés bactériennes du sol dégradent les hydrocarbures par de nombreuses voies cataboliques, rapportées dans le travail de Sierra-García *et al*., (2014). Le métabolisme aérobie des alcanes commence par l'incorporation terminale ou sub-terminale d'un atome d'oxygène par l'enzyme hydroxylase (codée par *alk*B). Une fois oxydés en alcool primaire, les étapes d'oxydation subséquentes par les alcools et les aldéhydes déshydrogénases convertissent les composés en acides gras qui peuvent ensuite être métabolisés par la β-oxydation et le cycle de l'acide citrique **(Figure 2)** (Moreno et Rojo, 2019).

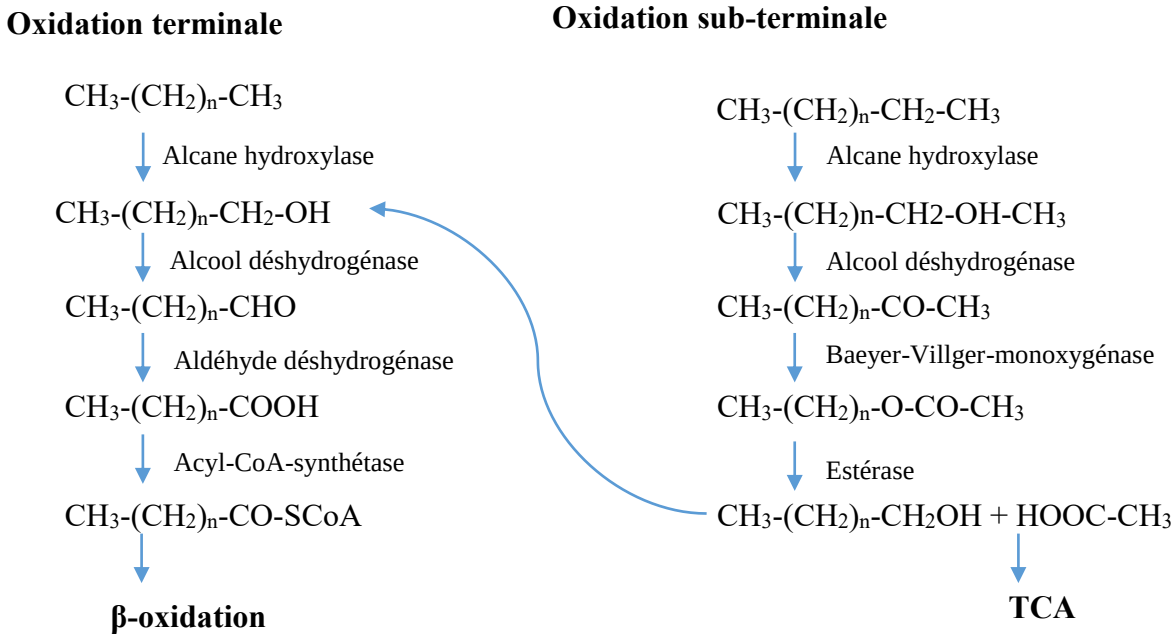

Figure 2. Voies métaboliques possibles pour la dégradation des alcanes par l'oxydation terminale et sub-terminale (Moreno et Rojo, 2019).

TCA : Cycle de l'acide tricarboxylique (Cycle de Krebs).

La dégradation aérobie des hydrocarbures aromatiques polycycliques (HAP) commence par l'action d'oxygénases, qui introduisent un atome d'oxygène dans les cycles aromatiques. La

dégradation des HAP est bien décrite pour le phénanthrène et le naphtalène (Ghosal *et al*., 2016) ainsi que pour le pyrène (Vila *et* Grifoll, 2009).

De nombreux facteurs affectent la bioremédiation, tels que ; la disponibilité des polluants, le type d'hydrocarbure, les micro-organismes, les conditions environnementales (le pH, la température, la teneur en eau, la salinité, la disponibilité en oxygène, les nutriments) et les propriétés physicochimiques du sol (Das et Chandran, 2011).

Le biosurfactant joue un rôle essentiel pour améliorer la disponibilité des hydrocarbures aux microbes (Lin *et al*., 2016). La capacité de la dégradation microbienne des HAP diminue avec l'augmentation de leur poids moléculaire (Varjani, 2017). Les hydrocarbures pourraient être dégradés par une souche individuelle ou un consortium appartenant au même genre ou à des genres différents (Kumari *et al*., 2018). De nombreuses recherches ont démontré que le consortium bactérien dégradant les hydrocarbures pourrait être plus efficace que les bactéries individuelles (Varjani, 2017).

I.2.2. Remédiation par les plantes : Phytoremédiation

La phytoremédiation comprend un groupe de technologies émergentes de remédiation biologique qui repose sur l'utilisation de plantes pour éliminer les polluants de l'environnement ou pour les rendre inoffensifs (Ojuederie et Babalola, 2017). Lorsqu'il s'agit de sols contaminés par les HAP, la phytoremédiation comprend quatre mécanismes : l'absorption directe ; la volatilisation, les sécrétions végétales et la décomposition enzymatique. La dégradation par les communautés microbiennes telluriques peut aussi avoir lieu (Chen *et al*., 2016). En phytoremediation, le choix de la plante est basé sur ses caractéristiques de production de biomasse, sa tolérance aux contaminants et le temps nécessaire pour atteindre une remédiation adéquate du sol (Balseiro-Romero *et al*., 2017). Les graminées ont un potentiel élevé de remédiation du sol en raison de leurs systèmes racinaires fibreux excessifs et de leur avantage concurrentiel à s'établir dans des conditions de stress (Chen *et al.*, 2016). Le ray-grass s'est révélé avoir un effet de rhizosphère élevé, ce qui peut se référer à son vaste système racinaire ramifié et à ses exsudats racinaires spéciaux (Ojuederie et Babalola, 2017). Sinon, en fonction de l'objectif fixé, du type de site contaminé et de polluants, une application de conception spécifique est effectuée pour traiter un problème environnemental. Les polluants peuvent avoir plusieurs destins, à savoir la phytostabilisation, la phytoextraction, la phytodégradation, la rhizodégradation, la phytovolatilisation ou l'évapotranspiration **(Figure 3)** (Pilon-Smits,2005).

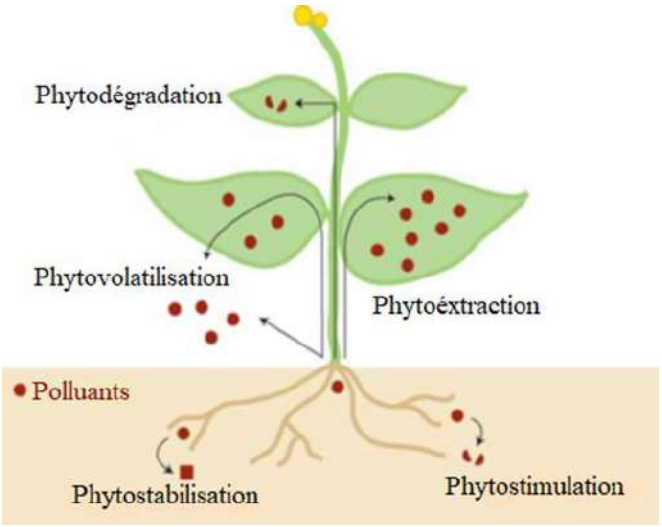

Figure 3. Destins possibles des polluants lors de la phytoremédiation (Pilon-Smits, 2005).

Les principaux avantages que présente la phytoremédiation par rapport aux autres techniques chimiques et physiques traditionnelles sont le coût relativement faible, la faible maintenance, l'application simultanée sur des sites pollués par des contaminants mixtes, respect de l'environnement et la réutilisations possibles du sol traité (Singh *et al*., 2003). Bien que plusieurs études ont montré que certaines plantes spécifiques ont une bonne capacité à dépolluer les sols contaminés par les hydrocarbures, certaines contraintes limitent le succès de la phytoremédiation, telles que le choix de la bonne plante, la nature des contaminants, la toxicité des contaminants pour la plante, la biodisponibilité des contaminants et le temps de remédiation (plus long) (Peng *et al*., 2009; Jeelani *et al*., 2017; Vangronsveld *et al.*, 2009). Malgré ces limites, la phytoremédiation reste une technologie prometteuse dont le développement est en augmentation depuis son émergence.

I.2.3. Remédiation par l'association plante-microbe

Bien que les plantes ont un potentiel de remédiation des xénobiotiques organiques, les polluants au-dessus d'une certaine concentration leur deviennent toxiques, conduisant à une diminution de la remédiation (Germaine *et al*., 2009). Pour améliorer la production de la biomasse végétale dans les sols pollués, les bactéries dégradant les polluants et favorisant la croissance des plantes peuvent être des candidats prometteurs de la phytoremédiation (Glick, 2010). La remédiation par le système plante-microbe est définie comme l'utilisation de plantes et de leurs micro-organismes associés pour séquestrer, dégrader ou stabiliser les contaminants. Les plantes peuvent établir des associations bénéfiques avec des micro-organismes dans leurs environnements naturels (Afzal *et al*.,

2019). Ces bactéries peuvent coloniser l'environnement externe ou interne de la plante (Santoyo *et al*., 2016). Les bactéries épiphytes sont celles qui vivent à l'extérieur de la plante, et à la surface des feuilles, tandis que celles qui occupent les racines des plantes sont appelés bactéries rhizosphériques. Les bactéries endophytes sont celles qui se développent à l'intérieur des tissus végétaux (Afzal *et al*., 2019).

Les bactéries associées aux plantes peuvent contribuer à améliorer la biodégradation des contaminants, car elles peuvent mettre à la disposition des plantes des composés qui les protègent en diminuant les niveaux d'hormones de stress, leur fournissent des nutriments clés et les protègent contre les agents phytopathogènes (Santoyo *et al*., 2016; Ojuederie et Babalola, 2017). À ce jour, les bactéries associées aux plantes ont été utilisées avec succès pour éliminer les hydrocarbures pétroliers (Singh *et al*., 2003; Cebron *et al*., 2009; Germaine *et al*., 2009; Vangronsveld *et al*., 2009). La phytoremédiation plante-microbe est devenue une stratégie durable de restauration des sols.

a. Rhizoremédiation

La rhizosphère est un système composé d'une zone de sol riche en nutriments, d'une variété de métabolites primaires (acides organiques, glucides et acides aminés) et de métabolites secondaires (alcaloïdes, terpènes et composés phénoliques), qui interfèrent d'une manière ou d'une autre avec la microflore de la rhizosphère (Venturi et Keel, 2016). Il est également nécessaire de mettre en évidence la libération d'oxygène par les racines des plantes, en aérant les pores, ce qui rend la rhizodégradation possible (Thomas *et al*., 2019). En effet, les exsudats, les nutriments et l'oxygène libérés par les plantes dans leurs racines sont absorbés par les micro-organismes rhizosphériques qui transforment une partie du polluant en métabolites secondaires libérés dans l'environnement rhizosphérique. L'autre partie des composés organiques est utilisée comme source d'énergie **(Figure 4)** (dos Santos et Maranho, 2018).

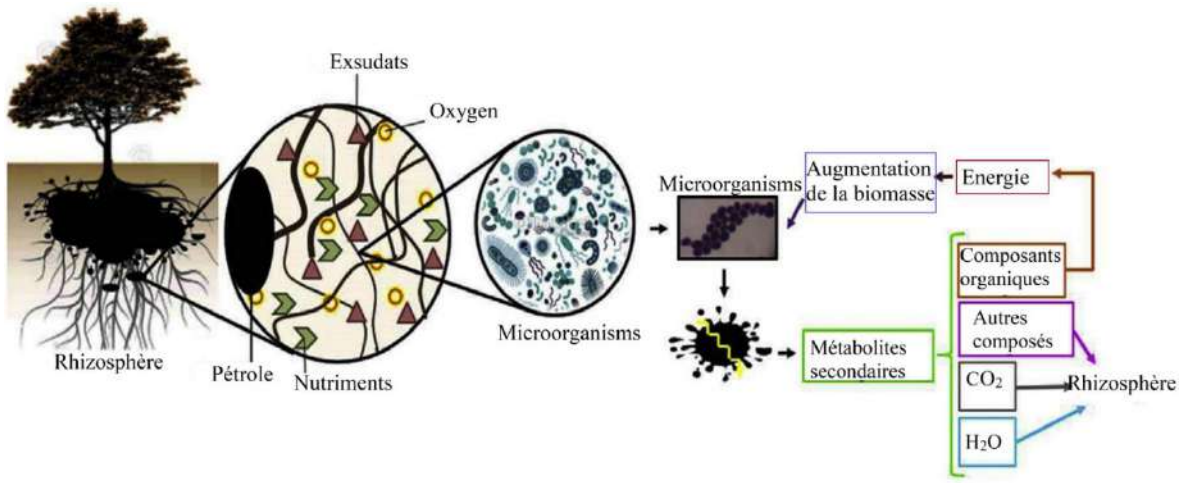

Figure 4. Les mécanismes de la rhizodégradation dans les sols contaminés par des hydrocarbures pétroliers (dos Santos et Maranho, 2018).

Au cours de la dernière décennie, pour éliminer les polluants organiques de l'environnement, les scientifiques ont fait recours aux plantes inoculées de bactéries dégradant les hydrocarbures ou produisant des PGP (Chapman, 2007; Germaine *et al*., 2009; Vangronsveld *et al*., 2009). Seules les bactéries qui ont certaines caractéristiques, comme la motilité et la production de polysaccharides, peuvent coloniser la rhizosphère (Lopes *et al*., 2016).

L'inoculation de plantes avec des souches bactériennes (PGPB) a montré une amélioration de la germination, de la vigueur des semis et de la croissance des plantes dans des conditions de stress (Balseiro-Romero *et al*., 2017). L'amélioration de la croissance des plantes par les bactéries rhizosphériques pourrait résulter de l'un des mécanismes suivant : Chélation de fer, production d'antibiotiques, production d'enzymes lytiques, fourniture de nutriments, fixation d'azote atmosphérique, réduction des niveaux d'hormones de stress et solubilisation des minéraux (Glick, 2010). Les bactéries peuvent affecter la croissance des plantes en utilisant un ou plusieurs des caractères cités.

Balseiro-Romero *et al*. (2017) ont constaté que l'inoculation des plantes avec des souches bactériennes PGP améliore la croissance des plantes mais pas l'activité oxydante ou la teneur en nutriments dans les conditions de stress. Au contraire, elles diminuent le stress oxydatif dû à l'augmentation de la tolérance et à l'adaptation aux conditions de stress. Les bactéries dégradant les contaminants peuvent donc améliorer l'adaptation des plantes aux contaminants en détoxifiant les sols contaminés, grâce à la minéralisation de ces composés (Gkorezis *et al*., 2016). Child *et al*., (2007) ont

constaté que la minéralisation du pyrène par la souche *Mycobacterium* sp. KMS a augmenté en présence de plantes dans la rhizosphère.

b. Endophyte-plante remédiation

La plupart des bactéries associées aux plantes proviennent du sol. Elles peuvent ensuite migrer de la rhizosphère vers le rhizoplan de leur hôte. Certaines bactéries du rhizoplan pénètrent dans les racines des plantes, et certaines d'entre elles peuvent se déplacer vers les parties aériennes (Frank *et al*., 2017).

Les bactéries endophytes sont considérées comme des bactéries rhizosphériques spécifiques qui possèdent la capacité de coloniser l'intérieur des racines des plantes (Afzal *et al*., 2019). Cette colonisation commence par les racines en reconnaissant certains composés des exsudats racinaires. La colonisation nécessite l'adhésion bactérienne à la surface des cellules racinaires, puis les bactéries commencent à pénétrer à l'intérieur en utilisant des mécanismes spécialisés (Walker *et al*., 2003). L'utilisation de bactéries endophytes dans la bioremédiation a été largement étudiée au cours des deux dernières décennies (Afzal *et al*., 2014). Les rapports sur la capacité des bactéries endophytes à dégrader différents polluants (les hydrocarbures pétroliers et les métaux lourds) ont été largement diffusés (Chen *et al*., 2016; Gkorezis *et al*., 2016; Bourceret *et al*., 2018).

A travers les racines et les feuilles, les hydrocarbures peuvent atteindre le système vasculaire et les espaces intercellulaires, réduisant ainsi la croissance des plantes ou induisant leur mortalité (Arellano *et al*., 2017). Les contaminants pénètrent dans le xylème des plantes, et les bactéries dégradantes résidant dans le xylème sont les meilleures candidates pour réduire la phytotoxicité et éviter l'évapotranspiration des contaminants (Thijs *et al*., 2016) **(Figure 5)**.

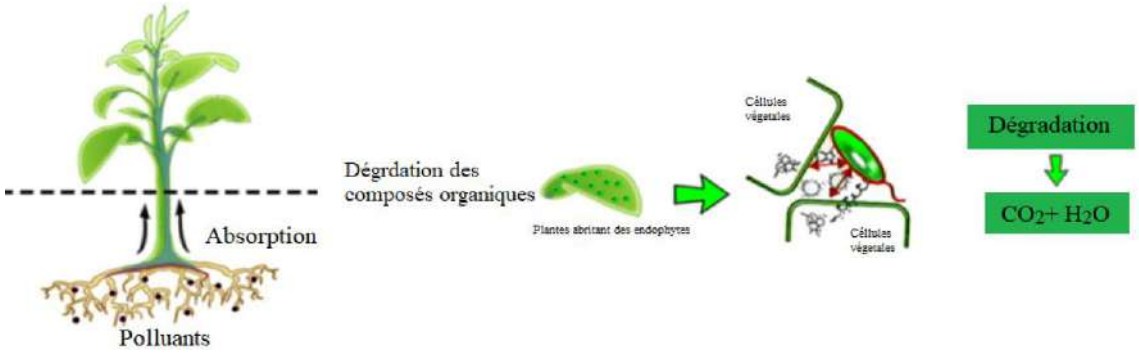

Figure 5. Le rôle des endophytes dans la dégradation des composés organiques dans les sols contaminés (Ijaz *et al*., 2016).

Les bactéries endophytes ayant la capacité de dégrader les HPT peuvent être isolées, puis ré inoculées dans leur hôte ou dans une autre plante hôte (Lumactud *et al*., 2016). Plusieurs recherches ont révélé que les plantes peuvent héberger diverses bactéries endophytes, notamment celles qui ont la capacité de dégrader les hydrocarbures (Peng *et al*., 2009; Babu *et al*., 2013). Wang *et al*. (2011) ont décrit une souche du genre *Dietzia* capable d'utiliser des *n*-alcanes, des composés aromatiques et du pétrole brut comme seules sources de carbone. Rares sont les rapports sur des bactéries capables de dégrader simultanément les n-alcanes et les HAP (Zhang *et al*., 2011).

Bien que de nombreuses études aient rapporté le succès de l'inoculation de bactéries endophytes dans le sol dans le cas de la biodégradation des polluants, la colonisation reste un obstacle majeur dans l'analyse d'une inoculation efficace (Germaine et *al*., 2009). Plusieurs aspects liés à l'inoculation bactérienne tels que la nature des polluants, le type de sol, les espèces végétales, la densité de l'inoculum et les méthodes d'inoculation ont fait l'objet de recherches approfondies et de débats (Afzal *et al*., 2011 ; Khan *et al*., 2013 ; Zheng *et al*., 2018). L'inoculation microbienne favorise l'activité des enzymes du sol, l'élimination des HAP et la croissance des plantes. Les bactéries endophytes peuvent donc présenter un intérêt particulier en tant que bio inoculants, car elles ont l'avantage de proliférer dans les tissus végétaux, faisant ainsi face à une moindre concurrence pour les nutriments et étant protégées du stress environnemental élevé dans les sols pollués (Sturz *et al*., 2000).

Plusieurs méthodes ont été rapportées pour introduire les bactéries dans les plantes, notamment l'inoculation des graines, de la rhizosphère et du sol (Compant *et al*., 2019). L'inoculation microbienne efficace des graines est la première étape pour améliorer la phytoremédiation, tout comme il est important de maintenir la viabilité des microbes inoculés et leur colonisation (Rilling *et al*., 2019). De nombreuses études rapportent que l'inoculation directe des graines ou du sol est utilisée en phytoremédiation à l'aide des dégradeurs spécifiques (Germaine *et al*., 2009; Thion *et al*., 2013; Ijaz *et al*., 2016).

Les facteurs environnementaux ; biotiques et abiotiques ont été récemment étudiés. L'effet des facteurs biotiques a été résumé dans la survie et la migration des micro-organismes hydrocarbonoclastes, tandis que les facteurs abiotiques tels que la nature physicochimique et la concentration des contaminants, la disponibilité des nutriments et les propriétés du sol peuvent influencer le processus de biorestauration (Santoyo *et al*., 2016). La biodisponibilité des contaminants peut être influencée par les propriétés du sol, telles que sa texture, la taille des particules, sa teneur en matière organique, sa teneur en eau, son pH et sa structure (Jung *et al*., 2008). Les contaminants des

sols anciennement pollués sont de plus en plus impossibles à assimiler et à biodégrader par les micro-organismes telluriques (Thomas *et al*., 2019). Les plantes et les microbes qui leur sont associés sécrètent des enzymes et des exsudats qui agissent comme du surfactant leur permettant d'augmenter la disponibilité de ces contaminants (Bais *et al*., 2006; Cébron *et al*., 2011).

Il existe plusieurs bactéries endophytes dégradant les hydrocarbures dans la nature. L'utilisation de bactéries endophytes indigènes, individuellement ou en consortium, comme un outil améliorant la dégradation des polluants dans les écosystèmes terrestres et aquatiques est désormais connue. Il est approuvé qu'un seul micro-organisme ne dégrade pas efficacement tous les HAP en raison de leurs structures chimiques complexes. Les consortiums microbiens auraient la capacité de mieux dégrader le mélange d'HAP (Chen *et al*., 2016).

La phytoremédiation assistée par les microbes a été bien documentée ainsi que son potentiel en tant qu'une stratégie efficace et peu coûteuse pour éliminer les hydrocarbures des sols pollués (Thijs *et al*., 2016). Il n'est pas surprenant que les bactéries endophytes, isolées des plantes poussant dans des sols contaminés par des hydrocarbures pétroliers, aient une capacité de dégradation des hydrocarbures car ces composés sont d'origine naturelle.

I.3. Rôle de l'association plante-endophyte dans la phytoremediation

Les bactéries endophytes pourraient affecter positivement les plantes de manière directe et/ou indirecte. Elles peuvent améliorer la croissance et le développement des plantes en modulant la croissance et en assurant la nutrition dans des conditions de stress (Egamberdieva *et al*., 2015). Selon Afzal *et al*., (2014), les bactéries endophytes ayant les activités classiques PGP et dégradation des polluants, donnent de meilleurs résultats que celles n'ayant qu'une de ces deux activités. De plus, l'activité de dégradation des contaminants pourrait être considérée comme une caractéristique pour améliorer la croissance des plantes, car les contaminants, en général, affectent négativement le développement des plantes et leur élimination sera bénéfique (Cruz-Morales *et al*., 2016).

La présence des caractères PGP dans les bactéries endophytes dégradant les hydrocarbures peut leur permettre d'être sélectionnées comme une source prometteuse d'amélioration de la phytoremédiation des sols contaminés (Khan *et al*., 2013). Cette amélioration est assurée grâce à l'amélioration de l'adaptation et de la croissance des plantes qui leur est conférée par les caractères PGP des bactéries endophytes (Afzal *et al*., 2014).

Les bactéries promotrices de croissance des plantes peuvent agir comme des biofertilisants en augmentant la disponibilité des nutriments par la fixation de l'azote et la solubilisation des phosphates.

Elles peuvent aussi se comporter comme des phytostimulants produisant des phytohormones améliorant la croissance des plantes tels que l'indole 3-acétique acide (IAA) ou le 1-amincyclopropane-1-carboxylate (ACC) désaminase. Elles peuvent également agir comme des agents de lutte biologique produisant des sidérophores, des antibiotiques ou des composés antifongiques (Balseiro-Romero *et al*., 2017).

L'azote (N_2) est l'un des nutriments limitants la croissance des plantes. il doit d'abord être réduit en ammoniaque avant d'être utilisé par ces dernières, qui sont incapables de réduire l' N_2 atmosphérique (Ågren *et al*., 2012; Santoyo *et al*., 2016).

Les inoculants endophytes fournissant les besoins des cultures en azote permettent l'augmentation des rendements (Puri *et al*., 2018). Ces bactéries ayant une activité nitrogénase peuvent fixer l'azote atmosphérique et le fournir aux plantes (Egamberdieva *et al*., 2015). Les chercheurs ont rapporté que les bactéries endophytes fonctionnent mieux que les bactéries de la rhizosphère. Elles permettent aux plantes de se développer dans un sol limité en azote et d'améliorer leur croissance et leur état phytosanitaire, en mettant à leur disposition l'azote qu'elles ont accumulé (Afzal *et al*., 2019).

Le phosphore est également l'un des facteurs limitant la croissance de la plante (Shrivastava et Kumar, 2015). Il se présente sous forme d'un micronutriment insoluble dans le sol. Il est donc indisponible pour les plantes (Afzal *et al*., 2019). Selon le pH du sol, le phosphate existe sous forme de phosphate tricalcique, de phosphate d'aluminium ou de phosphate de fer (Sharma *et al*., 2013).

Les bactéries endophytes peuvent solubiliser le phosphate précipité en utilisant différents mécanismes tels que l'acidification, la chélation, l'échange des ions et la dissolution par les enzymes (Afzal *et al*., 2019).

L'acidification du sol par les endophytes conduit à la libération d'un anion de phosphate monovalent à partir de sa forme minéral, par la substitution d'un proton (H^+) (Alori *et al*., 2017). Ainsi, les bactéries solubilisant le phosphate adoptent ce mécanisme libérant des acides organiques par l'oxydation des sources de carbone organique (Suleman *et al*., 2018).

Diverses bactéries endophytes solubilisant le phosphate sont isolées des plantes cultivées dans des sols contaminés par les hydrocarbures. Parmi ces bactéries, on cite *Rhizobium, Serratia, Pseudomonas, Xanthomonas. Bacillus, Clavibacter, Curtobacteriu,* et *Rhodococcus* (Kukla et *al*., 2014; Pawlik *et al*., 2017; Wu *et al*., 2019).

Le fer est un élément nécessaire pour de nombreux processus physiologiques comme la transpiration et la respiration (Afzal *et al*., 2019). Il est l'un des éléments les plus abondants dans la

terre, mais, il n'est généralement pas disponible pour les plantes et les micro-organismes, car dans la nature, il se présente principalement sous les formes insolubles de l'ion ferr (III) (Fe^{3+}), d'hydroxydes de fer ($Fe(OH)_2$) et d'oxyhydroxydes de fer $Fe(OH)_3$ (Jacoby *et al*., 2017).

Afin d'obtenir le fer pour leur croissance et leur développement, certaines bactéries endophytes synthétisent des molécules de liaison de faible poids moléculaire appelées sidérophores (chélateurs). Ces derniers, agissant comme des agents solubilisant fer en se liant au Fe^{3+} des minéraux ou de composés organiques insolubles, le rendant ainsi disponible (Compant *et al*., 2010). De plus, les bactéries productrices de sidérophores peuvent stimuler la croissance des plantes, soit directement en améliorant la nutrition en fer, soit indirectement, en limitant sa disponibilité pour les phytopathogènes, conduisant à l'inhibition de leur croissance (Ramakrishna *et al*., 2019).

Les sidérophores, intervenant dans la synthèse des auxines, peuvent également favoriser la croissance des plantes (Lopes *et al*., 2016).

Les phytohormones produites par les bactéries associées aux plantes stimulent leur croissance. Les auxines, les cytokinines et les gibbérellines peuvent être considérées comme des agents prometteurs de croissance et de développement des plantes (Egamberdieva *et al*., 2015).

L'une des phytohormones les plus étudiées est l'acide indole-3-acétique (AIA) qui est une auxine importante pour plusieurs processus physiologiques, y compris la signalisation inter cellulaire, la régulation du développement des plantes et l'induction des systèmes de défense des plantes (Afzal *et al*., 2019). De plus, l'AIA peut augmenter le nombre de poils absorbants, le nombre de racines latérales et la surface racinaire totale, conduisant à une amélioration de l'exsudation racinaire et de l'absorption des minéraux du sol (Kong *et al*., 2015).

Les cytokinines et les gibbérellines jouent un rôle important dans la régulation de la croissance et du développement des plantes. Les cytokinines sont impliquées dans la régulation du transport des métabolites, la division cellulaire et la synthèse des protéines. Alors que les gibbérellines sont impliquées dans la division cellulaire et l'allongement, l'activation des membranes et la synthèse des enzymes amylolytiques (Tanimoto, 2005).

L'éthylène est une hormone végétale qui contrôle la croissance et les processus physiologiques dans des conditions de stress abiotique et biotique, comprenant l'initiation de la division racinaire, la sénescence foliaire, la nodulation racinaire, l'allongement cellulaire et le transport des auxines (Afzal *et al*., 2019). Elle est synthétisée en réponse aux contraintes environnementales. L'augmentation de sa

production inhibe l'allongement des racines, le développement des racines latérales et la formation des poils absorbants (Santoyo *et al*., 2016).

Les bactéries endophytes produisent la 1-aminocyclopropane-1-carboxylate (ACC) désaminase qui est une hormone clé pour le clivage de l'1-aminocyclopropane-1-carboxylate (ACC), un précurseur de l'éthylène (Egamberdieva *et al*., 2015). Les bactéries productrices d'ACC désaminase hydrolysent l'ACC exsudé en α-cétobutyrate et en ammoniac, et l'utilisent comme une source d'azote (Glick, 2010) **(Figure 6).**

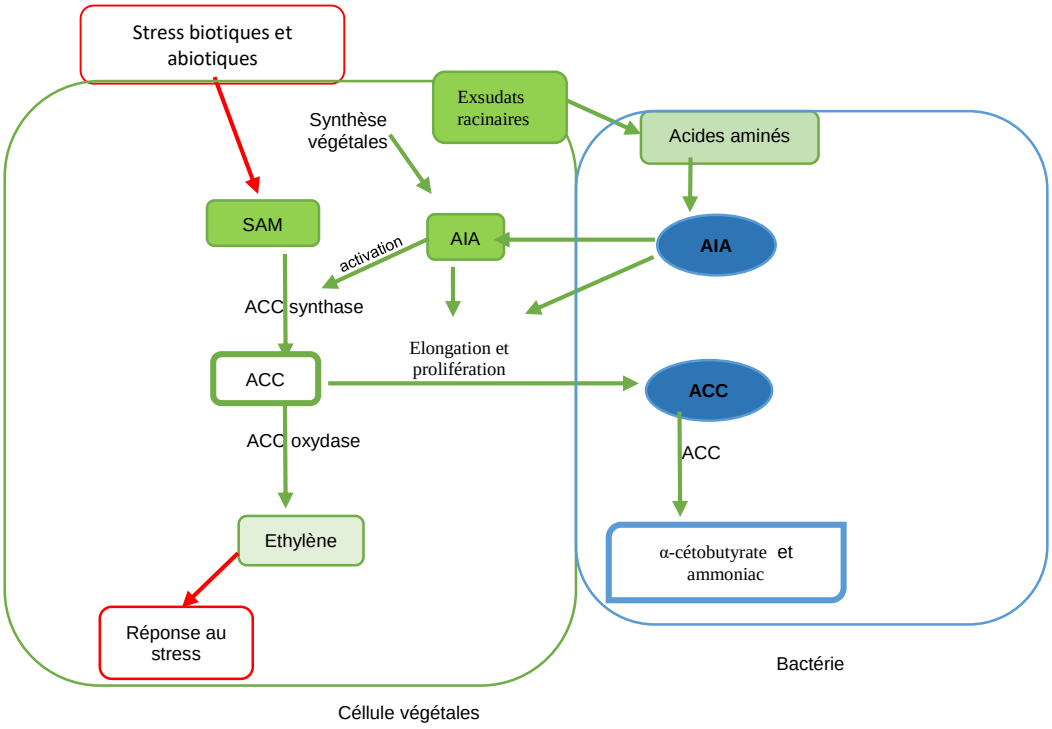

Figure 6. Représentation schématique du rôle de l'AIA et l'enzyme ACC désaminase dans l'amélioration de la croissance des plantes.

SAM (S-denosyl méthionine). AIA (l'acide indole-3-acétique). ACC (1-aminocyclopropqne-1-décarboxylate)

Les bactéries endophytes ayant une activité ACC désaminase, stimulent la croissance des plantes conduisant à des racines plus étendues, améliorant ainsi l'efficacité de la phytoremédiation (Arshad *et al*., 2007). L'étude du potentiel de différentes espèces de graminées inoculées par des bactéries PGP possédant l'ACC désaminase, utilisée en phytoremédiation d'un sol pollué par la créosote a révélé une amélioration de la production de la biomasse traduite par une augmentation de la densité des racines et des tiges (Huang *et al*., 2004).

Les bactéries (endophytes ou les rhizobactéries) ayant, à la fois, l'aptitude à dégrader les polluants et à promouvoir la croissance des plantes donnent de meilleurs résultats que celles qui ont une des deux activités seulement. De plus, l'activité de dégradation des contaminants pourrait être considérée comme un trait favorisant la croissance des plantes, car les contaminants, en général, affectent négativement le développement des plantes ; par conséquent, leur élimination leur sera bénéfique (Ijaz *et al*., 2016).

La détermination des activités PGP a une grande pertinence pour évaluer l'utilité réelle des bactéries associées aux plantes dans la bioremédiation. En effet, la présence des caractères PGP dans les bactéries dégradant les hydrocarbures peut être à l'origine de leur choix pour une bonne phytoremédiation (Khan *et al*., 2013; Afzal *et al*., 2014a; Afzal *et al*. 2019) **(Figure 7).**

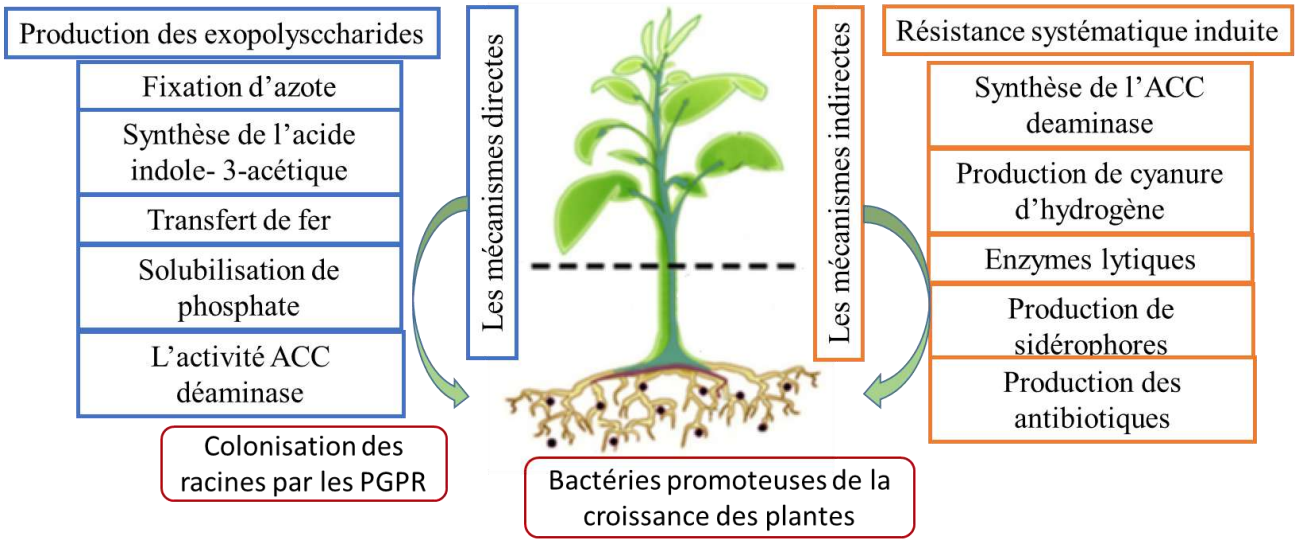

Figure 7. Mécanismes utilisés par les bactéries favorisant la croissance des plantes sous les conditions de stress (Afzal *et al*. 2019).

Objectifs

La présente étude a pour principaux objectifs :

- L'isolement des actinobactéries endophytes à partir des racines stériles des plantes poussant dans des sols contaminés par le pétrole brut au niveau de deux sites d'exploitation pétrolière (Hassi Messaoud et Haoud Berkaoui, Ouargla, Algérie).

- L'évaluation de la capacité des isolats à dégrader les hydrocarbures pétroliers et à produire des métabolites favorisant la croissance des plantes (PGP).

- Le choix d'une souche prometteuse et l'étude de sa capacité à remédier les sols contaminés par le pétrole brut Algérien.

- L'inoculation d'une plante modèle (*Zea mays*) avec la souche sélectionnée et l'évaluation de leurs efficacités à remédier les sols contaminés par le pétrole brut.

Nouveauté de la thèse

Les microorganismes ont prouvé leur efficacité dans la remédiation et la restauration des sols pollués. Ils ont été utilisés en culture pure ou sous forme d'un consortium pour restaurer des milieux contaminés par un ou plusieurs polluants. Cette technologie, appelée bioremédiation fait appel, dans le cas où les agents dépolluants sont des bactéries à des protéobactéries et des gammaprotéobactéries connues pour la rapidité et la facilité de leur isolement et de leur culture.

Par ailleurs, les plantes ont également été largement utilisées comme agents dépolluants. Leur intérêt dans le processus de remédiation est double, car en plus de la dépollution, ces plantes, souvent pérennes (arbres et arbustes) permettent la création d'un environnement vert agréable.

Au cours des dernières années, la remédiation par les microorganismes associés aux plantes (plantes-microbes) a été largement étudiée. Cette méthode a été appliquée sur des sols anciennement contaminés par des hydrocarbures pétroliers ou par des métaux lourds.

Avant notre étude, aucun travail sur la bioremédiation des sols pollués par du pétrole brut léger en utilisant le complexe plantes-microbes n'a été publié. Par ailleurs, la particularité des actinobactéries, connues pour leur versatilité métabolique et leur capacité à dégrader divers polluants, est à l'origine du choix porté sur ces microorganismes pour la construction du complexe plante-microbe orienté vers le traitement des sols contaminés par les hydrocarbures pétroliers. Cet axe n'a fait l'objet d'aucune étude antérieure.

I. Overview

A huge quantity of petroleum hydrocarbons enters the environment whether accidentally or due to human activities. Their toxicity and their destructive properties of natural ecosystems represent a major environment problem (Moliterni *et al*., 2012). The US Environmental Protection Agency, (U.S. EPA 1986) classified these compounds as priority environmental pollutants Therefore, crude oil pollution of soil is a worldwide environmental problem which it is necessary to resolve immediately (Koshlaf *et al*., 2016).

Algeria is one of the biggest petroleum producers. The two important exploitation sites are situated in Ouargla (Hassi Messaoud and Haoud Berkaoui). Thus, it is one of the regions exposed to petroleum soil contamination.

In the last two decades, a particular attention has come to be placed on the study of phytoremediation as alternative of physicochemical methods, known as expensive and destructive to the environment (Li *et al*., 2012). However, phytoremediation is an efficient, environmentally friendly and cost-effective approach (Bisht *et al*., 2014). It is used in the field of remediation of various environmental contaminants such as petroleum hydrocarbons, herbicides, explosives and heavy metals (Li *et al*., 2012).

Some plant species have shown the capacity to deal with relatively high concentrations of organic chemicals, which may be taken up, translocated, metabolized, and/or volatilized. In addition, plants may stimulate the degradation of xenobiotics in the rhizosphere (rhizodegradation) releasing plant root exudates (Alvarez *et al*., 2012; Álvarez *et al*., 2015).

On the other hand, endophytic bacteria colonize the inner tissues of living plants without causing any harm, establishing a harmonious relationship with the host plant (Tan et Zou, 2001; Kidd *et al*., 2017). The phytoremediation can be enhanced by the use of endophytes to cleanup organic and inorganic contaminants in soil and water (Pilon-Smits, 2005).

Current efforts are now focused on the endophytic bacteria with the ability to degrade organic contaminants and/or improve plant growth (Khan *et* al. 2013). Several studies have reported many endophytic bacteria which present an important role in the degradation of different hydrocarbon compounds. These microorganisms are Gram negative bacteria such as *Pseudomonas* and *Brevundimonas* (Phillips *et al*., 2008; Peng *et al*., 2013; Zhang *et al*., 2014) and Gram positive bacteria, including actinobacteria (Kukla *et* al., 2014; Singh et Sedhuraman, 2015).

Actinobacteria are a group of bacteria that play an important role in metabolizing complex organic matter in nature, removing effectively the xenobiotic compounds such as petroleum hydrocarbons (Barabás *et al*., 2001). Furthermore, Endophytic actinobacteria isolated from healthy surface-disinfected plant tissues play an important role in the metabolization of complex polymers such as organic and inorganic toxics into more readily assimilable nutrients (Doumbou *et al*., 2005). They are also well-known as producers of a wide range of plant growth promoting activities (PGP) (Subramaniam *et al*., 2016). Those properties make them promising candidates to enhance the phytoremediation of polluted soils. Bacteria from the phylum Actinobacteria seem to have the potential to use different metabolic pathways (Polti *et al*., 2011).

Furthermore, the genus *Streptomyces* is known for the production of more than 60% of bioactive metabolites, they were usually explored for antimicrobial activities (Goldman et Green, 2009). Few studies indicate that some strains of genus *Streptomyces* isolated from soil have the ability to degrade hydrocarbons (Ferradji *et al*., 2014). Nowadays, no information is available so far about the action of endophytic *Streptomyces* in the phytoremediation of petroleum-impacted soils.

Because of the potential role of actinobacteria to use different complex compounds, the study of the hydrocarbonoclastic endophytic actinobacteria associated with plants grown in contaminated soil may provide valuable information about the potential economic and environmental benefits of using endophytic actinobacteria in the phytoremediation.

II. Structure of the thesis

The manuscript of the thesis is composed of three chapters. It is thus structured:

General introduction; which consists of a brief literature review focused on petroleum hydrocarbons and their toxicity, bioremediation and phytoremediation technologies as well as the role of endophyte-plant partnership in the phytoremediation. The objectives and the original contributions of thesis are specified in this introduction.

Experimental layout; which presents the different experimental activities adopted during the achievement of the different parts of our study.

Chapter I; presents the results and the discussions of the research work about the isolated endophytic actinobacteria that tolerate volatile petroleum hydrocarbons and their ability of biodegradation of Algerian crude oil and their PGP production.

Chapter II; dedicates to the assessment of the ability of *Streptomyces* sp. Hlh1 to remediate contaminated soils with different concentrations of Algerian crude oil. The toxicity of the end metabolites is also evaluated in this chapter.

Chapter III; reports the results and the discussions of the efficiency of the remediation process by the partnership Plant-*Streptomyces* sp. Hlh1 in artificial contaminated soil with crude oil or polycyclic aromatics hydrocarbons (PAH) under lab scale conditions.

Conclusion; which comes to wind up the work done and highlight the contemplated perspectives at laboratory scale and field scale for better understanding of undertaking theme

It should be noted that this thesis is in the form of Thesis-Articles. It begins with a general introduction written in French and translated into English. Each chapter is represented by a scientific article published in a journal of rank A, preceded by a detailed summary written in French. A general conclusion of the executed published research written in French and translated into English.

General introduction

I. General introduction

Petroleum, in Latin “rock oil”, which is a dark, sticky and viscous liquid (Varjani, 2017). It is composed of carbon and hydrogen, also may contains nitrogen, sulfur and oxygen (Balachandran *et al*., 2012). It is called crude oil, once extracted from subsurface and transported to refineries where transformed to other products (Ugochukwu et Fialips, 2017). The location of oil field, the age and the depth of the well determine the crude oil composition (Varjani, 2017). It can be classified as light, medium or heavy based on the proportions of heavy molecular weight components (Xu et Lu, 2010).

Total petroleum hydrocarbons (TPH) is a term used to describe a large family of heterogeneous compounds that are found in crude oil and whose main chemical constituents are carbon and hydrogen atoms. TPH can be divided to four groups (fractions) of petroleum hydrocarbons (Bojes et Pope, 2007); aliphatics, aromatics (contains one or more rings), resins and asphaltenes (Abdel-Shafy et Mansour, 2016).

Aliphatic hydrocarbons represent the predominant fraction of crude oil, they are saturated or unsaturated, linear or branched open-chain such as alkanes, iso-alkanes, cycloalkanes (naphthenes), terpenes and steranes (Zhang *et al*., 2011). According to their molecular weight, they are classified to four classes: gaseous alkanes, low molecular weight aliphatic (C_8-C_{16}), medium molecular weight aliphatic (C_{17}-C_{28}), and high molecular weight aliphatic (>C_{28}) (Varjani, 2017).

Aromatics are hydrocarbons contains one or more ring and classified to two classes, namely; monocyclic aromatic hydrocarbons (MAH) contain only one ring and polycyclic aromatic hydrocarbons (PAH) usually contain more than one benzene ring. PAH constitute of two or three rings are known as light PAH, while those constitute of four or more rings are known as heavy PAH (Abdel-Shafy et Mansour, 2016). Resins and aspahltenes are polar compounds, with nitrogen, sulfur, oxygen atoms and trace metals (Varjani, 2017). They may contain alkyl groups which participate to their resistance to biodegradation (Zhu *et al*., 2010).

I.1. Pollution source and its impact on the environment

The term contamination refers to the presence of a substance where the concentration should not be at or above the baseline level. In other term, a contaminant is an undesired material although it does not have to be necessarily harmful. While, pollution refers to the presence of a harmful contaminant to the organisms or the infrastructure (Chapman, 2007).

Petroleum pollution in the environment may result through spillages and leakage of these products from underground tanks, steamers, unplugging of oil wells, abandoned oil refinery sites in soil, ground water and ocean (Varjani, 2017).

Hydrocarbon compounds are persistent, recalcitrant and highly toxic pollutants, as they cause permanent damage to the environment (Das et Chandran, 2011). They are hemotoxic, carcinogen and teratogenic compounds. Volatile organic compounds (VOC) are extremely toxic to human-being (Ghosal *et al*., 2016). Polycyclic aromatic hydrocarbons (PAH) cause international problem because of their strong ecotoxic properties (Chen *et al*., 2016).

Algeria is one of the top three producers of oil in Africa. It is considered as the most export country of petroleum to Europe and Eurasia (US Energy Information Administration, 2019). It has three main export terminals in Algeria and five refineries, three are in Skikda, Algiers and Arzew, and two in the Saharan cities of Hassi Messouad and Adrar.

The main source of oil pollution in Algeria waters are the effluents of the on-shore refineries and the various discharges and runoffs from the oil export terminals (Carpent et Kostianoy, 2019). While, most of the soil pollution sources in Algeria result from the spillages and leakage from underground tanks, unplugging of oil wells **(Figure 1).**

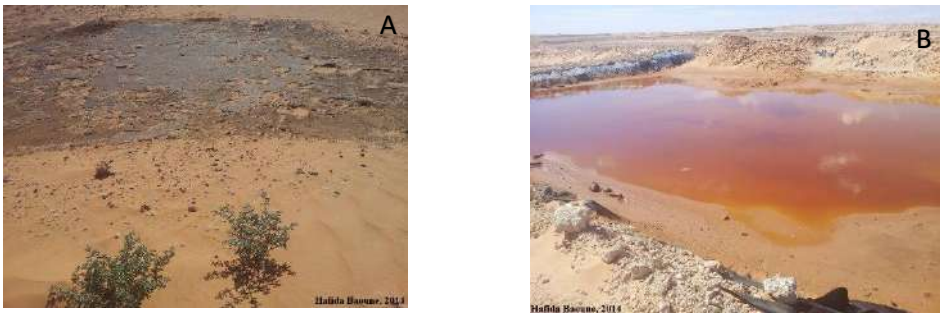

Figure 1. Sample pictures of pollution source aged (A) and recent (B) in Houad Barkaoui (Taken in 2014) (Baoune, 2014).

Hydrocarbons can have various effects on humans and living organisms. Depending on exposure time, health effects could be acute after single exposure or chronic after a repeated exposure (Abdel-Shafy et Mansour, 2016). In addition, contaminants concentrations, specific toxicity of some PAH, the route of exposure and the characteristics of exposed individuals such as sex and ages, could influence significantly the overall adverse effect (Rengarajan *et al*., 2015).

I.2. Hydrocarbons removal

Several decontamination methods based on physico-chemical processes were performed to clean-up petroleum hydrocarbons pollutants from the environments. The expensive cost, the limited efficiency, environment damages caused by those treatments led to search for another technique, more efficient, environmentally friend, versatile and economic. The use of plant and microorganisms to decontaminate polluted soil (Bioremediation) is turned out to be the most promising (Fingas 2011; Varjani 2017).

PAH have a strong adsorption to the soil particles, which decreases their accessibility and availability to microorganisms, especially in aged polluted soils (Chen *et al*., 2016). Great efforts have been dedicated to search for efficient and reliable strategies to remediate hydrocarbons

I.2.1. Microbial biodegradation

Microbial biodegradation or bioremediation treatment is the use of microorganisms to degrade or to transform pollutants to harmless compounds (Varjani, 2017).

Hydrocarbon degrading microbes have been found in habitats ranging from polar soils to marine environments (McGenity *et al*., 2012). In natural environments, many microbial species are recognized as promising candidates with the desired degradation activity to degrade PAH (Chen *et al*., 2016).

The phylogenetic diversity of hydrocarbonclastic bacteria is enormous; several recurrent groups are found in the most studies of phytoremediation, such as *Pseudomonas*, *Burkholderia*, *Arthrobacter*, *Flavobacterium*, *Sphingomonas*, *Sphingobium*, *Rhodococcus*, *Mycobacterium*, *Acinetobacter*, *Alcaligenes* species (Popp *et al*., 2006; Chi *et al*., 2018).

Biodegradation of hydrocarbons starts from simple and ends to complex compounds in the following order; linear alkanes, branched alkanes, low molecular weight alkyl aromatics, monoarmatics, cyclic alkanes, polyaromatics then asphaltenes (Varjani, 2017). A set of different enzymes are involved in the biodegradation of pollutants. In other words, hydrocarbons attack by bacteria could be carried out through different mechanisms: cell microbial attachment to the substrates and/or biosurfactants or bioemulsifiers production (Karigar et Rao, 2011). Soil bacterial communities degrade hydrocarbons via numerous different catabolic pathways are reported in the work of Sierra-García *et al*., (2014). Aerobic metabolism of alkanes starts with terminal or sub-terminal incorporation of oxygen into the hydrocarbon by hydroxylase enzyme (encoded by *alk*B). Once oxidized to primary

alcohol, subsequent oxidation steps by alcohol and aldehyde dehydrogenases convert the compounds to fatty acids that may then metabolized by β-oxidation and the citric acid cycle **(Figure 2)** (Moreno et Rojo, 2019).

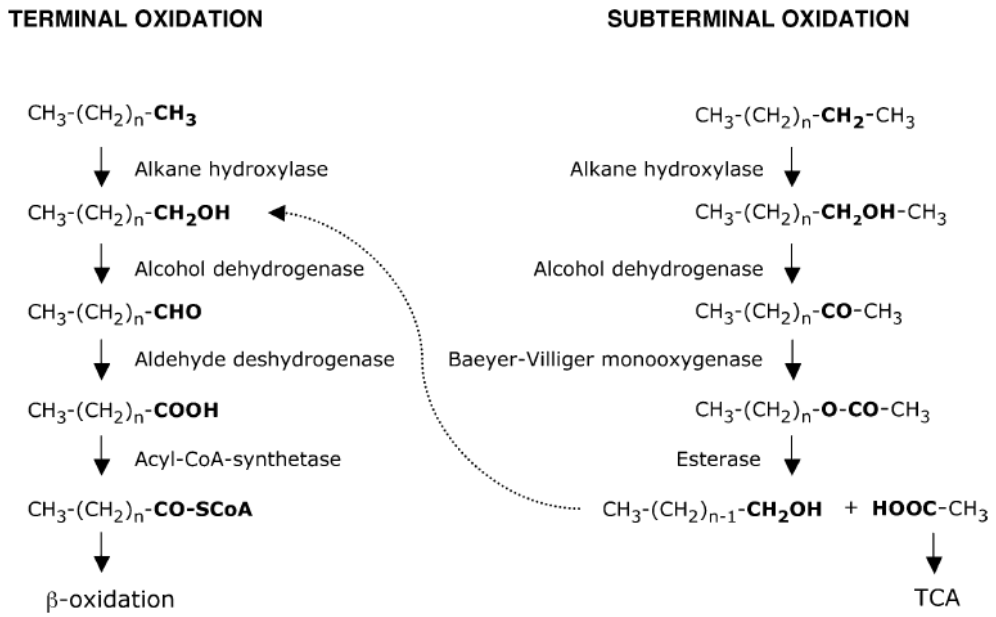

Figure 2. Possible metabolic pathways for the degradation of alkanes by terminal and sub-terminal oxidation (Moreno et Rojo, 2019).

TCA: TCA : tricarboxylic acid cycle (Krebs cycle).

Aerobic degradation of polycyclic aromatic hydrocarbons (PAH) begins by the action of oxygenases, which introduce oxygen to the aromatic rings. The PAH degradation have been well described for phenanthrene and naphthalene (Ghosal *et al.*, 2016) as well as pyrene (Vila et Grifoll, 2009).

Many factors affect the bioremediation, such as; pollutants availability, type of hydrocarbons, microorganisms, environmental conditions (pH, temperature, water content, salinity, oxygen availability, nutrients) and physiochemical properties of the soil (Das et Chandran, 2011).

Biosurfactant play a critical role to enhance the availability of hydrocarbons pollutants to microbes (Lin *et al*., 2016). The microbial degradation ability of PAHs decreased with the increase of their molecular weight (Varjani, 2017). Hydrocarbons could be degraded by individual strain or consortium of strains belonging to the same or different genera (Kumari *et al.,* 2018). Numerous research studies demonstrated that hydrocarbon degrading bacterial consortium could be better than individual bacteria (Varjani, 2017).

I.2.2. Remediation by plants: Phytoremediation

Phytoremediation comprises a group of emerging biological remediation technologies that based on the use of plants to remove pollutants from the environment or to make them harmless (Ojuederie et Babalola, 2017). Phytoremediation of PAH contaminated soil include four mechanisms: direct absorption; volatilization, plant secretions and enzyme decomposition. The degradation by soil microbial communities can also take place (Chen *et al*., 2016). In phytoremediation, the choice of the plant is based on its biomass production characteristics, contaminant tolerance and time necessary to reach the adequate soil clean-up (Balseiro-Romero *et al*., 2017). Grass plants have a high potential for soil phytoremediation due to their excessive, fibrous root systems and their competitive advantage to establish under stress conditions (Chen *et al*., 2016). Ryegrass were found to have high rhizosphere effect which may refer to their extensive branched root system and their special root exudates (Ojuederie et Babalola, 2017). Otherwise, depending on the goal to be achieved, the type of contaminated site and pollutants of concern, a specific design application is made to treat a certain environmental issue. The pollutants could have several fates, namely phytostabilization, phytoextraction, phytodegradation, rhizodegradation, phytovolatilization, and evapotrospiration **(Figure 3)** (Pilon-Smits, 2005).

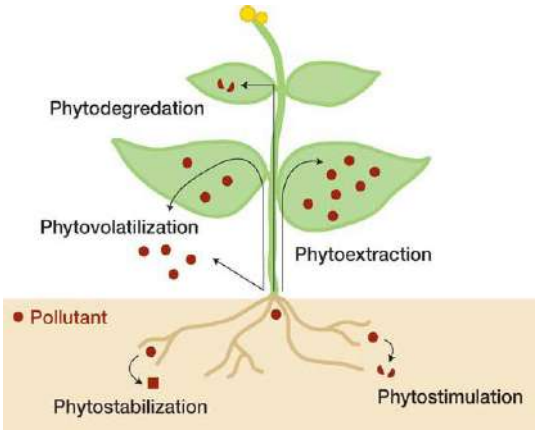

Figure 3. Possible fates of pollutants during the phytoremediation: the pollutants are represented by red circles (Pilon-Smits, 2005).

The main advantages of phytoremediation comparing to other traditional chemical and physical technics are the relatively low cost, low maintenance, applicable to remediate simultaneously sites with mixed contaminates, less environmental impact and the possible reuses of the treated soil (Singh *et al*., 2003). Although several studies found that some specific plants have good capacity to clean up

contaminated soils with hydrocarbons Some constraints restrict the phytoremediation success, such as the choose of the proper plant, the nature of the contaminants, the toxicity of contaminants to the plant, the contaminant bioavailability and the longer restoration time (Vangronsveld et *al.*, 2009) (Peng et *al*., 2009; Jeelani et *al*., 2017). In spite of these limitations, phytoremediation still a promising technology, whose development is increasing since its emergence.

I.2.3. Plant-microbe remediation

Although, plants have potential for remediating organic xenobiotic, pollutants above a certain concentration become toxic to them, conducting to a low remediation (Germaine *et al*., 2009). To enhance plant biomass in polluted soils, pollutants degrading and plant growth promoting bacteria can be promising candidates for phytoremediation (Glick, 2010). Plant-microbe remediation is defined as the use of plants and their associated microorganisms to sequester, degrade, or stabilize contaminants, plants can establish beneficial associations with microorganisms in the natural environments (Afzal *et al*., 2019). Those bacteria can colonize both the external or the internal environment of the plant (Santoyo et *al*., 2016). Epiphytic bacteria are those live outside the plant, on the leaf surface. While, those occupying plant roots within the soil are called rhizospheric bacteria. Endophytic bacteria are those thrive inside plant tissues (Afzal *et al*., 2019).

Plant-associated bacteria can contribute to enhance the biodegradation of contaminants as they can benefit them by synthesizing compounds that protect them by decreasing stress phytohormone levels, providing key plant nutrient and protecting them against plant pathogens (Santoyo *et al*., 2016; Ojuederie et Babalola, 2017). To date, plant-associated bacteria has been successfully applied to remove petroleum hydrocarbons (Singh *et al*., 2003; Cebron *et al*., 2009; Germaine *et al*., 2009; Vangronsveld *et al*., 2009). Phytoremediation using plant-microbe has emerged as a sustainable soil restoration strategy.

a. Rhizoremediation

The rhizosphere is a complex system of nutrient-rich soil zone, the variety of primary metabolites (organic acids, carbohydrates and amino acids) and secondary metabolites (alkaloids, terpenes and phenolic compounds) interferes in some way with the rhizosphere microfolora (Venturi et Keel, 2016). It is also necessary to highlight the release of oxygen by the roots of the plants, aerating the pores, which facilitates the rhizodegradation (Thomas *et al*., 2019). In fact, the exudates, nutrients

and oxygen released by plants in their roots are absorbed by the microorganisms which transform a part of these pollutants into secondary metabolites which will be released into the rhizospheric environment. The other part of organic compounds will be used as a source of energy **(Figure 4)** (dos Santos et Maranho, 2018).

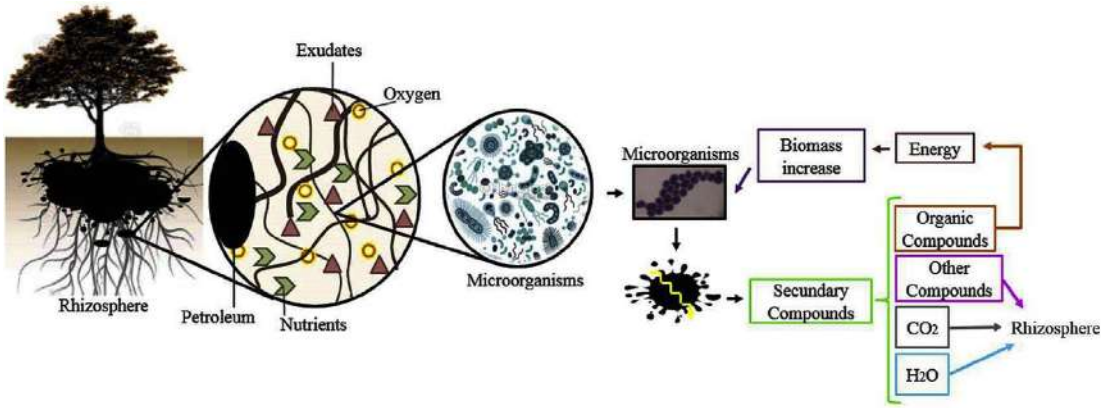

Figure 4. Rhizodegradation mechanisms in soil contaminated with petroleum hydrocarbons (dos Santos et Maranho, 2018).

In the last decade, in order to remove organic pollutants from the environment, scientists have used plant inoculated with hydrocarbons degrading or plant growth promoting bacteria (Chapman, 2007; Germaine *et al*., 2009; Vangronsveld *et al*., 2009). Only bacteria that have some traits like motility and polysaccharide production could colonize plant rhizosphere (Lopes *et al*., 2016).

Inoculation of plants with PGP bacterial (PGPB) strains showed enhancement in germination, seedling vigor, and plant growth under stress conditions in soil (Balseiro-Romero *et al*., 2017). The enhancement of plant growth by the rhizospheric bacteria may results from any one of a variety of the following mechanisms: chelating of iron, antibiotic production, lytic enzymes production, providing nutrients, atmospheric nitrogen fixation, reducing stress hormone levels and minerals solubilization (Glick, 2010). Bacteria can affect plant growth by using one or more of these traits.

Balseiro-Romero *et al*. (2017) found that the inoculation of plants with PGP bacterial strains improve plants growth but not oxidative activity or nutrient content under stress conditions. Contrary, they decrease the oxidative stress due to the increase of tolerance and the adaptation to stress conditions. Contaminants degrading bacteria may improve plant's adaptation to contaminants by detoxifying contaminated soils through mineralization of these compounds (Gkorezis *et al*., 2016). Child et *al*., (2007) found that the mineralization of pyrene by *Mycobacterium* sp. KMS strain increased in the presence of plants within the rhizosphere.

b. Endophyte -plant in remediation

Most of plant-associated bacteria comes from the soil environment, they may migrate from the rhizosphere subsequently to the rhizoplane of their host. Some rhizoplane bacteria penetrate into plant roots, and some of them may move to aerial plant parts (Frank *et al*., 2017).

Endophytic bacteria are considered like a specific rhizospheric bacteria that possess the ability to colonize the interior of plant roots (Afzal *et al*., 2019). This colonization begins from the roots by knowing some compounds in the root exudates. The colonization requires the bacterial adhesion to the root cell surface, they start to penetrate into the interior using specialized mechanisms (Walker *et al*., 2003). The use of endophytic bacteria for the bioremediation has been extensively explored in the last two decades (Afzal *et al*., 2014). Reports on the ability of endophytic bacteria to degrade different pollutants (petroleum hydrocarbons and heavy metals) have been extensively spread (Chen *et al*., 2016; Gkorezis *et al*., 2016; Bourceret *et al*., 2018).

Through roots and leaves, hydrocarbons can achieve vascular system and intercellular spaces, reducing plant growth or inducing their mortality (Arellano *et al*., 2017).Contaminants enter the xylem of plants, and the degrading bacteria residing the xylem are the best candidates to reduce the phytotoxicity and to avoid evapotranspiration of contaminants (Thijs *et al*., 2016) **(Figure 5)**.

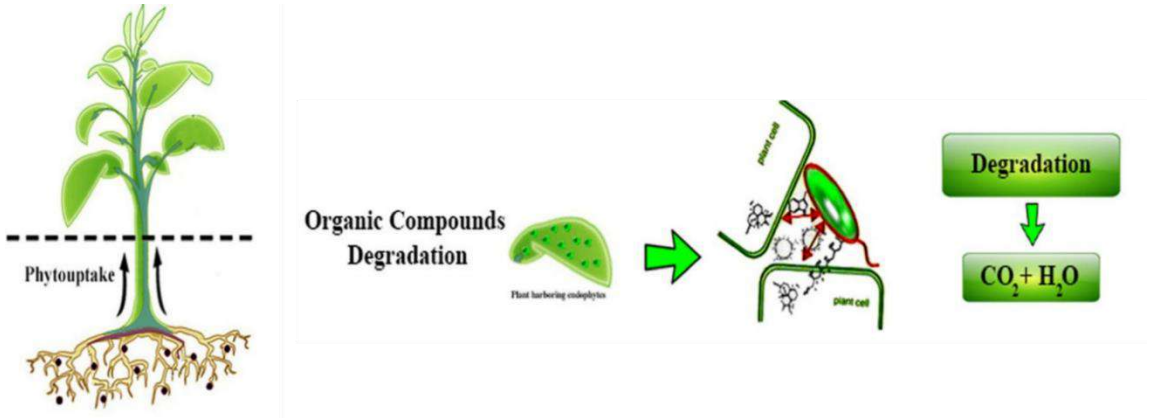

Figure 5. Role of endophytes to degrade organic compounds in contaminated soils (Ijaz et *al*., 2016).

Endophytic bacteria with TPH degradation ability can be isolated, and thereafter re-inoculated in their host or other host plant (Lumactud *et al*., 2016). Several researchers have revealed that plants may host diverse endophytic bacteria, especially those having the ability to degrade hydrocarbons (Peng *et al*., 2009; Babu *et al*., 2013). Wang et *al*. (2011) have described a strain from the genus *Dietzia* able to use *n*-alkanes, aromatic compounds, and crude oil as sole carbon sources. Rare reports about bacteria with the ability to degrade simultaneously *n*-alkanes and PAH (Zhang *et al*,. 2011).

Although, many studies have reported the success of the inoculation of endophytic bacteria into the soil, for the biodegradation of pollutants, the colonization is still a major obstacle in analyzing an effective inoculation (Germaine *et al*., 2009). Several aspects related to the bacterial inoculation such as pollutants nature, soil type, plant species, inoculum density and inoculation methods have been intensively researched and deeply debated (Afzal *et al*. 2011; Khan *et al*. 2013; Zheng *et al*. 2018). Microbial inoculation promotes soil enzyme activity, PAH removal and plant growth. Endophytic bacteria may be of particular interest as bioinoculant since they have the advantage of proliferating within plant tissues, facing less competition for nutrients and being protected from the high-stress environment of polluted soils (Sturz *et al* ., 2000).

Several methods have been reported for introducing bacteria into plants, especially, the inoculation of seeds, rhizosphere and soil (Compant *et al*., 2019). The effective microbial inoculation to seeds is the first step to improve phytoremediation, as it is so important to sustain the viability of the inoculated microbes and their colonization (Rilling *et al*., 2019). Many studies report that the direct inoculation to seeds or soil are used in phytoremediation using specific degraders (Germaine *et al*., 2009; Thion *et al*., 2013; Ijaz *et al*., 2016).

Environmental factors; biotic and abiotic have been investigated lately. The effect of biotic factors has been resumed on the survival and the immigration of hydrocabonclastic microorganisms, whereas, the abiotic factors such as the physicochemical nature and concentration of contaminants, nutrients availability and soil properties can influence on the bioremediation process (Santoyo *et al*., 2016). The bioavailability of contaminants can be influenced by soil properties, such as its texture, particle size, its organic matter content, its water content, its pH, and its structure (Jung *et al*., 2008). Contaminants of aged contaminated soil are increasingly unavailable for uptake and biodegradation by soil microorganisms (Thomas *et al*., 2019). Plant and microbes have shown to secret enzymes and exudates that act as surfactant and may increase the availability of those contaminants (Bais *et al*., 2006; Cébron *et al*., 2011).

There are several hydrocarbons degrading endophytic bacteria available in nature. The use of individual indigenous endophytic bacteria or consortium as tool to enhance the rate of degradation of pollutants in both terrestrial and aquatic ecosystems. It is approved that single microorganism does not efficiently degrade all PAH due to their complex chemical structures. Microbial consortia have the ability to better PAH mixture (Chen *et al*., 2016).

Microbial-assisted phytoremediation has been well documented and its potential as an effective and inexpensive strategy to remove hydrocarbons from polluted soils (Thijs *et al*., 2016). It is not surprisingly that endophytic bacteria isolated from plants grown in petroleum hydrocarbons contaminated soils have hydrocarbon degradation ability as these compounds are naturally occurring.

I.3. Role of endophyte-plant in phytoremediation

Endophytic bacteria could positively affect plant in direct and/or indirect way. They can improve plant growth and development by modulating growth, getting nutrients in stressed conditions (Egamberdieva *et al*., 2015). According to Afzal *et al*. (2014), endophytic bacteria having classic PGP and pollutant degradation activities performed better than those bacteria having only one of these activities. Moreover, the contaminant degrading activity could be considered itself as a plant-growth promoting trait, because contaminants, in general affect negatively the plant development consequently, the elimination of the toxics will benefit them (Cruz-Morales *et al*., 2016).

The presence of PGP traits in hydrocarbon degrading endophytic bacteria can select them as a promising resource to enhance phytoremediation of contaminated soil (Khan *et al*., 2013). This enhancement is insured due to the improvement of plant adaptation and growth which is conferred by endophytic bacterial PGP traits (Afzal *et al.*, 2014).

Plant growth promoting bacteria may act as biofertilizers by increasing the availability of nutrients such as nitrogen fixation and phospahate solubilization. They can also behave as phytostimulants by producing phytohormones reponsibles of improving plant growth such as IAA or ACC deaminase and hydrocarbons degraders decreasing the pollutants toxicity. They also may proceed as biocontrol agents by producing siderophores, antibiotics or antifungal compounds (Balseiro-Romero *et al*., 2017).

Nitrogen (N_2) is one of the limiting nutrients for the plant growth. It must first be reduced to ammonia to be used by those later, which cannot reduce atmospheric N_2 (Ågren *et al*., 2012; Santoyo *et al*., 2016).

Endophytic inoculants supplying nitrogen requirement allowing the increase of plant yield (Puri *et al*., 2018). Endophytic bacteria having nitrogenase activity can fix atmospheric nitrogen and provide it to plants (Egamberdieva *et al*., 2015). Researchers have reported that endophytic bacteria perform better than rhizospheric bacteria. They allow to plant to develop in nitrogen limited soil and improve its growth and health, providing to them nitrogen they have accumulated (Afzal *et al*., 2019).

Phosphorous is one of the most commonly factor limiting the growth of the plant (Shrivastava et Kumar, 2015). It presented in a form of insoluble micronutrient in soil. Thus, it is unavailable for plant (Afzal *et al*., 2019). Depending on the pH of the soil, the phosphate either exists as tricalcium phosphate, aluminum phosphate, or iron phosphate (Sharma *et al*., 2013).

Endophytic bacteria can solubilize precipitated phosphate using different mechanisms such us, acidification, chelation, ion exchange and enzymes dissolution of phosphates (Afzal *et al*., 2019).

The acidification of the soil lead to the release of monovalent phosphate anion from mineral phosphate by proton (H^+) substitution (Alori *et al*., 2017). Thus, phosphate solubilizing bacteria adopt this mechanism releasing organic acids through oxidation of organic carbon sources (Suleman *et al*., 2018).

Diverse endophytes of phosphate-solubilizing bacteria have been isolated from plants grown in contaminated soils with hydrocarbons, including *Rhizobium*. *Serratia*, *Pseudomonas*, *Xanthomonas*. *Bacillus*, *Clavibacter*, *Curtobacterium*, *Rhodococcus* (Kukla *et al*., 2014; Pawlik *et al*., 2017; Wu *et al*., 2019).

Iron is necessary element for the many physiological processes like transpiration and respiration (Afzal *et al*., 2019). It is one of most abundant elements on earth, but, is mostly unavailable for direct assimilation by plants and microorganisms, because in nature it occurs principally as Fe^{3+}, ferrous hydroxides $Fe(OH)_2$, and ferric oxyhydroxides $Fe(OH)_3$ (Jacoby *et al*., 2017).

In order to obtain iron for their growth and development, some endophytic bacteria synthesize low-molecular weight iron-binding molecules called siderophores (chelators). These later bind Fe^{+3} from minerals or organic insoluble compounds, making it available (Compant *et al*., 2010). In addition, siderophores producing bacteria can stimulate plant growth either directly by improving plan iron nutrition or indirectly by limiting its availability for phytopathgens, leading to the inhibition of their growth(Ramakrishna *et al*., 2019).

Siderophores may also increase auxins synthesis, which in turn enhance the plant growth-promoting (Lopes *et al*., 2016).

Phytohormones produced by plant-associated bacteria often stimulate plant growth. Auxins, cytokinins and gibberellins can be considered as causal agents for improving plant growth and development (Egamberdieva *et al*., 2015).

The most investigated phytohormones produced by plant-associated bacteria is the indole-3-acetic acid (IAA) which is an important auxin for several physiological process, including cell-cell

signaling, regulation of plant development, and induction of plant defense systems (Afzal *et al*., 2019). Moreover, IAA can increase the number of absorbent root hairs, the number of lateral roots and the total surface of roots, leading to enhancement of root exudation and mineral uptake from the soil (Kong *et al*., 2015).

Cytokinins and gibberellins play an important role in regulating plant growth and development. Cytokinins are involved in the regulation of metabolites transport, cell division, protein synthesis. While Gibberellines are involved in cell division and elongation, activation of membranes and amylolytic enzymes synthesis (Tanimoto, 2005).

Ethylene is a plant hormone to control growth and physiological processes under both abiotic and biotic stress conditions, including root initiation, leaf senescence, root nodulation, cell elongation, nodulation, auxin transport (Afzal *et al*., 2019). It is synthesized as a response to environmental stresses. The increase of ethylene production inhibit root elongation, development of lateral roots and formation of root hair (Santoyo *et al*., 2016).

Endophytic bacteria produce 1-aminocyclopropane-1-carboxylate (ACC) deaminase which is a key hormone for the cleavage of ACC, a precursor of ethylene (Egamberdieva *et al*., 2015). ACC deaminase producing bacteria hydrolyze the exuded ACC into α-Ketobutyrate and ammonia, and utilize it as nitrogen source (Glick, 2010) **(Figure 6).**

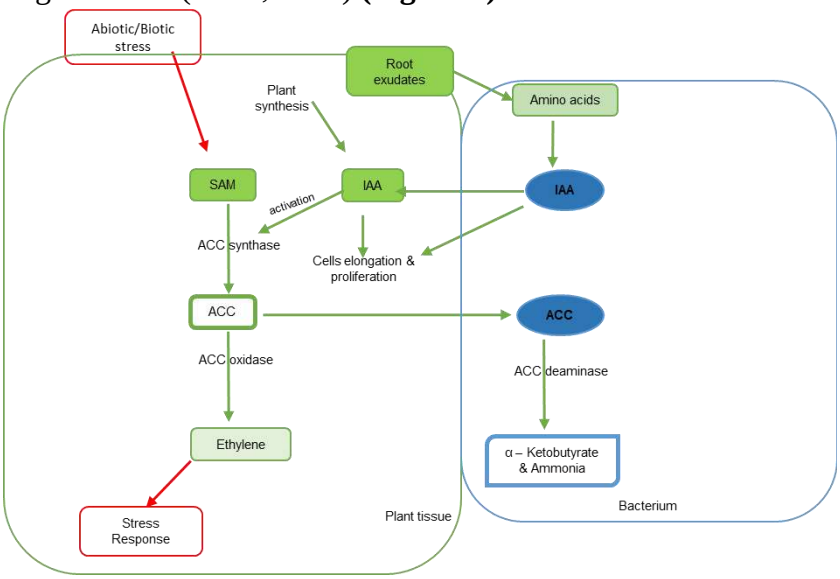

Figure 6. Schematic representation of the role of IAA and ACC deaminase to enhance the plant growth.

SAM (S-denosyl methionine), IAA (indole-3-acetic acid), ACC (1-qminocyclopropqne-1-decarboxylate).

Endophytic bacteria having ACC deaminase activity, stimulated plant growth leading to more extensive roots consequently enhancing phytoremediation efficiency (Arshad *et al*., 2007). The study of the potential of different grass species inoculated with PGP bacteria containing ACC deaminase used for phytoremediation of a creosote polluted soil revealed that an enhancement of biomass production translated with increase in the root and shoot densities (Huang *et al*. (2004).

Bacteria (endophytes or rhizobacteria) having at the same time the ability to degrade pollutants and to promote plant growth give better results than bacteria that have one of the two activities. In addition, the degradation activity of contaminants could be considered as a promoting plant growth trait, since contaminants, in general, affect negatively plant development; therefore, the elimination of toxins will benefit them (Ijaz *et al*., 2016).

The determination of PGP activities has great relevance for assessing the real usefulness of plant associated bacteria in bioremediation. In fact, the presence of PGP characters in hydrocarbon degrading bacteria may be the origin of their choice for phytoremediation (Khan *et al*., 2013; Afzal *et al*., 2014a; Afzal *et al*., 2019) (**Figure 7)**.

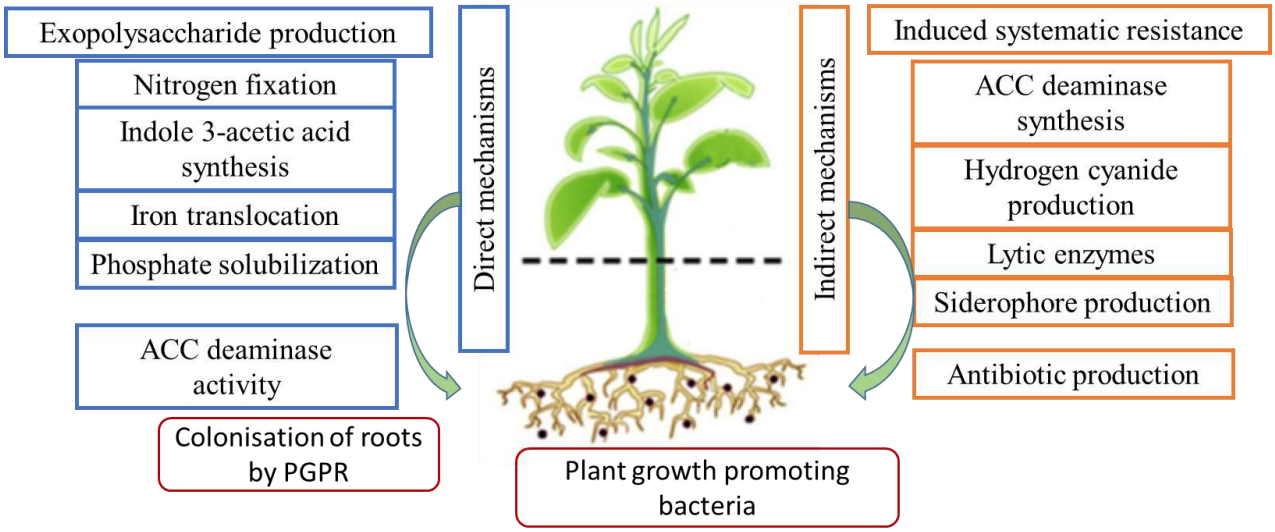

Figure 7. Mechanisms used by plant growth promoting bacteria under stress conditions.

Objectives

The main objectives of the present study are:

- The isolation of endophytic actinobacteria from surface sterilized roots of plants grown in contaminated soil with crude oil in two petroleum exploitation sites (Hassi Messaoud and Haoud Bekaoui, Ouargla, Algeria).

- The evaluation of the ability of the isolates to degrade petroleum hydrocarbons and to produce plant growth promoting metabolites (PGP).

- The selection of the promising strain and the study of their capability to remediate soil contaminated with Algerian crude oil.

- The inoculation of a model plant (*Zea mays*) with the selected strain and the evaluation of their efficiency to clean-up a petroleum hydrocarbons contaminated soils.

Novelty of the project

Microorganisms have proven their efficiency in the remediation and the restoration of polluted soils. They have been used as a single culture or as a consortium to restore contaminated environments with one or more pollutants. This technology is called bioremediation appeals in the case where the depolluting agents are bacteria belonging to proteobacteria and gammaproteobacteria known for their fast and easy isolation and culture.

In addition, plants have also been widely used as depolluting agents. Their interest in the remediation process is twofold, because in addition to depollution, these plants, often perennial (trees and shrubs) allow the creation of a pleasant green environment.

In the past few years, the remediation by microorganisms associated plants (plant-microbes) has been widely studied. This method has been applied on aged contaminated soils with petroleum hydrocarbons or heavy metals.

Prior to our study, no work on the bioremediation of polluted soils with light crude oil using plant-microbe has been published. In addition, the feature of actinobacteria, known for their metabolic versatility and their ability to degrade various pollutants is the origin of the choice made on these microorganisms for the construction of the complex plant-microbe oriented towards the treatment of contaminated soils by hydrocarbons. This axis has not been the subject of any previous study.

Plan expérimental

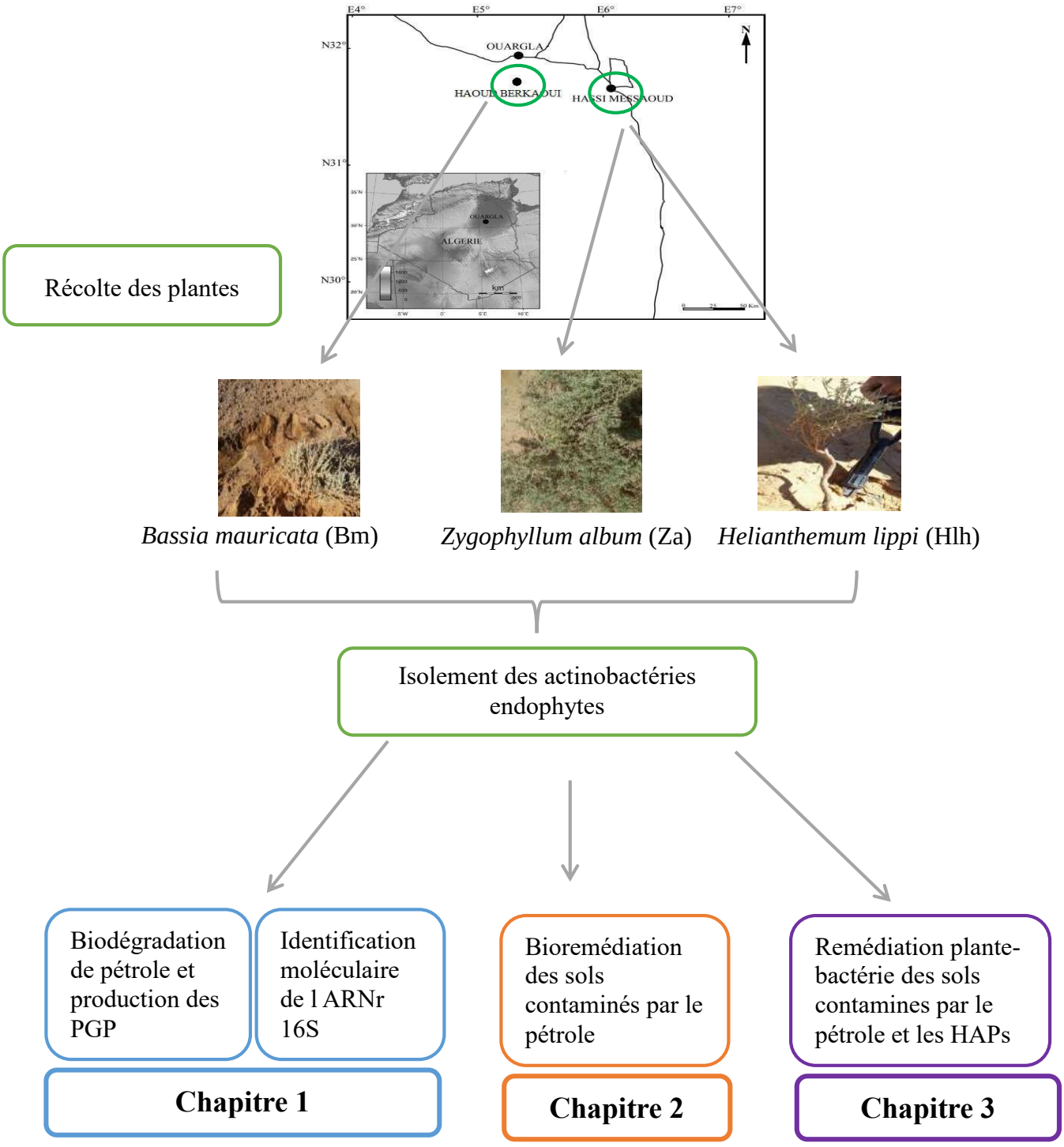

Figure 8. Schéma expérimental global du travail.

I. Plan expérimental

Les méthodes expérimentales utilisées dans ce travail sont résumées dans la **Figure 8**. Trois espèces végétales (De Février à Mai 2015) ; *Helianthemum lippii* (Hl), *Zygophyllum album* (Za) et *Bassia mauricata* (Bm) poussant dans des sols pollués par le pétrole brut ont été récoltées à Haoudh el-Hamra, la base nord de la raffinerie de pétrole Hassi-Messouad et Haoud Berkaoui, situées dans la région de Ouargla, au sud-est de l'Algérie **(Figure 1, Article 1, Supplementary material)**. La sélection des plantes est basée sur leur croissance dans les sols pollués par le pétrole.

Pour isoler les actinobactéries endophytes, les racines des plantes échantillonnées sont utilisées. Un protocole de stérilisation de surface des racines est effectué afin d'éliminer la flore épiphyte de la surface. Les racines sont ensuite coupées et placées dans des boites de Pétri contenant différents milieux de culture spécifiques pour les actinobactéries. L'ensemble des boites sont incubées à 28°C pendant 4 à 8 semaines.

La capacité des isolats à dégrader le pétrole brut est évaluée initialement en testant leur capacité à tolérer ses composés volatils. Les isolats sont ensemencés perpendiculairement à un creux rempli de pétrole brut sur un milieu minéral supplémenté de 10% de glucose. Après 7 jours d'incubation, la croissance microbienne est utilisée comme un paramètre qualitatif. L'eau stérile est utilisée comme témoin.

Les isolats ayant toléré les composants volatils du pétrole brut sont examinés pour leur capacité à utiliser ce substrat comme seule source de carbone et d'énergie. Ils sont cultivés dans 20 ml de milieu minimal supplémenté de 1% de pétrole brut comme seule source de carbone et d'énergie et incubées pendant 7 jours à 30 ° C sous agitation de 180 rpm. Les flacons non inoculés ont été utilisés afin d'évaluer la perte abiotique non liée à la dégradation par nos isolats. A la fin de l'incubation, le pétrole brut résiduel est extrait par extraction liquide-liquide et analysé par chromatographie en phase gazeuse équipée d'un détecteur à ionisation de flamme (GC-FID).

Les isolats dégradant le pétrole brut sont vérifiés pour leur aptitude à produire des métabolites améliorant la croissance des plantes (PGP). Ce paramètre est évalué par la solubilisation du phosphate, la production de l'acide indole-3-acétique (AIA), la fixation de l'azote, la production de ACC désaminase et de sidérophores. De plus, la production de biosurfactants est déterminée par l'activité hémolytique et l'activité émulsifiante (l'indice d'émulsion *E*24) en utilisant le kérosène comme substrat.

Les isolats prometteurs sont identifiés par le séquençage de l'ARN 16S. L'acide nucléique est extrait et amplifié en utilisant les amorces 27F et 1492R. L'analyse phylogénétique est réalisée avec le logiciel MEGA 7.

La souche jugée prometteuse est identifiée comme étant *Streptomyces* sp. Hlh1 et est utilisée pour étudier sa capacité à remédier un sol contaminé par du pétrole brut. Des sols non pollués ont été prélevés, séchés et tamisés. Des pots en verre sont remplis avec 200 g de sols stériles et non stériles, additionnés de 2%, 5% et 10% de pétrole brut en trois répititions. Les échantillons des sols sont inoculés avec *Streptomyces* sp. Hlh1 et incubés pendant 4 semaines à 30 °C. L'humidité est maintenue à 20% en utilisant de l'eau distillée stérile.

Après incubation, des échantillons de sols sont utilisés pour déterminer leur teneur en pétrole brut résiduel par GC-FID. Les autres échantillons sont utilisés pour évaluer la toxicité des métabolites finaux de dégradation et ceci en déterminant le nombre de graines da laitue (*Lactuca sativa*) germées ainsi que la longueur de la racine et l'hypocotyle.

La croissance de *Streptomyces* sp. Hlh1 dans les sols contaminés stériles est aussi évaluée en terme Unité Formant Colonie (UFC) après incubation.

Le phénanthrène, l'anthracène et le pyrène sont utilisés comme des modèles pour étudier la capacité de *Streptomyces* sp. Hlh1 à dégrader les hydrocarbures aromatiques polycycliques (HAP), dans un milieu liquide minimal en tant que seules sources de carbone et d'énergie. Les HAP résiduels sont extraits et quantifiés par la chromatographie en phase liquide à haute performance (HPLC).

Pour l'expérience de phytoremdiation au laboratoire, les échantillons de sols contaminés par le pétrole brut à raison de 20 g. Kg^{-1} ou des HAP individuels (2 mM) sont placés dans des pots en verre à raison de 200g/pot. Des graines de maïs (*Zea mays*) inoculées avec *Streptomyces* sp. Hlh1 au niveau de leur rhizosphère sont cultivées dans ces pots placés dans une chambre à température contrôlée de 25°C, 16:8 heures lumière : obscurité et 65% d'humidité relative pendant 15 jours. La croissance des plantes est évaluée en mesurant la longueur des racines et des tiges, la biomasse fraîche et sèche, la teneur en chlorophylle et en caroténoïde, ainsi que l'indice de tolérance aux contaminants. Le pétrole brut et les HAP résiduels sont extraits et quantifiés par GC-FID et HPLC, respectivement.

CHAPITRE I

Dégradation de pétrole par les endophytes Streptomyces spp. Isolées des plantes poussant dans des sols contaminés au sud de l'Algérie.

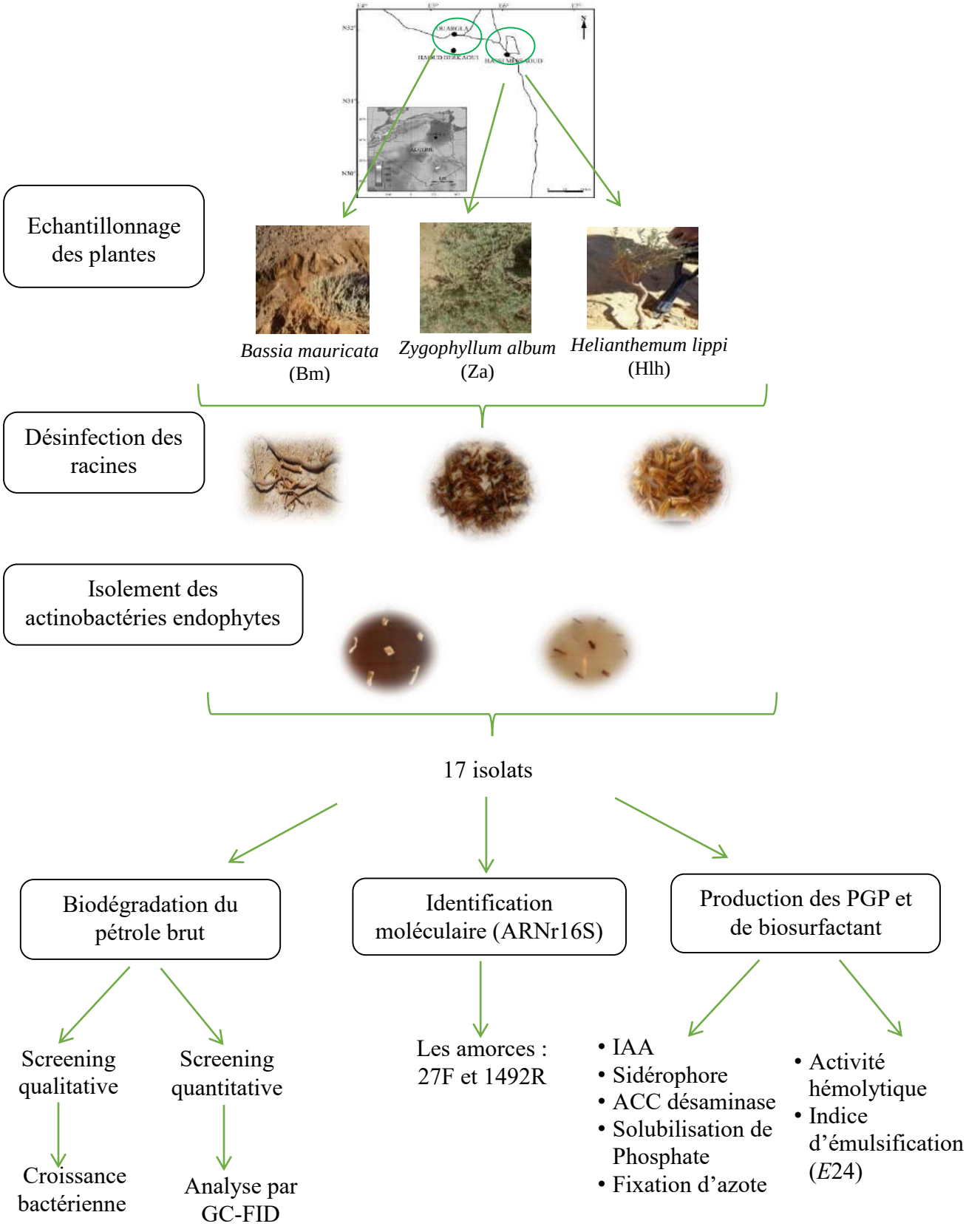

Figure 9. Dispositif expérimental du chapitre 1.

"Petroleum degradation by endophytic *Streptomyces* spp. isolated from plants grown in contaminated soil of southern Algeria" (Article publié en Septembre 2017 dans la revue Ecotoxicology and Environmental Safety**)**

Chapitre I : Dégradation de pétrole par les endophytes *Streptomyces* spp. Isolées des plantes poussant dans des sols contaminés au sud de l'Algérie.

Le présent chapitre est consacré à la recherche des actinobactéries endophytes capables de dégrader les hydrocarbures pétroliers et de produire des PGP et du biosurfactant. Cette étude est rendue possible grâce aux endophytes des plantes poussant dans les milieux pollués du sud est Algérien. En effet, la double adaptation de ces plantes aux conditions environnementales contraignantes et au polluant sont des atouts à l'origine du choix de ces plantes et de leurs endophytes.

La méthodologie adoptée pour la réalisation de cette partie de travail est résumée dans la **Figure 9.**

Dans la présente étude, 17 actinobactéries endophytes sont isolées à partir des racines provenant des plantes récoltées des sites contaminés par du pétrole brut. Le nombre d'isolats diffère d'une plante à l'autre **(Tableau 1, Article)**. La concentration du polluant dans le sol et l'espèce hôte considérée peuvent influencer la communauté microbienne endophyte (Peng *et al*. 2013). La majorité des isolats sont obtenus sur le milieu HV et des milieux à faible concentrations des nutriments. De tels milieux permettent la croissance et la sporulation des actinobactéries avant que les champignons à croissance rapide les masquent.

Une analyse qualitative de la tolérance au pétrole a montré que tous les isolats sont tolérants aux composés volatils du pétrole brut utilisé. Les souches Hlh1, Hlh8, Hlh9 et Zah8 ont présenté une croissance similaire en présence et en absence du pétrole. Elles sont donc considérées comme des souches hautement tolérantes au pétrole ; tandis que les isolats Hlh5 et Bmb4 ont présenté une plus faible croissance en présence du pétrole, par rapport à celle observée sans pétrole (souches à faible tolérance au pétrole). Les souches restantes ont montré une très faible tolérance **(Figure 2, Supplementary material)**. Cela pourrait probablement être dû à la présence de certains hydrocarbures volatils toxiques, qui affectent leur croissance (Leahy et Colwell, 1990).

L'analyse phylogénétique en séquençant l'ARNr 16S a montré que les souches tolérantes au pétrole appartenaient au genre *Streptomyces* **(Figure 1, Article)**. Les résultats obtenus montrent que, aucune des souches ayant une similitude avec les nôtres n'a été signalée capable de dégrader le pétrole.

Le genre *Streptomyces* est le plus souvent isolé à partir des plantes (Qin *et al*., 2011). En outre, des recherches précédentes ont indiqué que les plantes Sahariennes indigènes d'Algérie étaient riches en *Streptomyces* (Goudjal *et al*. 2016).

L'aptitude des isolats à dégrader le pétrole est évaluée en milieu liquide MM additionné de 10 000 ppm de pétrole brut. Le pétrole résiduel est quantifié par GC-FID et la biomasse bactérienne produite est mesurée en poids sec.

Selon les résultats de l'analyse GC-FID, cinq souches pourraient utiliser et dégrader efficacement le pétrole brut. Les taux de la dégradation les plus importants sont observés avec *Streptomyces* sp. Hlh9 (98%), tandis que *Streptomyces* sp. Hlh8 et *Streptomyces* sp. Zah8 ont dégradé 85,70% et 85,57% respectivement, *Streptomyces* sp. Hlh1 a dégradé que 57,14% alors que *Streptomyces* sp. Bmb4 n'a dégradé que 8,9%. La biomasse bactérienne a varié entre 0,18 et 0,43g/l **(Figure 2, Supplementary material)**.

Les travaux expérimentaux présentés fournissent, pour la première fois, un criblage systématique de la capacité des *Streptomyces* endophytes à dégrader le pétrole. D'autres genres d'actinobactéries endophytes ont été rapportés doués de cette aptitude, notamment *Rhodococcus* et *Micobacterium* (Kukla *et al*., 2014). Bien que *Streptomyces* sp. Hlh5 ait montré une tolérance au pétrole dans le milieu solide ainsi qu'une légère croissance en milieu liquide, son aptitude à dégrader le pétrole n'a pas été observée dans le milieu liquide. Par conséquent, la tolérance n'est pas associée à la capacité de dégradation du pétrole (Polti *et al*., 2007).

La concentration résiduelle des fractions hydrocarbonées est analysée **(Tableau 2, Article)**. Les souches *Streptomyces* sp Hlh1, Hlh8 et Hlh9 isolées de la même plante, présentent le même profil de dégradation. Elles dégradent toutes efficacement les *n*-alcanes de C_6 à C_{30}, alors que les HAP ne sont dégradés que par *Streptomyces* sp. Hlh1. *Streptomyces* sp. Zah8 présente un très large profil de dégradation des hydrocarbures. Elle est capable de dégrader tous les *n*-alcanes et les HAP. En revanche, *Streptomyces* sp. Bmb4 n'a dégradé que les *n*-alcanes de C_7 à C_{17}.

Trois *Streptomyces* retrouvés dans les sols de la Mitidja (au nord de l'Algérie) ont pu dégrader des fractions aliphatiques (C_{11}-C_{30}) présentes dans le pétrole brut utilisé et aussi le naphtalène comme un hydrocarbure individuel (Ferradji *et al*. 2014).

Dans la présente étude, toutes les souches s'avèrent aptes à dégrader efficacement les *n*-alcanes et les hydrocarbures aromatiques. Cependant, les HAP persistent plus dans l'environnement en raison de de leur taille moléculaire et de leur lente dégradation par peu de microbes (Wei *et al*. 2017). Il a

également été démontré que les fractions aliphatiques sont plus susceptibles dêtre dégrader que les fractions aromatiques. Par conséquent, la dégradation du pétrole brut dépend de sa composition et des conditions environnementales (Yanto et Tachibana, 2013). Cela devrait confirmer que les plantes poussant dans des sols pollués par le pétrole constituent un réservoir de microorganismes dégradant les hydrocarbures (Khan et *al.,* 2013).

Les bactéries endophytes produisant les métabolites PGP améliorent l'adaptation et la croissance des plantes, et par conséquence, améliorent la phytoremédiation des sols contaminés par des hydrocarbures (Afzal *et al*., 2014a). Dans notre étude, les souches étudiées produisent au moins un PGP testé **(Tableau 3, Article)**. Les souches *Streptomyces* sp. Hlh1 et *Streptomyces* sp. Bmb4 ont montré la capacité à produire tous les métabolites PGP testés. La production de l'indole-3-acétique acide (IAA) a varié de 11 à 22 µg mL^{-1}. L'IAA est une phytohormone responsable du développement des racines, stimulant la prolifération des cellules végétales, l'élongation des cellules végétales et induisant la transcription de l'ACC désaminase (Duca *et al*. 2014 ;Glick 2014).

La production de sidérophore et la fixation de l'azote ont été observées chez cinq isolats. La production de sidérophores peut être associée à la capacité de fixation de l'azote (Coombs et Franco 2003).

Streptomyces sp. Hlh1, Hlh9 et Bmb4 sont capables de solubiliser le phosphate inorganique et de produire une ACC désaminase. La solubilisation du phosphate améliore la fertilité des sols par la dégradation des composés organiques(Subramaniam *et al*., 2016). Par ailleurs, l'ACC désaminase est une enzyme clé qui stimule la croissance des plantes en réduisant le niveau d'éthylène produit dans les conditions de stress(Glick, 2014). Les bactéries endophytes possédant à la fois la capacité de dégradation des contaminants et de production des PGP donnent de meilleurs résultats que celles qui possèdent qu'une seule de ces activités. Par ailleurs, l'activité de dégradation des contaminants pourrait être considérée, elle-même comme l'un des métabolites PGP, car les contaminants ont généralement un effet négatif sur la croissance (Cruz-Morales *et al*., 2016).

Cinq souches ont montré une activité hémolytique, alors que seules les *Streptomyces* sp. Hlh1, Hlh9 et Bmb4 ont montré une activité émulsifiante. La valeur de l'indice d'émulsification E_{24} la plus élevée est celle de biosurfactant produit par *Streptomyces* sp. Bmb4.

Le principal facteur limitant la biodégradation des polluants est souvent leur faible disponibilité. Les microorganismes producteurs de biosurfactants peuvent augmenter la disponibilité de ces

polluants (Calvo *et al*., 2009). Des études antérieures ont démontré que tous les producteurs de biosurfactants ne possèdent pas d'activité hémolytique (Youssef *et al*., 2004).

La recherche de l'aptitude de *Streptomyces* endophytes à dégrader le pétrole n'a fait l'objet d'aucune étude antérieure. Les résultats de notre travail ont confirmé la possibilité d'isoler des actinobactéries endophytes possédant des caractéristiques favorisant la croissance des plantes (PGP), ainsi qu'une capacité à dégrader le pétrole. Parmi les actinobactéries isolées, cinq ont montré la capacité de dégrader le pétrole jusqu'à 98% de la concentration initiale après sept jours en milieu liquide minimal. Elles peuvent également produire diverses métabolite favorisant la croissance des plantes (PGP). Ces propriétés ouvrent des perspectives prometteuses pour leur application en tant qu'agents potentiels de bioremédiation des sols contaminés par le pétrole.

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Petroleum degradation by endophytic *Streptomyces* spp. isolated from plants grown in contaminated soil of southern Algeria

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ABSTRACT

Petroleum hydrocarbons are well known by their high toxicity and recalcitrant properties. Their increasing utilization around worldwide led to environmental contamination. Phytoremediation using plant-associated microbe is an interesting approach for petroleum degradation and actinobacteria have a great potential for that. For this purpose, our study aimed to isolate, characterize, and assess the ability of endophytic actinobacteria to degrade crude petroleum, as well as to produce plant growth promoting traits. Seventeen endophytic actinobacteria were isolated from roots of plants grown naturally in sandy contaminated soil. Among them, six isolates were selected on the basis of their tolerance to petroleum on solid minimal medium and characterized by 16S rDNA gene sequencing. All petroleum-tolerant isolates belonged to the *Streptomyces* genus. Determination by crude oil degradation by gas chromatorgraph-flame ionization detector revealed that five strains could use petroleum as sole carbon and energy source and the petroleum removal achieved up to 98% after 7 days of incubation. These isolates displayed an important role in the degradation of the n-alkanes (C_6-C_{36}), aromatic and polycyclic aromatic hydrocarbons.

All strains showed a wide range of plant growth promoting features such as siderophores, phosphate solubilization, 1-aminocyclopropane-1-carboxylate deaminase, nitrogen fixation and indole-3-acetic acid production as well as biosurfactant production. This is the first study highlighting the petroleum degradation ability and plant growth promoting attributes of endophytic *Streptomyces*. The finding suggests that the endophytic actinobacteria isolated are promising candidates for improving phytoremediation efficiency of petroleum contaminated soil.

1. Introduction

Petroleum is a complex mixture of different hydrocarbon and non-hydrocarbon compounds, it represents the major source of energy and the primary raw material for industry and daily life (Riazi, 2005).

A huge quantity of petroleum hydrocarbons enters the environment whether accidentally or due to the human activities causing a common problem in the world due to their toxicity and destructive properties to the natural ecosystems (Moliterni et al., 2012).

Algeria has very important petroleum production refineries. The principal petroleum extraction companies are situated in the south, Ouargla city. Therefore, it is one of the regions exposed to petroleum soil contamination.

Among a variety of strategies used for hydrocarbon remediation, includes physicochemical methods which are expensive and destructive for the environment (Bisht et al., 2014). Today, phytoremediation is recognized as a promising technology that has attracted worldwide attention because it is an efficient, environmentally friendly and cost-effective approach (Li et al., 2012). It is used in the field of remediation of various environmental contaminants such as petroleum hydrocarbons, herbicides, explosives and heavy metals. This technology can be enhanced by the use of endophytic microbes to clean organic and

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inorganic contaminants in soil and water (Pilon-Smits, 2005).

Endophytic microorganisms are those that live inside plant tissues, without causing any harm to host plants or environments (Tan and Zou, 2001). Current efforts are now focused on the endophytic bacteria with the ability to degrade organic contaminants and/or improve plant growth (Khan et al., 2013). Several studies have reported many endophytic bacteria which present an important role in degrading different hydrocarbon compounds. Among these microorganisms are Gram negative bacteria such as *Pseudomonas* and *Brevundimonas*, (Peng et al., 2013; Phillips et al., 2008; Zhang et al., 2014) and Gram positive bacteria, including actinobacteria (Kukla et al., 2014; Singh and Sedhuraman, 2015).

Endophytic actinobacteria recovered from healthy surface-disinfected plant tissues play a significant role in the cleavage of complex polymers including organic and inorganic toxics into more readily assimilable nutrients (Doumbou et al., 2001). They are also well-known producers of a wide range of plant growth promoting activities (PGP) (Subramaniam et al., 2016), which become promising candidates to enhance the phytoremediation of polluted soils. Bacteria from the *phylum Actinobacteria* seem to have the potential to use different metabolic pathways (Polti et al., 2011).

Furthermore, the genera *Streptomyces* is known for the production of more than 60% of bioactive metabolites, they were usually explored for antimicrobial activities (Goldman and Green, 2009). There are a few reports which indicate that *Streptomyces* isolated from soil has the ability for hydrocarbons degradation (Ferradji et al., 2014). No information is available so far about the action of endophytic *Streptomyces* in the phytoremediation of petroleum-impacted soils.

Because of the potential role of actinobacteria to use different complex compounds, the study of the hydrocarbonoclastic actinobacteria associated with plants from contaminated soil may provide valuable information about the potential economic and environmental benefits of using endophytic actinobacteria in phytoremediation. To the best of our knowledge, this work provides a novel insight that describes endophytic *Streptomyces*, their petroleum degradation potential, their PGP features and emulsification ability. Therefore, the objectives of this study were (1) the isolation and the identification of petroleum-degrading endophytic actinobacteria from selected Saharian plants (2) the evaluation of their ability to use and degrade Algerian crude oil petroleum (3) the assessment of PGP features and (4) the evaluation of emulsification capacity.

2. Materials and methods

2.1. Sample collection

Ouargla city is known for sandy soil and arid to a hyperarid climate with mean rainfall of 100 ± 50 mm a^{-1} (Hamdi-Aïssa et al., 2004). Specimens of healthy plants obtained from different areas of Ouargla, Algeria (Supp. Fig. 1), were used to isolated endophytic actinobacteria. The plants were selected based on their growth in petroleum contaminated soil. One specimen of each plant was collected. *Helianthemum lippii* (Hl) was collected from Haoudh el-Hamra (31° 53′ 38.1″N 6° 0′ 6.3″E), *Zygophyllum album* (Za) from the north base of oil refinery of Hassi Messouad (31° 39′ 43.6″N 6° 3′ 21.2″E) and *Bassia mauricata* (Bm) from Haoud Berkaoui (31° 50′ 16.0″N 5° 3′ 8.7″E). To the best of our knowledge, there is no record that these plants have been previously studied in order to isolate endophytic actinobacteria.

2.2. Isolation of endophytic actinobacteria

Five healthy root samples were harvested from each plant. The roots were washed in tap water to remove adhering soil particles. Tissue surfaces were sterilized by sequential immersion in 70% (*v/v*) ethanol for 5 min, 0.9% (*v/v*) of Sodium hypochlorite (NaClO) for 20 min, then, the root samples were divided aseptically into thin discs (0.2–0.5 cm) and soaked into 10% (*w/v*) of sodium bicarbonate ($NaHCO_3$) solution for 10 min in order to reduce the opportunity for emergence of endophytic fungi from the tissue, followed by washing with sterile distilled water for three times to remove surface sterilization agents (Verma et al., 2009). Finally the roots were placed onto different media, Humic acid-vitamin B (HV) (Hayakawa and Masayuki, 1987), yeast extract-casein hydrolysate (YECD) (Coombs and Franco, 2003), tap water-yeast extract agar (TWYE) (Crawford et al., 1993) and starch-casein agar (SC) (Kuster and Williams, 1964). All media were amended with nystatin (100 μg mL^{-1}) and nalidixic acid (50 μg mL^{-1}) to suppress the growth of fungi and gram-negative bacteria, respectively. The inoculated plates were incubated at 28 °C for 4–8 weeks. To confirm the surface disinfection process was successful, 100 μL of water from the final rinse was plated out on Petri-plates of yeast extract-mal extract agar and were incubated at 28 °C for 4 weeks. No contamination was found.

Isolates from *Helianthemum lippii*, *Zygophyllum album* and *Bassia mauricata* were named as Hlh, Zah and Bmb respectively, followed by a number.

2.3. Screening for crude oil petroleum biodegradation

A qualitative assay was performed to evaluate the tolerance of the isolates to Algerian crude petroleum according to the protocol of Benimeli et al. (2003). Rectangular troughs were cut in the center of minimal medium (MM) agar (containing in g L^{-1}: L-asparagine, 0.5; K_2HPO_4, 0.5; $MgSO_4{\cdot}7H_2O$, 0.2; $FeSO_4{\cdot}H_2O$, 0.01; glucose, 10.0) and filled with crude petroleum. Petroleum was sterilized by filtration (0.22 μm). The strains were inoculated by streaking them perpendicular to the troughs; Petri dishes were sealed with parafilm to avoid petroleum evaporation. After 7 days of incubation at 30 °C, microbial growth was evaluated and used as a qualitative parameter. Growth control was carried out using a medium without crude oil petroleum. Qualitative analysis of growth in presence of petroleum was systematized using 4 categories: from no-growth strain (no-petroleum -tolerant strain) to very good growth (highly-petroleum -tolerant strain) (Benimeli et al., 2003). The petroleum tolerant strains were selected for further assays.

The ability of the selected strains to degrade petroleum was evaluated in a liquid medium. The strains were cultivated in malt-yeast extract medium in a rotary shaker at 180 rpm for three days at 30 °C. Biomass was harvested by centrifugation (10,000 for 10 min at 4 °C), washed twice with sterile distilled water, culture suspensions were homogenized using a 10 cc syringe with 21 G1 1/2needle. The homogenized culture was inoculated at a final concentration of 0.01 g L^{-1} in 20 mL of liquid MM (without glucose) supplemented with 1% of sterile crude petroleum. Previously, the protein concentration of the homogenized culture was determined as explained below. In two milliliters sterile Eppendorf tube containing 1.3 g of glass beads (425–600 μm), 1.5 mL of the homogenized culture was added for the cell lysis. The process included six cycles of high shaking using a vortex for 10 min and cooling for 5 min between each shaking (Bélanger et al., 2011). Protein quantification was determined in the supernatant according to (Bradford, 1976). Inoculated MM with glucose as carbon source was used as growth control, and uninoculated MM was used to evaluate the abiotic loss of petroleum. Cultures were incubated at 220 rpm for 7 days at 30 °C. All experiments were performed in triplicate. The biomass was estimated after centrifugation (10,000 rpm for 10 min at 4 °C) by washing the pellets with sterile distilled water and drying to constant weight at 105 °C. The supernatants were used to determine residual petroleum concentration, from the inoculated and uninoculated flasks (Benimeli et al., 2007).

2.4. Petroleum characterization using gas chromatography (GC-FID)

The hydrocarbons from the culture supernatants were recovered through a liquid-liquid extraction process: 10 mL of pentane was added

to 10 mL of supernatant, after shaking, the mixture was decanted and the organic phase was recovered. The method recovery efficiency was 94%. Then, 5 mL of each sample was mixed with 1 g of Na_2SO_4 (99.99%) to dry them (EPA, 2012). Next, samples were filtered and the recovered liquid phase was analyzed using a chromatograph Varian 3800 GC, with FID detector, and capillary column VF-5 ms (30 m, 0.25 mm, 0.2523 µm). The injector and detector temperatures were 200 °C and 300 °C, respectively. The run parameters were: 45–100 °C, increasing 5 °C min^{-1}, then a second ramp from 100 to 275 °C increasing 8 °C min^{-1}. The final temperature (275 °C) was keeping during 5 min. Internal control was performed with cyclohexanone. The control was prepared with 1 µL of cyclohexanone and 5 mL of pentane. In addition, 1 µL of cyclohexanone was added to each sample. The concentration of cyclohexanone was determined and the recovery was calculated at 80–90%. Calibration was performed with the original petroleum at a concentration of 1%, 2% and 5%.

2.5. Screening of endophytic actinobacteria for plant growth promoting metabolites production

All isolates were cultivated on malt-yeast extract medium for two days at 180 rpm and 30 °C. 10 µL of washed culture at a final concentration of 0.01 g L^{-1} was used to determine PGP characteristics of the strains.

Phosphate solubilization ability was determined in PVK medium according to (Nautiyal, 1999). The production of indole-3-acetic acid (IAA) was determined by inoculating the strain on 10 mL of liquid MM containing 2 mg mL^{-1} of L-tryptophan and incubated at 30 °C with shaking at 180 rpm for five days at darkness (Khamna et al., 2010). The amount of IAA was determined by a colorimetric method, by mixing 1 mL of culture supernatant with 1 mL of Salkowski reagent (Glickmann and Dessaux, 1995), followed by 30 min incubation in the dark. Colour intensity was determined as A540 using a spectrophotometer. The concentration of IAA was quantified using IAA standard curve.

Growth on N-free medium was determined using a method described by Franco-Correa et al. (2010). 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity was carried out by streaking the isolated on MM agar (without L-asparagine) containing 3 mM of ACC per liter (MM-ACC agar) as a sole nitrogen source. The plates were incubated at 28 ± 2 °C in the dark for 7 days. Growth and sporulation of the strains on MM-ACC agar were taken as an indicator of the efficiency of selected isolates to utilize ACC and to produce ACC deaminase (El-Tarabily, 2008).

Siderophores production was screened according to the method described by Schwynan and Neilands (1987), which is based on the chelation of the iron by the siderophore producing a change in the colour of the dye of blue to orange.

2.6. Screening methods for biosurfactant

Production of biosurfactant was determined by hemolytic activity (Carrillo et al., 1996) and the emulsifying activity was carried out on LB (containing in g L^{-1}: peptone, 10.0; yeast extract, 5.0; NaCl, 10.0; glucose, 1.0), after 7 days of incubation at 30 °C. The emulsification index (E24) was calculated by using the following equation $E24$ = (Height of emulsion formed / Total height of solution) × 100 (Kukla et al., 2014).

2.7. Phylogenetic analyses of selected strains

The identification of isolates was carried out by the sequencing of a fragment of the gene 16S rDNA. The isolates were grown in 20 mL of ISP2 (containing in g L^{-1}: glucose 4.0; Malt extract, 10.0; yeast extract, 4.0) at 30 °C and 180 rpm for 24–48 h. Biomass was harvested by centrifugation (10,000 rpm for 5 min). DNA extraction was carried out using genomic DNA extraction kit (Promega, USA) according to the protocol supplied by the manufacturer. The DNA was used as a template for PCR amplification, the primers used were 27 f (5′-AGA GTT TGA TCC TGG CTC AG-3′) and p1492r (5′-TAC GGC TAC CTT GTT ACG ACT). All amplification products were checked by electrophoresis on 1% agarose gels.

The amplified fragments were purified and sequenced by Macrogen (Korea). The 16S rDNA gene sequences of actinomycete strains have been deposited in GenBank.

Sequences belonging to the same species or closely related species of type strains, available through the public databases, were aligned and a similarity matrix was calculated using MEGA 7 programme package (Kumar et al., 2016). The phylogenetic analysis was carried out using MEGA 7 software by the neighbor-joining method with 1000 bootstrap (Saitou and Nei, 1987) and plotted with the MEGA 7 software package.

2.8. Statistical analysis

Data analysis was conducted using the R (3.2.2) program for windows. Data were represented as the mean ± standard deviation (SD) of the triplicates samples.

3. Results and discussion

3.1. Isolation and selection of petroleum degrading endophytic actinobacteria

Endophytic actinobacteria have been isolated from different kind of plants, including medicinal plants and crop (Coombs and Franco, 2003; Qin et al., 2009). In the present study, seventeen endophytic actinobacteria were isolated from the internal tissue of surface-sterilized roots of plants growing at sites contaminated by crude petroleum. The surface sterilization protocol was checked by spreading out the last washing water of root samples on ISP2 medium. After 15 days of incubation, no growth was observed, revealing that the surface sterilization protocol was effective to remove the epiphytic microorganisms and the obtained isolates can be considered to be true endophytic actinobacteria.

The number of isolates in the three plants was quite different (Table 1). The endophytic community may be influenced by the pollutant concentration in the soil and the host plant species (Peng et al., 2013). Also, we observed that the isolation efficiency was influenced by the culture medium used. In the present study, the majority of isolates were obtained on TWYE (47.05%) followed by HV (35.29%), then YECD medium (11.76%) (Table 1). However, HV was the only medium that allowed isolating actinobacteria from the three plants. Starch casein agar yielded the lowest number of isolates (5.88%). Previous research has shown that low-nutrient media permit the growth and sporulation of actinobacteria before fast growing fungi mask slow growing actinobacteria (Coombs and Franco, 2003; Qin et al., 2009).

Many endophytic bacteria have exhibited hydrocarbon and metal tolerance (Babu et al., 2013; Peng et al., 2013). Qualitative assay of growth in the presence of petroleum indicated that all isolated actinobacteria were tolerant to 20% of crude petroleum oil (Supp. Fig. 2).

Moreover, the strains Hlh1, Hlh8, Hlh9 and Zah8 have exhibited similar growth in both contaminated and uncontaminated medium,

Table 1
Number of endophytic isolates of actinobacteria recovered from the roots of the three collected plants.

Plants / Medium	*Helianthimum lippi* (Hl)	*Zygophyllum album* (Za)	*Bassia mauricata* (Bm)	Total (%)
HV	4	1	1	35.29
TWYE	7	1	–	47.05
YECD	2	–	–	11.76
SCA	1	–	–	5.88

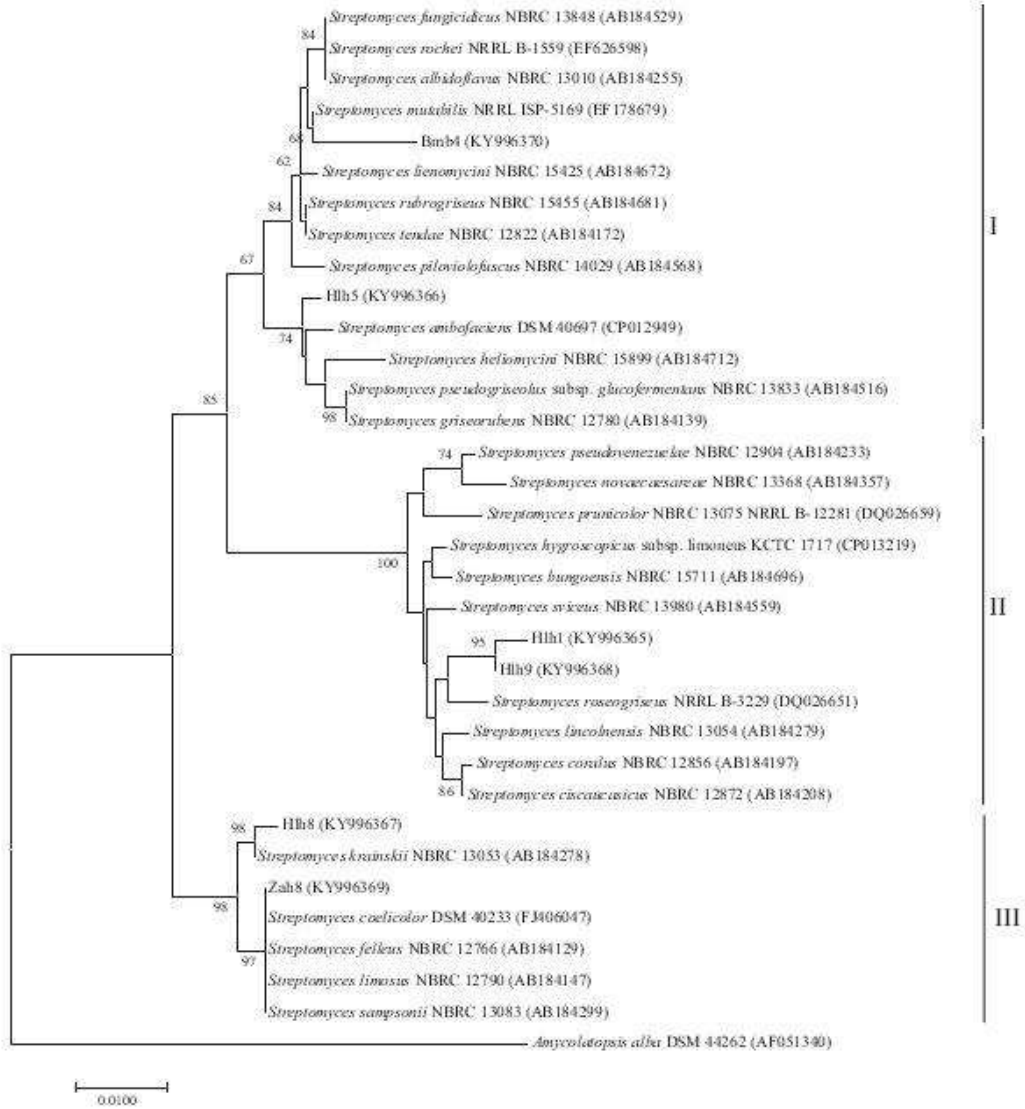

Fig. 1. Phylogenetic tree of selected actinobacteria inferred using the Neighbor-Joining method. The evolutionary distances were computed using the Jukes-Cantor method. Evolutionary analyses were conducted in MEGA7. Accession numbers of 16s rDNA sequences are given in parentheses.

thus, they were considered highly petroleum tolerant strains; whereas Hlh5 and Bmb4 were displayed a little growth in comparison to the growth observed in the uncontaminated medium (low-petroleum tolerant strains). The remaining strains displayed a very low tolerance. This could probably be because of the presence of some toxic volatile hydrocarbons which affect their growth, as reported previously by (Leahy and Colwell, 1990) who reported that some volatile petroleum hydrocarbon compounds could influence microbial growth.

The petroleum concentrations used in the qualitative screening assured the selection of high tolerance strains. Although all endophytic isolates were able to grow in the presence of petroleum, some isolates showing a strong tolerance, therefore, the best six strains were selected for further assays.

3.2. Phylogenetic analysis of endophytic actinobacteria

The taxonomic position of the selected strain was elucidated through phylogenetic analysis using their 16S rDNA sequences (Fig. 1).

A phylogenetic tree showed that all the strains found in this study belong to *Streptomyces* genera. Furthermore, previous studies reported that among actinobacteria, *Streptomyces* is the most frequently isolated genera (Qin et al., 2011). Furthermore, (Goudjal et al., 2016) indicated that Saharian native plants in Algeria were rich in *Streptomyces*. The overall 16S rDNA G+C content was ranged from 58.8 to 59.9 mol%.

The 16S rDNA sequences were compared to the corresponding sequences of 28 culture collection strains, and *Amycolatopsis alba* was used as an outgroup. According to the phylogenetic analysis, the strains were associated with three clusters. Inside the cluster I, the isolate Bmb4 was closely associated to *Streptomyces mutabilis* and *S. rochei*, with 98.9% and 98.6% of identity, respectively. The isolate Hlh5 was highly related to *Streptomyces ambofaciens* (99.6%) and *Streptomyces pseudogriseolus* subsp. *glucofermentans* (99.4%). Within cluster II, the isolates Hlh1 and Hlh9 were closely associated among them with 99.5% of identity; also, they showed 98.5% and 99.0% of identity with

Streptomyces sviceus, respectively. In cluster III, the isolate Hlh8 was closely associated to *Streptomyces krainskii* (99.8%). The isolate Zah8 showed 100% of identity with four *Streptomyces* species: *S. coelicolor*, *S. felleus*, *S. limosus* and *S. sampsonii*. Although they showed a high identity, further work needs to be done to assign species. Moreover, none of the strains that related to the new isolates found in this study showed the ability to utilize petroleum.

It was not possible to perform an ecological analysis on the diversity of the endophytic actinobacteria found in these plants because there are no previous reports on this subject. However, there have been some reports regarding the diversity and ecology of the endophytic bacterial population from plants grown in contaminated soil and the use of them to degrade organic pollutants (Kukla et al., 2014; Peng et al., 2013). In addition, there is no study on endophytic *Streptomyces* isolated from other plants growing in areas contaminated with crude petroleum.

3.3. Determination of petroleum degradation ability

The petroleum biodegradation by the actinobacteria was evaluated in MM liquid medium supplemented with 1% of Algerian light petroleum (10,000 ppm) as sole carbon and energy source. It was evaluated by the biomass produced and the petroleum degradation after 7 days of incubation.

According to the results of the GC-FID analysis, five strains could effectively utilize and degrade petroleum at different rates (Fig. 2). The highest petroleum removal was observed by *Streptomyces* sp. Hlh9 and *Streptomyces* sp. Zah8 (from 10,000 ppm to 250 and 1443 ppm, respectively). *Streptomyces* sp. Hlh8 and *Streptomyces* sp. Hlh1 could degrade 85.70% and 57.14% of petroleum respectively, while *Streptomyces* sp. Bmb4 degraded 8.91% of petroleum. Biomass production ranged from 0.18 to 0.43 g L^{-1} among the different strains (Fig. 2). No variations of petroleum concentration were observed in uninoculated control, so there was no proof of noticeable contribution of abiotic loss during the petroleum degradation.

Numerous studies have reported the use of endophytic bacteria for the biodegradation of organic pollutants in the environment (Khan et al., 2013). However, experimental work presented here provides, for the first time, a systematic screening on endophytic *Streptomyces* able to degrade petroleum.

Kukla et al. (2014) reported other genera of endophytic actinobacteria, including *Rhodococcus* and *Microbacterium* for their potential hydrocarbon degradation ability. Furthermore, endophytic *Nocardiopsis* able to degrade diesel was isolated from *Hibiscus rosasinensis* (Singh and Sedhuraman, 2015).

Although *Streptomyces* sp. Hlh5 displayed petroleum tolerance in the solid medium as well as a slight growth in liquid medium, none petroleum degradation was observed. Therefore, the tolerance was not associated with petroleum degradation ability (Polti et al., 2007). Similarly, previous reports have demonstrated that some hydrocarbons are able to support the growth of actinobacteria even no degradation was observed (Bourguignon et al., 2014).

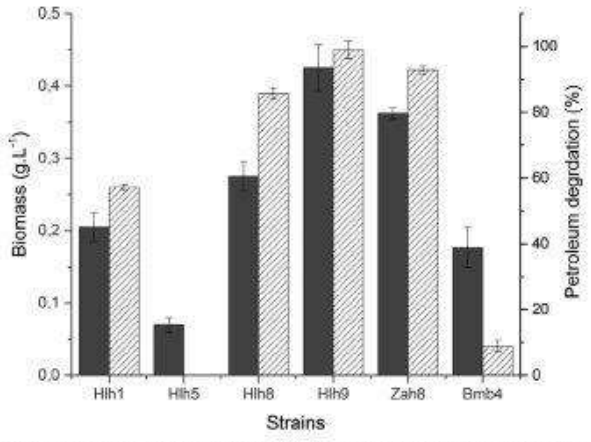

Fig. 2. () Growth of strains in MM liquid medium supplemented with 1% of crude petroleum expressed in g L^{-1}. () Degradation of petroleum by selected strains.

Table 2
Algerian crude petroleum composition and utilization of hydrocarbons by the selected isolates.

Hydrocarbons	Petroleum composition (ppm)	Petroleum degradation (%)				
		Hlh1	Hlh8	Hlh9	Zah8	Bmb4
C6	0.01	96.37	100	100	0.00	0.00
C7	77.12	100	97.49	100	99.36	64.52
C8	82.53	91.27	100	100	99.48	20.43
C9	520.79	99.75	96.91	99.61	99.62	75.88
C10	499.03	83.63	95.17	99.34	99.73	66.08
C11	608.06	73.85	93.91	99.33	99.37	43.40
C12	641.29	95.33	95.58	99.54	97.96	33.56
C13	641.29	97.09	98.41	99.50	99.10	3.89
C14	10.73	96.41	80.00	64.45	60.00	2.30
C15	552.59	94.84	98.40	99.59	97.40	87.00
C16	497.15	100	99.24	99.29	94.50	0.00
C17	467.54	100	98.40	99.66	98.48	99.44
C18	385.44	100	98.90	99.73	94.83	0.00
C19	312.01	35.26	98.00	99.52	94.37	0.00
C20	284.24	47.91	98.72	99.58	96.35	0.00
C21	251.00	37.14	98.14	99.59	96.92	0.00
C22	219.27	24.99	98.78	99.63	97.31	0.00
C23	73.30	39.16	92.32	99.47	89.81	0.00
C24	64.91	36.46	0.00	0.00	87.03	0.00
C25	25.17	50.68	92.32	99.47	88.76	0.00
C26	43.74	61.20	98.53	100	99.47	0.00
C27	23.98	5.72	93.42	100	92.44	0.00
C28	14.53	95.43	99.10	100	94.08	0.00
C29	0.43	38.46	100	100	72.58	0.00
C30	0.49	0.00	100	20.00	8.70	0.00
Benzene	1.93	0.00	0.00	0.00	0.00	0.00
Toluene	1.13	100	0.00	0.00	85.71	0.00
p-Xylene	37.98	100	4.56	10.00	93.68	78.73
o-Xylene	35.53	100	4.69	11.20	98.63	0.00
m-Xylene	32.59	99.54	7.71	14.2	97.30	7.78
Naphthalene	34.24	90.00	65.43	98.18	75.30	13.49
2-methyl naphthalene	32.35	70.08	0.00	0.00	87.90	14.43
1-methyl naphthalene	63.35	0.00	0.00	0.00	55.12	10.28
acenaphthylene	49.96	0.00	0.00	0.00	71.42	6.32
Acenaphtalene	34.10	0.00	59.00	100	45.14	0.00
Fluorene	70.82	76.34	29.21	96.43	0.00	0.00
Pristane	542.09	52.14	90.44	99.19	78.45	0.00
Phytane	385.44	83.6	29.69	100	0.00	19.39
Phenanthrene	2.91	47.91	43.61	54.00	0.00	0.00
Anthracene	4.59	37.67	17.99	10.00	0.00	0.00
Fluoranthene	10.16	43.75	0.00	0.00	42.66	0.00
Pyrene	1.12	48.38	0.00	0.00	0.00	0.00
benzo(a) anthracene	37.77	0.00	0.00	0.00	98.57	0.00
Chrysene	31.94	69.76	0.00	0.00	99.78	0.00
benzo(b) fluoranthene	12.89	0.00	0.00	0.00	92.83	0.00
benzo(k) fluoranthene	0.88	0.00	0.00	0.00	88.80	0.00
Benzo(a)pyrene	7.22	0.00	0.00	0.00	98.35	0.00

In fact, the hydrocarbon degrading actinobacteria, as published previously, were usually isolated from soil. Balachandran et al. (2012) have described that the hydrocarbon degrading *Streptomyces* ERI-CPDA-1 was able to degrade of 98.25% of petroleum at 0.01–0.1% within 7 days at pH 7 and 1–5 g L^{-1} of NaCl. Furthermore, Barabás et al. (2001) reported that *Streptomyces* isolated from Kuwait Burgan oil soil were able to utilize n-alkanes, kerosene and crude oil as sole carbon and energy sources.

The residual concentration of hydrocarbon fractions was analyzed

in the supernatant of culture medium. Four strains showed the ability to degrade a variety of hydrocarbons present in the crude petroleum. Moreover, the degradation of n-alkanes and aromatic hydrocarbons were significantly different between strains (Table 2). These compounds are the most frequent organic pollutants and are the main components in the crude petroleum (Zhang et al., 2011).

Streptomyces ssp. Hlh1, Hlh8 and Hlh9, isolated from the same plant, showed a similar petroleum degradation profile, they effectively degraded n-alkanes from C_6 to C_{30}, while, the degradation of aromatic hydrocarbons was better performed by *Streptomyces* sp. Hlh1. On the other hand, *Streptomyces* sp. Zah8 displayed a very broad hydrocarbon degradation profile, being able to degrade all n-alkanes and polycyclic aromatic hydrocarbons (PAH). Wang et al. (2011) have described *Dietzia* strain with the ability to use n-alkanes, aromatic compounds, and crude oil as sole carbon sources. There have been rare reports about bacteria with the ability to degrade simultaneously n-alkanes and PAH (Zhang et al., 2011). In contrast, *Streptomyces* sp. Bmb4 degraded only n-alkanes from C_7 to C_{17}. And also, none strain was able to degrade benzene (Table 2). Efficient degradation of n-alkanes and simple aromatic hydrocarbons, such as xylene, and naphthalene were observed in all the strains.

Similarly, three *Streptomyces* recovered from soils at Mitidja plain (North of Algeria) were able to degrade aliphatic fractions (C_{11}-C_{34}) present in crude oil and naphthalene as an individual hydrocarbon (Ferradji et al., 2014). In the present study, all the strains were effective degraders of n-alkanes as well as aromatic hydrocarbons and PAH. Although, PAH are being persistent in the environment as the increase of molecular size and their slow degradation by few microbes (Wei et al., 2017), *Streptomyces* sp. Hlh1 was the only strain that showed pyrene degradation as well as the PAH having three cycles or more. Sheng et al. (2008) found a pyrene-degrading *Enterobacter* sp. isolated from *Allium macrostemon* grown in PAH-contaminated soil.

It is also demonstrated that aliphatic fractions are more susceptible to degradation than aromatic, asphaltene and resin fractions. Therefore, the biodegradation of crude oil is affected by petroleum hydrocarbons composition and environmental conditions (Yanto and Tachibana, 2013).

However, it should be indicated that all plants in this study were grown in the contaminated soil. It would thus confirm that the endophytes isolated from plants growing in petroleum contaminated sites have displayed a natural ability for potential petroleum degradation (Siciliano et al., 2001).

3.4. PGP activities and biosurfactant production

Even though plants are autotrophic organisms, they absorb and metabolize hydrocarbons, but do not depend on them as sole carbon and energy source. Therefore, bacterial endophytes play an important role in the degradation of hydrocarbons absorbed by the plants in polluted environments (Khan et al., 2013). Bacterial PGP features enhance the adaptation and the growth of plants and consequently improve phytoremediation of hydrocarbon contaminated soil (Afzal et al., 2014). In our study, the actinobacterial strains were assessed for some plant growth promotion attributes like phosphate solubilization, growth on N-free media, ACC deaminase, siderophores and IAA production. Results summarized in Table 3 showed that the isolates were able to produce at least one PGP traits tested.

The production of IAA ranged from 11 to 22 µg IAA mL^{-1} after 96 h of incubation. *Streptomyces* sp. Bmb4 was the most efficient IAA producer, with 22.8 µg IAA mL^{-1} followed by *Streptomyces* sp. Zah9 which excreted 19 µg IAA mL^{-1}. The IAA is the most important phytohormone responsible for root development (Duca et al., 2014), stimulating plant cell proliferation, plant cell elongation and induce the transcription of ACC deaminase (Glick, 2014).

Five isolates were able to secrete siderophores into Chrome Azurol S (CAS) medium, as well as, they grew on the nitrogen-free media. There

Table 3
Plant growth promoting characteristics of the isolates.

Strains	IAA[a]	NFB	ACC	Siderophores[b]	PS
Hlh1	11.0 ± 0.6	+	++	20.5 ± 0.7	+
Hlh5	–	+	–	20.0 ± 1.8	–
Hlh8	–	+	–	14 ± 1.40	–
Hlh9	–	+	+++	4 ± 1.41	+
Zah8	19.0 ± 1.6	–	–	ng	–
Bmb4	22.8 ± 0.6	+	++	21.0 ± 0.0	+

+ : present; – : absent; ng: no growth was observed on CAS agar.
[a] In µg IAA mL^{-1} of medium. ± : standard deviation of three replicates.
[b] The halo diameter calculated by subtracting the colony diameter from the total halo size (mm).

is some evidence that siderophores production may associate with nitrogen fixation ability (Coombs and Franco, 2003). Additionally, siderophores stimulate plant growth by enhancing iron translocation from roots to shoots in the seedlings (Subramaniam et al., 2016). Tripathi et al. (2005) found that *Pseudomonas putida* KNP9 produces siderophore which increases the growth of mung bean in contaminated soil by cadmium and lead (Tripathi et al., 2005). Furthermore, only *Streptomyces* sp. Zah8 did not grow into CAS medium. These results were slightly similar to (Kukla et al., 2014) who found that the composition of the medium may not suitable or could inhibit microbial growth for some Gram positive bacteria.

Phosphate solubilization improves soil fertility through organic compounds degradation (Subramaniam et al., 2016). *Streptomyces* ssp. Hlh1, Hlh9 and Bmb4 were able to solubilize mineral phosphate producing a clear zone on screening agar plate. In fact, actinobacteria were rarely described for their ability to produce organic acid responsible of phosphate solubilization (Jog et al., 2014). These strains were also able to use ACC as sole nitrogen source. ACC deaminase producing *Streptomyces* has been previously reported by other authors (Dimkpa et al., 2009; El-Tarabily, 2008). Indeed, ACC deaminase stimulates plant growth reducing ethylene level produced by the plants under stressful conditions (Glick, 2014). Additionally, inoculation plants with petroleum degrading bacterial endophytes with ACC deaminase activity increases plant biomass, petroleum removal and therefore, improve phytoremediation of hydrocarbons contaminated soil (Afzal et al., 2012). Hong et al. (2011) found a petroleum degrading rhizobacterium *Gordonia* sp. S2RP-17 with ACC deaminase activity and siderophores that increase both total petroleum hydrocarbon removal and the growth of *Zee mays* in petroleum contaminated soil.

Streptomyces ssp. Hlh1 and Bmb4 showed all PGP traits whereas *Streptomyces* sp. Zah8 was only IAA producer. Sheng et al. (2008) have described one pyrene degrading endophytic *Enterobacter* able to produce IAA, siderophores and solubilize inorganic phosphate, demonstrating its potential to increase pyrene removal and improve plant growth in pyrene contaminated soil.

According to Afzal et al. (2014), endophytic bacteria having classic PGP and pollutant degradation activities performed better than those bacteria having only one of these activities. Moreover, the contaminant-degrading activity could be considered itself as a plant-growth promoting trait, because contaminants, in general, affect negatively the plant develop; consequently, the elimination of the toxics will benefit them (Cruz-Morales et al., 2016). In this sense, the determination of PGPs has great relevance to evaluate the actual usefulness of the endophytic actinobacteria in bioremediation. As reported in previous research, the presence of PGP traits in hydrocarbon degrading endophytic bacteria can select them as a promising resource to enhance phytoremediation of contaminated soil (Khan et al., 2013).

The major limiting factor for the biodegradation of pollutants is often their low availability. Biosurfactant-producing microorganisms can increase pollutant availability (Calvo et al., 2009). The five endophytic actinobacteria selected produced clear zones around the

colonies causing lysis of blood. However, the emulsification activity was positive only in *Streptomyces* sp. Hlh1, Hlh9 and Bmb4. The values of EI_{24} were ranged from 35% to 46%. The highest emulsification layer was formed by *Streptomyces* sp. Bmb4 (EI_{24} = 46.64%). Although Carrillo et al. (1996) recommended the hemolysis activity as a primary method to screen biosurfactant production, Youssef et al. (2004) reported that not all biosurfactant have hemolytic activity and compounds other than biosurfactants may cause hemolysis. Moreover, this method may exclude good biosurfactant producers. Thus, the hemolytic activity may not be an effective method for the screening of biosurfactant production. In this study, the production of biosurfactant was not found to be associated with the hemolysis activity. Consequently, the isolates exhibited biosurfactant production will be studied further to enhance their production in order to ensure their effect on the bioavailability of hydrocarbons.

4. Conclusion

There was no previous scientific study on *Streptomyces* from plant roots that can degrade crude petroleum. *Helianthemum lippii*, *Bassia mauricata* and *Zygophyllum album* were selected to investigate petroleum-degrading endophytic actinobacteria. The results of our study confirmed that it is possible to isolate endophytic actinobacteria with plant growth promoting features, as well as, petroleum degrading ability from desert plants grown naturally in sites contaminated with crude petroleum. Among seventeen isolates, five showed the ability to remove petroleum up to 98%, after 7 days in liquid medium. These strains belonged to *Streptomyces* genera. Furthermore, they were producers of various plant growth promoting features. To the best of our knowledge, this is the first report on the biodegradation of crude petroleum oil by endophytic *Streptomyces* spp. These properties open up promising perspectives for their application as potential agents for phytoremediation of a petroleum-contaminated ecological environment.

Conflict of interest

No potential conflict of interest was reported by authors in this study.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ecoenv.2017.09.013.

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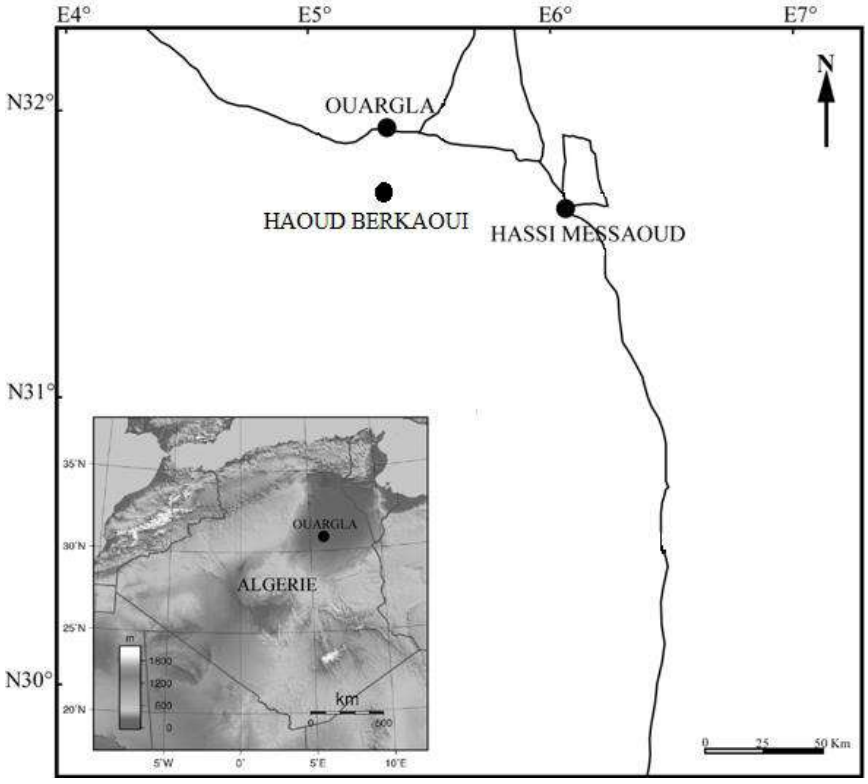
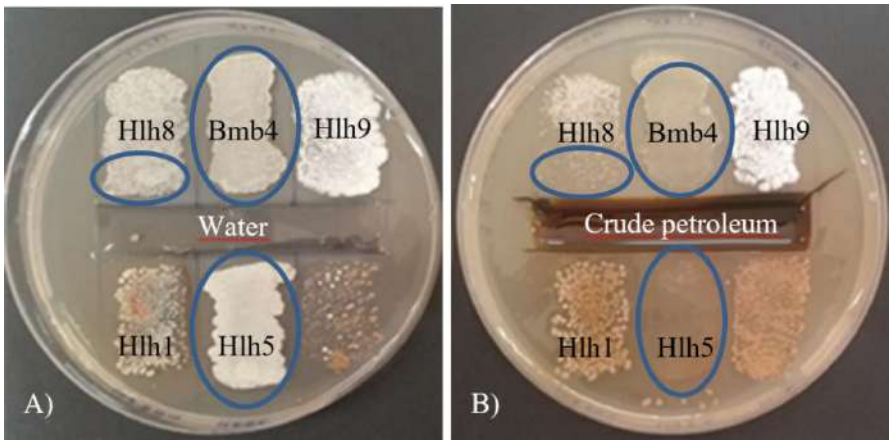
Chapitre I: "Petroleum degradation by endophytic *Streptomyces* spp. isolated from plants grown in contaminated soil of southern Algeria"



Supplementary Figure1. Localization of studied sites in Ouargla, South of Algeria (Baoune et *al*., 2018).



Supplementary Figure2. Qualitative tolerance assay. A) Growth control B) Growth in presence of crude petroleum. The surrounding areas indicate differences in the growth or sporulation of the selected actinobacteria by comparing the effects of water and petroleum (Baoune et *al*., 2018).

CHAPITRE II

Bioremédiation des sols contaminés par le pétrole en utilisant Streptomyces sp. Hlh1.

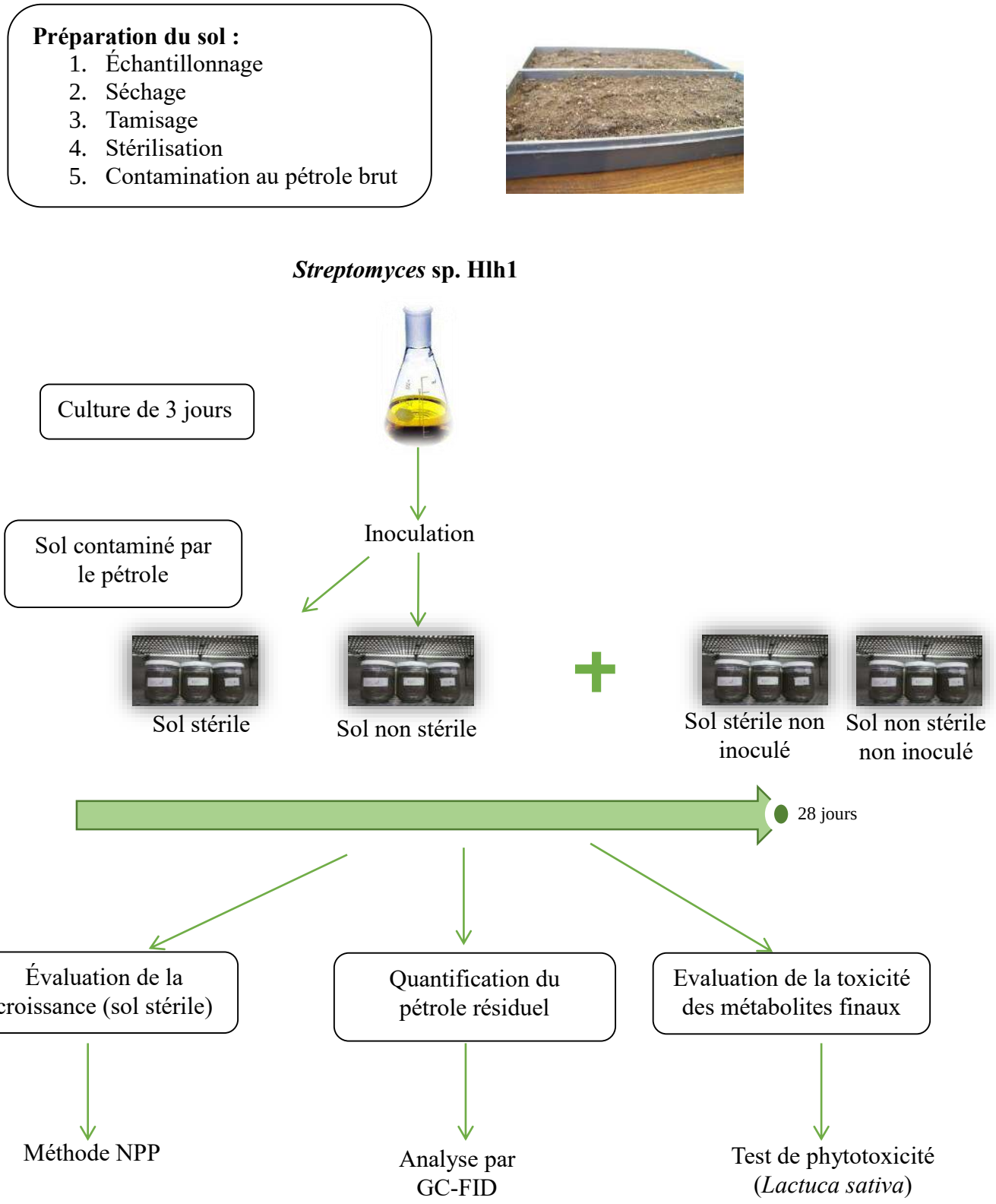


Figure 10. Dispositif expérimental du chapitre II.

"Bioremediation of petroleum-contaminated soils using *Streptomyces* sp. Hlh1" (Article publié en Septembre 2019 dans la revue Journal of Soil and Sediments)

Chapitre II : Bioremédiation des sols contaminés par le pétrole en utilisant *Streptomyces* sp. Hlh1.

Ce chapitre est consacré à l'étude de la capacité de la souche prometteuse préalablement sélectionnée, *Streptomyces* sp. Hlh1 impliquée dans le processus de remédiation des sols artificiellement contaminés par du pétrole brut. La toxicité des métabolites finaux est également évaluée.

La méthodologie adoptée pour la réalisation de cette partie de travail est résumée dans la **Figure 10**.

Des échantillons de sols stériles (SS) et non stériles (NS) sont artificiellement contaminées avec différentes concentrations de pétrole brut (2%, 5% et 10%). Ils sont placés dans des pots en verre à raison de 200g /pot et inoculés avec la souche *Streptomyces* sp. Hlh1 selon les codes suivants : ISS-2, ISS-5, ISS-10, INS-2, INS-5 et INS-10. D'autres échantillons non inoculés sont utilisées comme control (témoin) pour quantifier la perte abiotique du pétrole brut. Ils sont ainsi codés : (CSS-2, CSS-5, CSS-10, CNS-2, CNS-5 et CNS-10) **(Tableau 1, Article)**. L'ensemble des échantillons sont incubés à 28°C pendant quatre semaines.

L'efficacité de *Streptomyces* sp. Hlh1 dans la bioremédiation des sols contaminés par le pétrole brut est déterminée en quantifiant les hydrocarbures pétroliers totaux résiduels (HPT) par GC-FID.

Les résultats obtenus n'ont révélé aucune variation de la teneur en pétrole brut des sols stériles contaminés et non inoculés (SS). Par conséquent, aucune contribution des processus abiotiques dans l'élimination du pétrole n'a été observée.

L'absence de pertes abiotiques peut être expliquée par le système fermé utilisé. Ces résultats sont en accord avec ceux de Borah et Yadav (2017), dans lesquels ils ont indiqué que les pertes abiotiques étaient liées aux conditions environnementales.

Après 28 jours d'incubation, une diminution considérable de la concentration en pétrole brut est enregistrée dans les échantillons inoculés par rapport aux témoins (contrôles). Le niveau final de l'activité de dégradation a montré des différences hautement significatives ($p < 0,05$) entre les sols inoculés et non inoculés **(Fig 1, Article)**. Le contenu des HPT dans les traitements ISS-2, INS-2 et CNS-2 est réduit d'environ 35%, 55% et 48%, respectivement par apport à la concentration initiale, tandis que, les traitements ISS-5, INS-5 et CNS-5 ont montré une diminution d'environ 18%, 51% et

7%, respectivement, alors que les HPT des échantillons ISS-10, INS-10 et CNS-10 ont diminué de 42%, 27% et 12%, respectivement. De plus, une diminution significative des HPT est observée entre CNS et CSS. Ceci indiquerait que la flore microbienne autochtone contribue notablement à la dégradation du pétrole.

Dans ce travail, l'effet positif de la flore microbienne autochtone est observé principalement dans les sols contaminés avec 2% et 10% de pétrole, alors que, l'activité de *Streptomyces* sp. Hlh1 est prépondérante dans le sol contaminé avec 5 % de pétrole.

Il est possible que les faibles concentrations de pétrole stimulent faiblement le développement de *Streptomyces* sp. Hlh1 en comparaison au microbiote autochtone, alors que les concentrations très élevées réduisent le développement de *Streptomyces* sp. Hlh1, en diminuant la perméabilité de l'oxygène et en favorisant le développement du microbiote microaérophile (Borah et Yadav, 2017).

D'après les résultats obtenus, il est clair que *Streptomyces* sp. Hlh1 affecte la dégradation des hydrocarbures pétroliers entraînant une élimination plus efficace de ces derniers. En effet, les *Streptomyces* sont bien connus pour leurs caractéristiques de croissance microbienne, notamment la croissance des mycéliums, leur taux de croissance rapide, ainsi que la dissémination et la persistance des spores, qui les rend excellents pour s'adapter aux environnements rigoureux (Amoroso *et al*., 2013).

En terme de concentration, la dégradation des HPT est significativement affectée par la concentration initiale du pétrole dans le sol. L'efficacité de la dégradation dans les échantillons de sols inoculés diminue avec l'augmentation de la concentration initiale du pétrole. *Streptomyces* sp. Hlh1 et la flore microbienne indigène pourraient avoir une capacité d'élimination plus élevée à de faibles concentrations de pétrole brut.

L'étude de Abbasian *et al*., (2016) vient soutenir le fait que, la concentration de pétrole dans le sol est un facteur essentiel influant l'efficacité de la biodégradation et de la production de la biomasse microbienne.

D'autre part, la détermination de la croissance des microorganismes après l'inoculation dans un sol contaminé par le pétrole est un paramètre important pour déterminer leurs capacités de colonisation. Après 4 semaines d'incubation, la croissance microbienne est évaluée dans les sols contaminés stérile et inoculés par *Streptomyces* sp Hlh1. Il n'y a pas d'inhibition de la croissance dans les sols contaminés. De même, une différence statistiquement significative est observée dans la croissance bactérienne entre les différents traitements (p <0,05) **(Tableau 2, Article)**.

D'autres chercheurs ont indiqué que l'élimination des HPT des sols contaminés est liée à la taille et à l'activité de la population microbienne (Wu *et al*., 2017b).

L'analyse des fractions pétrolières par GC-FID est réalisée afin de déterminer la concentration des alcanes résiduels (C_6-C_{35}) et les 14 hydrocarbures aromatiques prioritaires pour chaque expérience de bioremédiation **(Tableau 3, Article)**. L'efficacité d'élimination des alcanes et des hydrocarbures aromatiques dans les sols inoculés est considérablement supérieure à celle des sols non inoculés. De plus, une diminution remarquable est observée dans tous les échantillons de sols inoculés avec *Streptomyces* sp. Hlh1, en comparaison avec les sols non inoculés.

Les *n*-alcanes sont moins toxiques et persistants en comparaison avec les hydrocarbures aromatiques (Zhao *et al.,* 2016). Leur niveau d'élimination est considérablement plus élevé que celui des HAP (Qin *et al* ., 2013; Li *et al*., 2016).

En revanche, dans la présente étude, on constate que les composés aromatiques sont consommés et dégradés de manière significative, suggérant que *Streptomyces* sp. Hl1 a une activité plus forte sur l'élimination de ces composés.

Nos résultats révèlent des différences significatives de la longueur des racines et des hypocotyles, l'indice de vigueur (VI) et le pourcentage de germination entre les différents traitements et le témoin.

Par ailleurs, l'inoculation avec *Streptomyces* sp. Hlh1 a amélioré la germination et la croissance de la laitue. Les hypocotyles de laitue ont présenté un développement élevé par rapport aux racines, ce qui indique que le contact direct des racines avec le substrat conduit à la réduction de leur taille (Saez *et al.,* 2014).

La production potentielle des métabolites toxiques peut survenir au cours de la bioremédiation. La laitue est considérée comme l'une des plantes les plus sensibles à ces composés pétroliers (Bamgbose et Anderson 2015). L'effet toxique sur la germination est considéré comme pertinent pour évaluer la toxicité des polluants (Banks et Schultz, 2005).

Les graines exposées à 2% de pétrole ont présenté des racines et des hypocotyles plus longs que celles ayant germé dans le sol non contaminé **(Fig 2, Article)**. La biodégradation des hydrocarbures à 2% peut générer des métabolites non toxiques, tandis que, à de fortes concentrations, la dégradation incomplète des hydrocarbures peut donner des métabolites plus toxiques, contraignant la croissance de la plante. De plus, les racines étaient nettement plus longues dans ISS que dans CSS (p <0,05), suggèrent l'efficacité de la bioremédiation **(Fig 2, Article)**.

L'indice de vigueur est plus élevé dans les sols inoculés par rapport aux sols non inoculés **(Tableau 4, Article)**. Ces résultats indiquent que *Streptomyces* sp. Hlh1 a pu réduire la toxicité des composés pétroliers générant des composés moins toxiques ou non toxiques. De ce fait, cette souche pourrait contribuer à la bioremédiation des sols contaminés par le pétrole.

Le pétrole brut est extrait des échantillons de sol artificiellement contaminés et inoculés par la souche endophyte *Streptomyces* sp. Hlh1. L'analyse des hydrocarbures a montré que la dégradation des composés aromatiques est nettement supérieure à celle des *n*-alcanes. De plus, les essais de la toxicité démontrent que les sols inoculés permettent une amélioration des paramètres biologiques des graines par rapport aux sols non inoculés. Ces résultats révèlent que la bioremédiation utilisant *Streptomyces* sp. Hlh1 représente une alternative prometteuse d'un point de vue biotechnologique, pour éliminer et détoxifier les composants pétroliers dans des environnements contaminés.

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SOILS, SEC 1 • SOIL ORGANIC MATTER DYNAMICS AND NUTRIENT CYCLING • RESEARCH ARTICLE

Bioremediation of petroleum-contaminated soils using *Streptomyces* sp. Hlh1

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Abstract

Purpose Bioremediation using microorganisms is a promising strategy to remediate soil with petroleum hydrocarbons. *Streptomyces* sp. Hlh1, an endophytic strain, has previously demonstrated the ability to degrade crude petroleum in liquid culture. To apply this strain at field scale, it is necessary to test its ability to colonize the soil, compete with native microbiota, and remove the petroleum hydrocarbons under unfavorable conditions. Herein, a study was conducted to evaluate the performance of *Streptomyces* sp. Hlh1 to remove crude petroleum from contaminated sterilized and non-sterilized soils.

Materials and methods Soils samples, contaminated with 2%, 5%, and 10% of petroleum, were inoculated with *Streptomyces* sp. Hlh1, and incubated at 30 °C for 4 weeks. At the end of bioremediation assays, the pollutant concentrations were determined by Gas chromatography flame ionization detector and the degradation rates were also calculated. The survival of the strain in the soil was estimated and the toxicity of metabolites was evaluated on *Lactuca sativa*.

Results and discussion *Streptomyces* sp. Hlh1 was able to grow and remove total petroleum hydrocarbons (TPH), n-alkanes, and aromatic hydrocarbons found in soil samples. In sterilized soil samples, *Streptomyces* sp. Hlh1 removed up to 40% of TPH at an initial concentration of 10%. Whereas, the maximum TPH removal reached was 55% in non-sterilized soil at an initial concentration of 2%. In addition, it was observed that the degradation of aromatic hydrocarbons was more active than n-alkanes. The strain grew well and produced high biomass in contaminated soil. Lettuce seedling was found to be the adequate bioindicator to assess the toxicity of petroleum end products. *Streptomyces* sp. Hlh1 performed a successful bioremediation, which was confirmed through the phytotoxicity test.

Conclusions The study shows the first insight of the contribution of free endophytic actinobacterial strain in the bioremediation of petroleum-contaminated soil; therefore, it suggests that *Streptomyces* sp. Hl1 can be usefully exploited at field scale.

Keywords Bacteria · Bioremediation · Detoxification · Persistent organic pollutants (POPs) · Polycyclic aromatic hydrocarbons (PAHs)

1 Introduction

In recent times, petroleum pollutants in the soil are a widespread problem throughout the world (Koshlaf et al. 2016) and often occur through various stages of petroleum exploration, accidents, transportation, and leakage (Ghoreishi et al. 2017). The US Environmental Protection Agency classified these compounds as priority environmental pollutants (U.S.

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EPA 1986). Therefore, crude oil pollution of soil is a worldwide environmental problem which it is necessary to resolve immediately (Koshlaf et al. 2016).

A number of physical and chemical strategies have been developed for the remediation of petroleum-contaminated sites. However, they are environmentally disruptive and expensive (Marchand et al. 2017). In the last two decades, stronger attention has come to be placed on the study of an alternative method, bioremediation, in which natural population of microbes with the appropriate metabolic abilities transforms or degrades toxic contaminants to less-toxic or non-toxic end products, reducing the harmful effect on health and environment (Sarkar et al. 2005; Das and Kumar 2016). This biotechnological process is a promising strategy for the removal of petroleum pollutants from contaminated environments and has been considered an efficient method, low-cost, and environmentally friendly (Wu et al. 2017b).

There are several reports available on isolation and identification of hydrocarbonoclastic microorganisms from petroleum-contaminated soil, water, and plants originally grown in contaminated soils, including members of *Pseudomonas*, *Rhodococcus*, *Bacillus*, *Acinetobacter*, *Streptomyces*, *Penicillium*, and *Chrysporium* (Khan et al. 2013; Varjani 2017; Baoune et al. 2018). It is therefore not surprising that microorganisms isolated from hydrocarbon-contaminated sites (soil, water, and plants) have hydrocarbons degrading ability as these compounds are naturally occurring (Varjani 2017).

Actinobacteria are a group of bacteria that play an important role in metabolizing complex organic matter in nature, thus removing effectively the xenobiotic compounds such as petroleum hydrocarbons (Barabás et al. 2001). Furthermore, their metabolic versatility allows the production of a wide range of metabolites with biotechnological importance (Alvarez et al. 2017). Genera of *Mycobacterium*, *Rhodococcus*, and *Gordonia* have been well known for their contribution to the remediation of contaminated soils with different petroleum hydrocarbon compounds (Bourguignon et al. 2016). Whereas, the genera *Streptomyces* are widely found in polluted soil by pesticides, heavy metals as well as hydrocarbons as abundant and promising candidates for bioremediation (Polti et al. 2014; Liao et al. 2015; Alvarez et al. 2017). However, little attention has been paid so far to their use for bioremediation of hydrocarbon-contaminated soils (Barabás et al. 2001; Liao et al. 2015).

The evaluation of the success of a bioremediation process should not be limited only to analytical methods, as they may have problems in the determination of compounds with low concentration or even standardized methods may not be available (Logeshwaran et al. 2018). In this sense, there are several complementary toxicity studies involving plant processes in order to verify bioremediation success and the effect of microbial metabolites produced during the pollutant removal (Montagnolli et al. 2015). The use of *Lactuca sativa* for toxicity tests is recommended because lettuce is a fairly sensitive crop to environmental stress, it requires short exposure time, and it has low cost (Banks and Schultz 2005).

In our previous research, an actinobacterial strain was isolated from roots of *Hilianthium lippi*, grown in contaminated soil, in the south of Algeria. The strain was identified as *Streptomyces* sp. Hlh1 using 16S rDNA analysis (Baoune et al. 2018). *Streptomyces* sp. Hlh1 showed the ability to degrade crude petroleum oil, as well as, it was found to be effective on the degradation of a wide range of n-alkanes from C_6 to C_{30}, and aromatic hydrocarbons. Studies in liquid systems allow selecting the most appropriate microorganisms for bioremediation processes, considering the ability to degrade contaminants; however, they do not provide information about the capacity of this microorganism to colonize the soil, compete with the native microbiota, or to degrade the pollutant under less favorable conditions (Polti et al. 2011; Huang et al. 2013; Guo et al. 2014; Aparicio et al. 2018; Saez et al. 2018). Therefore, it is vital to evaluate the performance of *Streptomyces* sp. HL1 in soil microcosms, both in the presence and the absence of native microbiota.

In the current research, our objectives were to evaluate the use of *Streptomyces* sp. Hlh1 to remediate soil contaminated with different concentrations of crude petroleum oil by studying its total petroleum hydrocarbons (TPH) and hydrocarbon fractions removal rate. In addition, the direct effect of this bioprocess on the growth parameters of lettuce (*Lactuca sativa* L.) was evaluated to determine the toxicity of the final products.

2 Experimental

2.1 Soil preparation

The non-polluted soil was obtained and prepared according to Polti et al. (2014). Glass pots were filled with 200 g of soil and kept at 20% of humidity using sterile distilled water (Polti et al. 2014). The soil was sterilized according to Polti et al. (2009). Both sterilized soil samples (SS) and non-sterilized soil samples (NS) were then contaminated with 2%, 5%, and 10% of crude petroleum.

2.2 Culture preparation and soil inoculation

A petroleum-degrading actinobacterium identified as *Streptomyces* sp. Hlh1 was previously isolated, characterized, and stored in our laboratory (Baoune et al. 2018). *Streptomyces* sp. Hlh1 was pre-cultured in Erlenmeyer flasks containing 30 mL of Tryptic Soy Broth (TSB containing in g·L^{-1}: tryptone, 15; soy peptone, 3; NaCl, 5; K_2HPO_4, 2.5; and

Table 1 Experimental condition and soil samples labels

Soil sample labels	Sterilized	Inoculum	Petroleum concentration (%)
ISS-2	Yes	*Streptomyces* sp. Hlh1	2
CSS-2	Yes	–	2
INS-2	Not	*Streptomyces* sp. Hlh1	2
CNS-2	Not	–	2
ISS-5	Yes	*Streptomyces* sp. Hlh1	5
CSS-5	Yes	–	5
INS-5	Not	*Streptomyces* sp. Hlh1	5
CNS-5	Not	–	5
ISS-10	Yes	*Streptomyces* sp. Hlh1	10
CSS-10	Yes	–	10
INS-10	Not	*Streptomyces* sp. Hlh1	10
CNS-10	Not	–	10

glucose, 2.5). The culture was incubated for 72 h at 30 °C on a rotary shaker (200 rpm). Bacterial culture was centrifuged (10,000 for 10 min at 4 °C) and washed twice with sterile distilled water (Polti et al. 2014). Experimental condition labels of soil samples are presented in Table 1. Sterilized and non-sterilized soil samples were each inoculated with *Streptomyces* sp. Hlh1 to a final concentration of 1 g·kg^{-1} of soil (wet weight). The glass pots were then incubated at 30 °C for 4 weeks. Also, uninoculated sterilized soil and uninoculated non-sterilized soil were used as controls. Furthermore, microbial growth was evaluated only in ISS treatments as CFU g^{-1} according to Polti et al. (2009). In all cases, samples were taken at the end of each assay to determine crude petroleum degradation.

2.3 Determination of petroleum hydrocarbons via GC-FID analysis

Ten grams of each individual sample was dissolved in 2 mL of pentane HPLC quality, phase separated, and percolated through 2 g of silica gel. Samples were analyzed and quantified by gas chromatography using a Varian 3800 GC, equipped with a split/splitless injector, a flame ionization detector, and a capillary column VF-5ms (30 m, 0.25 mm, 0.25 μm). The injector and the detector of temperatures were maintained at 250 °C and 330 °C respectively. The sample (1 μL) was injected in split mode and the column temperature was raised from 30 to 300 °C at a rate of 15 °C/min and a second ramp from 300 to 325 °C at a rate of 15 °C/min. The final temperature, at 325 °C, was maintained for 5 min.

2.4 Toxicity test

The toxicity assay was performed with lettuce (*Lactuca sativa*) to test the petroleum detoxification potential of *Streptomyces* sp. Hlh1; three parameters were assessed on lettuce seedlings: germination, root elongation, and hypocotyl elongation (Aparicio et al. 2015). For the assay, a sample (10 g) of contaminated soils, inoculated and non-inoculated by *Streptomyces* sp. Hlh1, was placed on the inner surface of a sterile Petri dish containing moist Whatman filter paper then a total of 30 seeds were placed on the surface of the soil. The non-contaminated soil was used as a control. Petri plates were then sealed and the seeds were left to germinate for 5 days at 22 ± 2 in darkness (Bidlan et al. 2004). At the end of incubation time, the number of germinated seeds was registered. The length of roots and hypocotyls was measured by using a millimeter scale. Vigor index [(mean root length + mean hypocotyl length) × (percent germination / 10)] was also calculated (Bidlan et al. 2004; Amoroso et al. 2013; Saez et al. 2014; Aparicio et al. 2015).

2.5 Statistical analysis

Data obtained from this study were analyzed using an analysis of variance (ANOVA) to compare significant differences among the treatments using Minitab 17 Statistical software. Fisher's test was done to see the significant differences among the treatments ($p < 0.05$). The error bars shown in the figures represent one standard deviation. All assays were performed in triplicate and the results are the average of them.

3 Results and discussion

3.1 TPH removal efficiency in soil samples

In a previous study, Baoune et al. (2018) demonstrated the ability of endophytic *Streptomyces* sp. Hlh1 to degrade 57% of crude petroleum in a minimal liquid medium, at an initial concentration of 1% after 7 days of incubation. For this reason, *Streptomyces* sp. Hlh1 was selected to study its potential

to contribute to bioremediation of petroleum-contaminated soils. Actinobacteria has been well known for their dominance during bioremediation of petroleum hydrocarbons, because of their ability to use a large variety of hydrocarbon compounds including crude oil, diesel oil, and aliphatic and aromatic hydrocarbons, especially the genera *Gordonia*, *Rhodococcus*, *Mycobacterium*, *Streptomyces*, and *Nocardia* (Pizzul et al. 2006; Shen et al. 2009; Balachandran et al. 2012; Seo et al. 2012; Luo et al. 2014; Liao et al. 2015). To the best of our knowledge, this is one of the first reports on the potential use of *Streptomyces* sp. in bioremediation of petroleum hydrocarbon-contaminated soils.

The effectiveness of *Streptomyces* sp. Hlh1 to bioremediate crude petroleum–contaminated soil was determined through total petroleum hydrocarbons (TPH) measurements in both SS and NS, using three petroleum concentrations (2, 5, and 10%). Figure 1a shows residual TPH concentration and petroleum removal efficiency after 28 days of incubation. No variations of petroleum concentration were observed in all CSS flasks; hence, no evidence of the noticeable contribution of abiotic processes to the petroleum removal was observed. Consequently, the bioavailable petroleum concentration detected in control flasks was considered as 100% to further calculations. The absence of abiotic losses may due to the use of a closed laboratory system in screw-capped flasks and incubated in a chamber. These results are consistent with the work of Borah and Yadav (2017), in which stated that abiotic losses were related to the environmental conditions during the bioremediation process.

After 28 days of incubation, the inoculated systems demonstrated a significantly decreasing in crude oil concentration compared to the control. The final level of bio-removal activity showed highly significant differences between inoculated and uninoculated soils. At the onset of experiments, TPH content was reduced from 20,048 to 13,053, 9060, and 10,474 mg·kg^{-1} soil in ISS-2, INS-2, and CNS-2, respectively, which translates to a TPH removal efficiency of 35%, 55%, and 48%. The TPH in ISS-5, INS-5, and CNS-5 was decreased from 98,901 to 85,373, 40,982, and 78,820 mg·kg^{-1} soil, respectively. This displays 18%, 51%, and 7% of TPH degradation. While the TPH in ISS-10, INS-10, and CNS-10 was reduced from 67,420 to 39,655, 57,080, and 59,196 mg·kg^{-1}, respectively. This represents 42%, 27%, and 12% TPH removal efficiency. Furthermore, a significant decrease of TPH was observed between in CNS, in comparison with CSS. This would indicate that there was of the noticeable contribution of autochthonous microbiota on petroleum removal. Autochthonous microbiota could grow on petroleum hydrocarbons as a carbon source. In this work, the positive effect of autochthonous microbiota was observed mainly in contaminated soil with 2 and 10% of petroleum, whereas when 5% was used, the activity of *Streptomyces* sp. Hlh1 was preponderant (Sarkar et al. 2005; Agnello et al. 2016). It is possible that low concentrations of

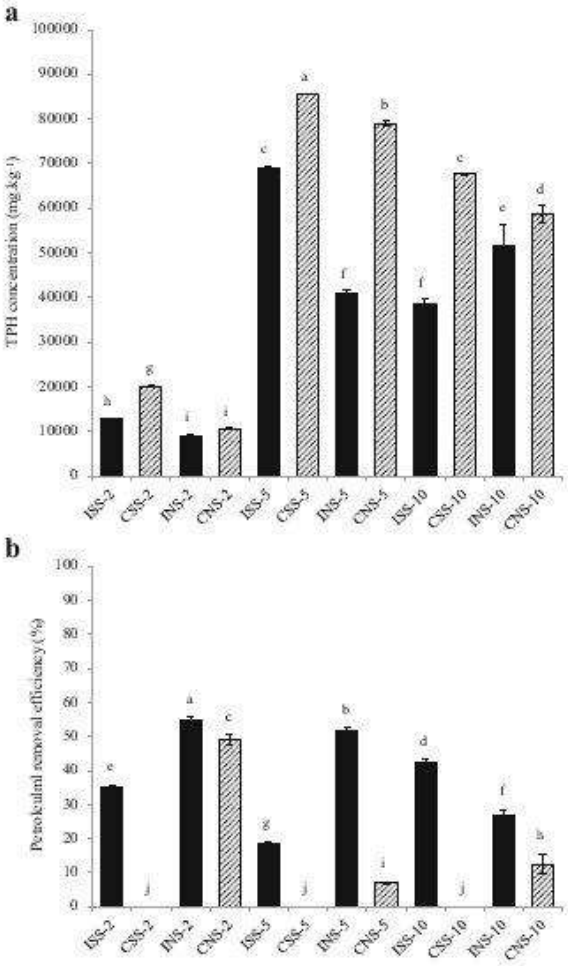

Fig. 1 (**a**) Concentrations of residual TPH in soil samples. (**b**) Petroleum removal efficiency in soil samples. Different letters represent a significant difference at ($p \leq 0.05$)

petroleum were not able to stimulate the development of *Streptomyces* sp. Hlh1 compared to the autochthonous microbiota, while very high concentrations reduce the development of *Streptomyces* sp. Hlh1 by decreasing the oxygen permeability and favoring the development of microaerophilic microbiota. In this sense, it is necessary to evaluate the toxicity of the treated soil because the anoxygenic degradation of hydrocarbons can generate metabolites of greater toxicity, such as phenol (Fuentes et al. 2014; Varjani 2017). Several groups of microbes and indigenous bacteria increase the remediation rate of hydrocarbons in contaminated soils (Borah and Yadav 2017). In addition, the added inoculum improved the degradation level in comparison with uninoculated treatments. From these results, it is clear that *Streptomyces* sp. Hlh1 affected the degradation of petroleum hydrocarbons, leading to more effective TPH removal. Consequently, the inoculation of petroleum-

contaminated soils with the suitable strain may increase the efficiency for biodegradation of petroleum pollutants. Indeed, *Streptomyces* are well known for their microbial growth characteristics, including mycelia growth, rapid growth rate, spores dissemination, and persistence, which make them excellent to adapt in the rough environments (Amoroso et al. 2013). Researchers have already demonstrated that the inoculation of petroleum-contaminated soil with native petroleum-degrading microorganisms enhances soil population activity and TPH degradation in crude oil–contaminated soils (Roy et al. 2014). Regarding the concentration, the degradation of TPH was significantly affected by the concentration of petroleum in the soil; the TPH removal was highly in CNS-2 which was 55% followed by 51% in CNS-5 while 42% and 35% in ISS-10 and ISS-2, respectively. Microorganisms cannot completely degraded petroleum hydrocarbons to CO_2 and H_2O, generating more or less complex residues (especially, persistent compounds and metabolites) (Qin et al. 2013). The difference of removal efficiency of organic contaminants is attributed to biotic and abiotic factors such as properties of contaminants, soil characteristics, plant exudates, and indigenous microbiota (Wang et al. 2017). Previous studies demonstrated that high hydrocarbon concentration improves the effect of fertilization, soil microbial population, and therefore TPH removal, whereas low hydrocarbon concentration had no significant effect on TPH removal (Margesin et al. 2007). Furthermore, the study of Abbasian et al. (2016) argued that petroleum concentration in soil is an essential factor influencing the biodegradation efficiency and microbial biomass. Nevertheless, little is known on the effect of hydrocarbon concentration on the removal efficiency (Margesin et al. 2007).

In other report, researchers working on bioremediation of aged petroleum-contaminated soils collected from China and augmented with microorganisms confirmed that the degradation rate of TPH with bioaugmentation by a TPH-degrading consortium of different bacterial strains reached 58% of TPH removal (20,200 $mg \cdot kg^{-1}$ initial TPH concentration) (Wu et al. 2017b). Also, Guarino et al. (2017) found that the degradation level of TPH obtained by using landfarming and bioaugmentation was more effective (85% TPH removal) than the use of landfarming or natural attenuation in a high concentration of TPH in the soil. Fan et al. (2014) studied the effect of biostimulation plus bioaugmentation with *Candida tropicalis* SK21 on the remediation of TPH in real contaminated soils and achieved 83% removal of TPH after 180 days. It has also been reported that the use of plant residues may stimulate the activity of degrading hydrocarbons bacteria, enhancing bioremediation of TPH and PAHs in contaminated soils (Shahsavari et al. 2013; Koshlaf et al. 2016).

Although, the degradation efficiency in inoculated non-sterilized soil samples decreases with the increase of the initial concentration of petroleum (Fig. 1b), indicating that *Streptomyces* sp. Hlh1 and the indigenous microbiota could have higher removal ability in a low concentration of crude petroleum oil. Since petroleum is toxic to soil microbes and limits the microbial biomass and diversity in soil (Liu et al. 2017). Nevertheless, *Streptomyces* sp. Hlh1 was found to be active on TPH removal in petroleum-contaminated soil with the highest concentration of petroleum used (10%).

On the other hand, the determination of microorganism growth after inoculation in petroleum-contaminated soil is an important parameter to determine the colonization ability of the inoculated strain. The microbial growth in ISS was evaluated at the end of the bioremediation assay (Table 3). After 4 weeks of incubation, there was no growth inhibition in contaminated soils. Likewise, a statistically significant difference was observed for the total counts of the bacterial growth between treatments ($p < 0.05$). The contaminated soil with 10% of petroleum showed the highest cell count 2.42×10^7 $CFU \cdot L^{-1}$. Moreover, the microbial growth showed a positive relation to TPH removal efficiency ($R^2 = 0.786$). Other researchers reported that the TPH removal from contaminated soils is related to the size and the activity of the microbial population. Wu et al. (2017b) have demonstrated that the better results of microbial growth could be obtained with inoculating the strain before the contamination of soils. Our results seem to be consistent with the work of Abbasian et al. (2016), who found that microbial growth decreases in high TPH concentrations (2.5% and 5%), while a slight increase is observed with 10% crude oil.

3.2 Removal of petroleum hydrocarbon fractions

TPH included *n*-Alkanes and PAHs. For that reason, the analysis of petroleum by GC-FID was performed in order to determine the concentration of residual alkanes (C6-C35) and 14 priority aromatic hydrocarbons for each bioremediation experiments (Table 2). By the end of the bioremediation process, the petroleum hydrocarbon fractions in the contaminated soils were significantly decreased compared with the original contaminated soils, indicating that all the hydrocarbons existing in the used petroleum were metabolized. Moreover, a marked decrease was observed in all inoculated soil samples with *Streptomyces* sp. Hlh1, in comparison with uninoculated soils. In addition, the removal efficiencies of alkanes and aromatics in the inoculated soil were considerably higher than that of the uninoculated soils. Furthermore, an increase of aromatic

Table 2 Microbial growth in sterilized soil samples after 28 days of incubation at 30 °C

Soil sample	Microbial growth ($CFU \cdot L^{-1}$)
ISS-2	$2.53 \cdot 10^6$
ISS-5	$2.75 \cdot 10^5$
ISS-10	$2.42 \cdot 10^7$

hydrocarbons concentration in INS-10 (from 7624 to 8712 mg·kg^{-1} soil) was observed, suggesting that the aromatic hydrocarbons containing more than one aromatic ring were not completely degraded. Van Gestel et al. (2003) reported that an increase of the secondary metabolites is related to the incomplete degradation of petroleum hydrocarbons. The n-alkanes are less-toxic and persistent than aromatics (Zhao et al. 2016) and generally, the removal level of alkanes is considerably higher than that of PAHs in crude petroleum (Qin et al. 2013; Li et al. 2016). Contrastingly, in the present study, the aromatics were significantly consumed and degraded, suggesting that *Streptomyces* sp. Hl1 had stronger activity on aromatics removal.

Several studies have shown that the success of the bioremediation process of soils contaminated with hydrocarbons is based on the choice of microorganisms with the suitable metabolic activities for the degradation of specific petroleum hydrocarbons (Borah and Yadav 2017). For instance, the actinobacteria *Nocardia* sp. SoB and *Gordonia* sp. SoCp were able to remove 75% of n-alkanes in artificially contaminated soils after 28 days of incubation (De Pasquale et al. 2012). Moreover, Chen et al. (2017) reported that bioaugmentation using endophytic bacteria carrying catabolic genes for the degradation of target contaminants improved petroleum removal. In the present study, similar results were found, since bioaugmentation with *Streptomyces* sp. Hlh1 increased the elimination of alkanes, and aromatics, improving the efficiency of oil remediation in contaminated soils.

In previous studies, it was found that the interaction between hydrocarbons and soil particles affected bioremediation because water and air are limiting factors in the removal of hydrocarbons (Zhao et al. 2016).

The first step in the aerobic pathway of the degradation of alkanes and aromatic hydrocarbons involves the presence of oxygen that is required for microbial activity (Gargouri et al. 2014). Therefore, it is necessary to ensure adequate oxygenation of the system, in addition, the oxygen level also depends on the soil moisture (Ghazali et al. 2004; Schjønning et al. 2011). For this reason, in this study, the soils inside the pots were mixed vigorously once a week, and soil moisture content was maintained at 20%, which is in agreement with previous reports (Polti et al. 2014; Wu et al. 2017a).

However, the comparison of our findings with other studies is nevertheless difficult, since different soils, strains, inoculum concentration, moisture content, nutrients, temperature, and incubation time for the bioremediation process were used, in which variable results were obtained.

3.3 Toxicity assay

The chemical analysis with GC-FID demonstrated the success of petroleum removal. However, a potential production of toxic metabolites may occur during the process. Lettuce is considered one of the most sensitive specimens for petroleum compounds (Bamgbose and Anderson 2015) and its toxic effect on germination is considered relevant to assess the toxicity of pollutants (Banks and Schultz 2005). Roy et al. (2014) confirmed that the degradation of crude oil by the inoculated bacteria decreases the phytotoxicity of remediated soil. Previously, the removal efficiency of toxic petroleum compounds with microbes was checked by toxicity bioassays of lettuce seeds, in which the decrease of pollutants is accompanied with the reducing of phytotoxicity (Gargouri et al. 2014). Hence, the obtained results confirmed that lettuce is a good bioindicator to evaluate the toxicity of petroleum compounds.

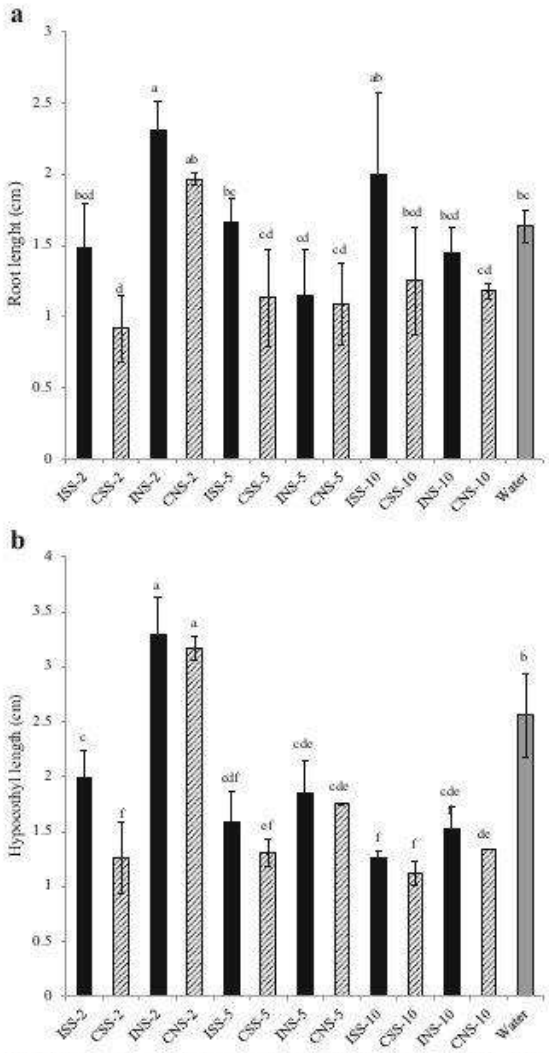

Fig. 2 Parameters of lettuce seedlings cultivated on soils and water (control): (**a**) root length, (**b**) hypocotyl length. Different letters represent a significant difference at ($p \leq 0.05$)

In a previous study, researchers reported that the toxicity of petroleum hydrocarbons on *Lactuca sativa* is related to its high solubility and the presence of volatile hydrocarbons that could easily penetrate into cells altering their structures (Montagnolli et al. 2015). For this purpose, it was evaluated that the effect of biodegradation metabolites on lettuce seeding (*Lactuca sativa*) developed in soils from different treatments.

The results of phytotoxicity test have been given in Fig. 2 and Table 4. It was observed that root and hypocotyl lengths, the vigor index (VI), and germination percentage differ significantly from control and each other. Simultaneously, soil inoculation with *Streptomyces* sp. Hlh1 improved the germination and growth of the seedlings. Lettuce hypocotyls showed high development than roots (Fig. 2), indicating that the direct contact of roots to explore the substrate and obtaining resources for correct growth was reduced (Saez et al. 2014). However, seeds exposed to 2% of petroleum showed longer roots and hypocotyls in comparison to non-contaminated soil. Similar results were observed in plants exposed to abiotic stress (Franco et al. 2011). On one hand, in abiotic stress, the elongation of roots may result from the research of water and nutrients, on the other hand, the high availability of nutrients may limit the growth of roots. The biodegradation of petroleum hydrocarbons at a concentration of 2% may generate non-toxic metabolites, while in high concentrations, the incomplete degradation of hydrocarbons may give less or toxic and does not allow the development of plants. Furthermore, the root:hypocotyl ratio was similar for seeds from soil without petroleum and with 2% (data not showed), confirming that petroleum concentration did not affect negatively the plant development.

Moreover, the root lengths were considerably longer in ISS than in CSS ($p < 0.05$), confirming the bioremediation efficiency (Fig. 2). Besides, as shown in Tables 3 and 4, the vigor index was higher in the inoculated soils compared to non-inoculated soils; these results indicate that petroleum hydrocarbons were effectively removed. The lower values of VI, observed in uninoculated CNS, suggested that the pollutants were not completely degraded or they were transformed to toxic metabolites by indigenous microbiota. These results are in agreement with the work of Saez et al. (2014).

The test of phytotoxicity did not show a toxic effect on germination, roots, and hypocotyls elongation in inoculated soils, indicating that *Streptomyces* sp. Hlh1 could reduce the toxicity of petroleum compounds generating less toxic or non-toxic compounds, and thereby this strain can contribute to petroleum-contaminated soil bioremediation.

Table 3 Concentration of residual hydrocarbon fractions and TPH ($mg \cdot kg^{-1}$) in different treatments. Different letters represent a significant difference at ($p \leq 0.05$)

Soil sample	Alkanes	Aromatics	TPH
ISS-2	5063^{h}	300^{e}	13053^{h}
CSS-2	11507^{e}	682^{e}	20048^{g}
INS-2	6153^{gh}	189^{e}	9060^{i}
CNS-2	7502^{g}	172^{e}	10474^{i}
ISS-5	34603^{c}	3301^{cde}	68901^{c}
CSS-5	35193^{c}	4318^{bcd}	85373^{a}
INS-5	14179^{ef}	988^{de}	40982^{f}
CNS-5	25219^{d}	1411^{abc}	78820^{b}
ISS-10	15936^{e}	2586^{cde}	39655^{f}
CSS-10	52895^{a}	7624^{ab}	67420^{c}
INS-10	40227^{b}	8712^{a}	57080^{e}
CNS-10	43136^{b}	5455^{abc}	59196^{d}

Table 4 Effect of different treatments on the germination percentage and seedling vigor of lettuce seeds. Different letters represent a significant difference at ($p \leq 0.05$)

Treatment	Germination (%)	Vigor index
ISS-2	77 ± 7^{e}	$26.63^{g'}$
CSS-2	76 ± 10^{e}	$16.71^{k'}$
INS-2	96 ± 2^{ab}	$53.54^{a'}$
CNS-2	87 ± 2^{bcd}	$45.11^{b'}$
ISS-5	84 ± 5^{cde}	$27.58^{g'}$
CSS-5	82 ± 12^{de}	$20.03^{j'}$
INS-5	96 ± 3^{ab}	$29.06^{e'}$
CNS-5	95 ± 4^{ab}	$27.20^{f'}$
ISS-10	92 ± 2^{abc}	$29.97^{d'}$
CSS-10	90 ± 3^{bcd}	$21.33^{i'}$
INS-10	100 ± 0^{a}	$29.70^{d'}$
CNS-10	91 ± 5^{abcd}	$22.86^{h'}$
Water (control)	100 ± 0^{a}	$41.16^{c'}$

Values indicate means of combined data of three replicates each

4 Conclusions

Crude petroleum oil was removed from artificially contaminated soil samples by using an inoculum of the endophytic *Streptomyces* sp. Hlh1. Hydrocarbon analysis showed that the degradation of aromatic compounds was significantly greater than n-alkanes. Toxicity assays demonstrated that seeds grown in bioremediated soils with *Streptomyces* sp. Hlh1 show an improvement in biological parameters compared to those grown in not inoculated soil samples.

These findings demonstrated that the bioremediation using *Streptomyces* sp. Hlh1 would represent a promising alternative from a biotechnological point of view, to remove and detoxify petroleum from contaminated environments.

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CHAPITRE III

Efficacité du complexe Zea mays- Streptomyces sp. Hlh1 dans la phytoremédiation des sols contaminés par les hydrocarbures pétroliers.

Préparation du sol :

1. Échantillonnage
2. Séchage
3. Tamisage
4. Stérilisation
5. Contamination par du pétrole brut ou des HAP

Préparation des graines :

1. Désinfection
2. Germination (3 jours)

***Streptomyces* sp. Hlh1**

Culture de 3 jours

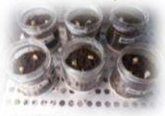
Inoculation

MM + HPA individuels

7 jours

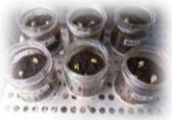
Quantification des HPA (HPLC)

Inoculation



Sol contaminé par le pétrole brut

Sol contaminé par les HPA



Sol contaminé par le pétrole ou les HAP

Sol contaminé par le pétrole ou les HAP

14 jours

Analyses de plantes

- La longueur des racines et des tiges
- Le contenue en caroténoïde et en chlorophylle
- La biomasse fraiche et sèche

Quantification des hydrocarbures

- Le pétrole brut par GC-FID
- Les HPA par HPLC

Figure 11. Dispositif expérimentale du chapitre III.

"Effectiveness of the *Zea mays*-*Streptomyces* association for the phytoremediation of petroleum hydrocarbons impacted soils " (Article publié en Août 2019 dans la revue Ecotoxicology and Environmental Safety**)**

Chapitre III : Efficacité du complexe *Zea mays*- *Streptomyces* sp. Hlh1 dans la phytoremédiation des sols contaminés par les hydrocarbures pétroliers.

Le succès de la souches sélectionnée *Streptomyces* sp. Hlh1 dans la remédiation des sols contaminés par le pétrole brut est à l'origine de son choix pour la phytoremédiation. Le présent chapitre consiste à l'étude de l'inoculation des plantes de maïs cultivé dans des sols contaminés par du pétrole brut ou des HAP par *Streptomyces* sp. Hlh1 d'une part et l'estimation de l'effet de l'inoculation sur la croissance de la plante utilisée d'autre part.

La méthodologie adoptée pour la réalisation de cette partie de travail est résumée dans la **Figure 11**.

Les résultats de l'étude de la dégradation du phenanthrène (Phe), de l'anthracène (Ant) et du pyrène (Pyr) par *Streptomyces* sp. Hlh1 sur milieu minimal liquide, montrent que cette souche est capable de dégrader 64%, 85% et 94% de ces composants respectivement **(Figure 1, Supplementary material)**.

Il semble évident que la dégradation des hydrocarbures ne dépend pas seulement du type de pétrole, mais aussi de leur présence dans un mélange ou individuellement. En effet, le travail effectué dans le chapitre I met en évidence un taux de dégradation des HAP contenus dans le pétrole brut plus faible que celui des fractions testées séparément dans le présent chapitre. Cette différence peut être expliquée par la disponibilité des fractions étudiées séparément par rapport au mélange d'hydrocarbures.

Le pétrole brut, généralement de composition complexe. En plus des hydrocarbures il est également constitué de composés azotés, d'oxygène, de soufre et de métaux lourds (Speight, 2014). Ces composés pourraient interférer dans la biodisponibilité de chaque composé. Cette capacité d'utiliser les hydrocarbures pétroliers pourrait être également liée à l'évolution sélective exercée par l'environnement à partir duquel les microorganismes sont isolés, conduisant à l'acquisition de capacités métaboliques pour survivre et se développer dans des environnements pollués. La plupart des études ont démontré que l'utilisation de microorganismes isolés de sites contaminés tend à

fonctionner plus efficacement que l'utilisation de microorganismes non adaptés aux contaminants (Fingas, 2011).

Dans les échantillons de sols stériles artificiellement contaminés par du pétrole brut ou des HAP individuels, Les résultats de la quantification du pétrole brut résiduel par GC-FID et des HAP résiduels par HPLC ont montré que toutes les concentrations de contaminants ont diminuées à la fin de l'expérimentation **(Fig 1. Article)**. Les hydrocarbures aromatiques polycycliques (HAP) montrent une diminution importante en présence des plantes non inoculées par rapport au contrôle (Sols sans plante), ce qui suggère que la culture de maïs, seule, pourrait être utilisée dans le traitement des sols contaminés par ces HAP. De plus, des différences significatives de la dégradation des contaminants sont constatées entre les traitements biologiques et les contrôles (p <0,05) dans le cas des HAP et non en présence du pétrole brut.

L'inoculation de *Streptomyces* sp. Hlh1 accélère la dissipation des hydrocarbures pétroliers dans les sols contaminés dans toutes les conditions testées. Le pétrole brut, le phénanthrène et l'anthracène sont dissipés plus efficacement du sol implanté et inoculé (70%, 88%, 73% respectivement), alors que le pyrène est éliminé jusqu'à 89% et 85% des sols plantés de maïs inoculé et non inoculé respectivement, sans aucune différence significative observée **(Fig 1. Article)**.

L'inoculation microbienne favorise l'activité enzymatique de dégradation, l'élimination des HAP et la croissance des plantes (Sturz *et al*., 2000). En dépit de la toxicité des hydrocarbures, qui est un facteur limitant, les plantes ne peuvent pas les dégrader facilement. Certaines plantes possèdent des mécanismes de tolérance permettant de tolérer, d'immobiliser et/ou d'accumuler des hydrocarbures dans leurs différentes parties, voire de les dégrader et de les éliminer (Li *et al*., 2012; Khan *et al*., 2013). Cette réponse dépend non seulement du type de plante, mais également de la concentration des hydrocarbures et du temps d'exposition (Arellano *et al*., 2017).

Les *n*-alcanes résiduels sont quantifiés. Leur élimination augmente en présence de plantes inoculées par rapport aux plantes non inoculées et aux contrôles **(Tableau 1, Article)**.

Les rapports *n*-C_{17} / pristane et *n*-C_{18} / phytane sont significativement plus faibles (p <0,05) en présence de plantes inoculées avec *Streptomyces* sp. Hlh1 par rapport au sol sans plantes ou implantés de plantes non inoculées **(Tableau 2, Article)**.

Les rapports *n*-C_{17} / pristane et *n*-C_{18} / phytane sont généralement utilisés comme un index pour l'estimation de la biodégradation des hydrocarbures. Puisque *n*-C_{17} et *n*-C_{18} sont des composés facilement dégradables et que le pristane et le phytane sont des composés relativement moins

dégradables, ces rapports sont plus élevés dans le cas des entrées des ruisseaux, tandis que les rapports faibles indiquent une dégradation importante des hydrocarbures pétroliers (Bajt, 2017; Rostami *et al*., 2019).

En terme général, les sols implantés améliorent nettement l'élimination des hydrocarbures aromatiques polycycliques par apport à ceux non implantés ($p<0,05$). Cet effet, non observé en présence du pétrole brut pourrait être dû aux effets toxiques des composés pétroliers à faible poids moléculaire (Iqbal *et al*., 2019).

Les plantes de maïs sont capables de tolérer les concentrations des hydrocarbures utilisées, et ont également contribué à l'élimination de pétrole brut et des HAP. De plus, une dégradation significative des contaminants est observée dans les sols implantés et inoculés en comparaison avec des sols autrement traités.

Il serait possible que l'effet de *Streptomyces* sp. Hlh1 pourrait être lié aux mécanismes de stimulation des plantes, qui excrètent des exsudats racinaires qui attirent les microbes de la rhizosphère. L'amélioration du taux d'élimination des contaminants organiques de la rhizosphère peut être due à l'augmentation de la densité microbienne et / ou de l'activité résultant de la libération d'exsudats racinaires des plantes (Ying *et al*., 2011; Becerra-Castro *et al*., 2013).

Le développement des plantes exprimé dans ce cas en fonction de la longueur des racines et des tiges, la teneur en caroténoïdes et en chlorophylle, la biomasse fraîche et sèche, est analysé dans toutes les conditions testées à la fin de l'expérimentation. Toutes les plantes ont poussé et aucune mortalité n'a été constatée dans les sols contaminés et non contaminés. Cependant, une forte réduction de la longueur des racines et des tiges est enregistrée dans les sols contaminés. Le pétrole brut semble influencer négativement le développement des racines des plantes de maïs **(Fig. 2, Article)**.

À travers les racines et les feuilles, les hydrocarbures peuvent atteindre le système vasculaire et les espaces intercellulaires, réduisant ainsi la croissance des plantes ou induisant leur mortalité (Arellano *et al*., 2017). Il est bien connu que dans les sols pollués par le pétrole, la réduction du développement des plantes est due à la nature toxique des hydrocarbures qui réduisent l'absorption d'eau et de nutriments (Kirk *et al*., 2005).

Les plantes inoculées par *Streptomyces* sp. Hlh1 présentent des racines et des tiges plus longues, ainsi que des teneurs plus élevées en chlorophylle et en caroténoïdes. Les plantes inoculées et non inoculées traitées au pyrène présentent des teneurs en chlorophylle et en caroténoïdes similaires **(Fig. 3, Article)**.

L'amélioration de la longueur des racines et des tiges des plantes de maïs par *Streptomyces* sp. Hlh1 pourrait être liée à la présence des métabolites PGP tels que l'idole acétique acide (IAA) et l'ACC désaminase. L'aptitude de *Streptomyces* sp. Hlh1à produire ces métabolites a déjà été signalée dans le chapitre I (Baoune *et al*., 2018). Il est bien connu que l'IAA et l'ACC désaminase contribuent à la croissance et au développement des plantes dans des conditions de stress. L'ACC désaminase est une enzyme clé responsable de la réduction des niveaux d'éthylène dans les plantes et, par conséquent, de la croissance et du développement des racines(Glick, 2014).

Le poids frais et le poids sec des plantes cultivées dans les différentes conditions expérimentales n'a pas montré de différences significatives, tandis que les plantes inoculées présentent des poids frais légèrement supérieurs à ceux des plantes non inoculées **(Tableau 1, Supplementary material)**.

Il ressort de cette étude que *Zea mays* est capable de remédier les sols contaminés par des hydrocarbures pétroliers et que sa capacité de remédiation est améliorée par son inoculation avec l'endophyte *Streptomyces* sp. Hlh1. De même, La meilleure croissance des plantes observée chez les plantes inoculées et cultivées dans des sols contaminés ouvre une possibilité exceptionnelle d'utiliser le maïs inoculé de *Streptomyces* sp. Hlh1 en tant que candidat prometteur pour une stratégie de phyto-restauration des sols contaminés par les hydrocarbures.

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Effectiveness of the *Zea mays-Streptomyces* association for the phytoremediation of petroleum hydrocarbons impacted soils

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ABSTRACT

Restoring polluted sites by petroleum hydrocarbons is a challenge because of their complexity and persistence in the environment. The main objective of the present study was to investigate the performance of plant-actinobacteria system for the remediation of crude petroleum and pure-polycyclic aromatic hydrocarbons (PAHs) contaminated soils. The endophytic strain *Streptomyces* sp. Hlh1 was tested for its ability to degrade model PAHs (phenanthrene, pyrene and anthracene) in liquid minimal medium. *Streptomyces* sp. Hlh1 demonstrated the ability to grow on PAHs as sole carbon and energy source, reaching hydrocarbons removal of 63%, 93% and 83% for phenanthrene, pyrene and anthracene, respectively. Maize plant was chosen to study the impact of *Streptomyces* sp. Hlh1 inoculation on the dissipation of contaminants and plant growth. Thus, maize seedlings grown in soils contaminated with crude petroleum and pure-PAHs were inoculated with *Streptomyces* sp. Hlh1. Results showed that the endophyte inoculation increased contaminants removal. Maximum hydrocarbons removal (70%) was achieved in inoculated and planted soil contaminated with crude oil, while 61%, 59%, and 46% of hydrocarbons dissipation were registered for phenanthrene, pyrene and anthracene, respectively. These degradations rates were significantly higher compared to non-inoculated systems in all the treatments evaluated. Further, it was revealed that hydrocarbons (C_8–C_{30}) were efficiently degraded in plant-*Streptomyces* Hlh1 system. Moreover, the inoculation with the actinobacteria resulted significant plant development and enhanced photosynthetic pigments compared to plants grown in the other experimental conditions. The present study provide evidence that the inoculation of maize plants with *Streptomyces* sp. Hlh1 play a remarkable role in the removal of petroleum hydrocarbons, enhancing plant development in contaminated soils.

1. Introduction

Over the last decades there was an important increase of petrochemical industries due to the exponential growth of the worldwide population and the extensive use of petroleum-based products (Koshlaf and Ball, 2017). As a result, petroleum wastes have been released into the environment, where they can dissolve or float in water or accumulate in the soil causing several damages (Aydin et al., 2017). Certainly, petroleum wastes produce major concern besides consuming considerable economic resources (Lalande et al., 2003).

Crude petroleum is a mixture of hydrocarbon and non-hydrocarbon compounds. According to Speight (2014), hydrocarbon compounds can be classified as follow: paraffins (linear or branched chains), naphthenes or cycloparaffins (containing one or more rings) and aromatics compounds (containing one or more aromatic ring which may be linked up with naphthenic rings and/or paraffinic side chains). Those compounds are deleterious for the environment and living organisms because of their lipophilic, mutagenic and carcinogenic properties (Fatima et al., 2015). Especially pyrogenic polycyclic aromatic hydrocarbons (PAHs), which are generated from the incomplete incineration of crude petroleum throughout industrial process, has been recognized as priority persistent organic pollutants (POPs) and the most hazardous for the environment, due to their toxic, mutagenic, carcinogenic properties and their recalcitrance (Aydin et al., 2017). PAHs compounds

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contain petrogenic (2–3 fused aromatic benzene rings) and pyrogenic (≥4 rings) in various different configurations. Their persistence and bioavailability in the environment are both related to their molecular size. For instance, the half-lives of PAHs in soils and sediments may range from 16 to 126 days for phenanthrene (three ring molecule), whereas it may range from 229 to 1400 days for pyrene (five rings) (Kanaly and Harayama, 2000). Further, high molecular weight PAHs may be adsorbed onto deep soil or sediments where oxygen molecule is rare, significantly reducing their possibilities of being biodegraded since aerobic conditions are considered essential for PAHs degradation (Qin et al., 2017).

A number of restoring technologies including biological and physico-chemical processes are available to reduce petroleum hydrocarbon concentrations in the environment (Sannino and Piccolo, 2013). Significant attention has been given to bioremediation, that mainly relies on the use of organisms with specific metabolic abilities in order to remove or minimize the toxic effect of organic and inorganic pollutants (de Almeida et al., 2017). Certainly, biological remediation is considered as an efficient approach to cleanup petroleum contaminated soils (Tiralerdpanich et al., 2018). Phytoremediation based on the interactions between plants and microorganisms, appears to be especially effective for this purpose (Soleimani et al., 2010). Particularly, plant-bacteria association has been considered as an effective partnerships to cleanup hydrocarbons impacted soils (Khan et al., 2013).

Some plant species have shown the capacity to deal with relatively high concentrations of organic chemicals, which may be taken up, translocated, metabolized, and/or volatilized. In addition, plants may stimulate the degradation of xenobiotics in the rhizosphere (rhizodegradation) releasing plant root exudates (Alvarez et al., 2012, 2015). On the other hand, endophytic bacteria colonize the inner tissues of living plants establishing a harmonious relationship with the host and causing positive effects on plant health (Kidd et al., 2017). In a phytoremediation scene, endophytic bacteria with specific metabolic capabilities could be able to degrade organic pollutants and to reduce both phytotoxicity and evapotranspiration of volatile compounds. Certainly, plant-associated bacteria have exceptional ability to improve plant growth and biomass production in polluted soils, due to their ability to produce plant growth-promoting (PGP) molecules (Fatima et al., 2016;Simon Sola et al., 2017, 2019; Soleimani et al., 2010).

Plant-bacteria association can be exploited for the remediation of polluted systems (Weyens et al., 2010) and is considered as an important component of phytoremediation technologies (Glick et al., 2007). Endophytes belonging to Gammaproteobacteria, Bacilli, Alphaproteobacteria, Flavobacteria and Actinobacteria have demonstrated potential to degrade different hydrocarbons (Afzal et al., 2014). In particular, actinobacteria play relevant ecological roles in the environment, and their ability to degrade complex polymers represents the reason why actinobacteria have received special attention as candidates for bioremediation of organic compounds (Alvarez et al., 2017). Thus, members of this phylum are being intensively studied by our research group in the Laboratory of Biotechnology of Actinobacteria because of their ability to bioremediate heavy metals, and petroleum hydrocarbons in single and mixed polluted systems (Alvarez et al., 2017; Aparicio et al., 2015; Baoune et al., 2018, 2019; Fuentes et al., 2010; Polti et al., 2009).

Previously, we have demonstrated that the endophyte *Streptomyces* sp. Hlh1 was capable of degrading light Algerian crude petroleum in both liquid and soil systems, and producing PGP molecules (Baoune et al., 2018). Based on these data, we hypothesized that the inoculation of maize plants exposed to petroleum hydrocarbons with *Streptomyces* sp. Hlh1, could be a successful phytotechnology to improve the phytoremediation process. In this context, the main purpose of the present study was to investigate the effectiveness of the plant-actinobacteria association to remove crude petroleum and pure-PAHs from contaminated soils.

2. Materials and methods

2.1. Chemicals and culture media

Light crude petroleum was obtained from refinery situated in Hassi Mesouad, City of Ouargla, Algeria (Baoune et al., 2018). PAHs used in this study were phenanthrene, anthracene and pyrene (> 99% purity, analytical grade), purchased from Sigma-Aldrich Co. (St.Louis, MO, USA). Stock solutions of PAHs (anthracene or phenanthrene 4.45 g L^{-1}; pyrene 5.81 g L^{-1}) were prepared by dissolving each PAH separately in acetone. Crude petroleum and pure-PAHs were filter sterilized prior to use.

Depending upon the objectives of the experiment, bacterial strain was cultured in one of the following media, all of which were sterilized by autoclaving at 121 °C for 20 min.

ISP2-agar medium containing (in g L^{-1}) malt extract, 10; yeast extract, 4; glucose, 4; agar, 20 (pH 7.0), was used for microbial maintenance.

Tryptic Soy Broth (TSB) containing (in g L^{-1}) tryptone, 15; soy peptone, 3; NaCl, 5; K_2HPO_4, 2.5; glucose 2.5, was used to prepare the bacterial inoculum.

Liquid minimal medium (MM) containing (in g L^{-1}) L-asparagine, 0.5; K_2HPO_4, 0.5; $MgSO_4.7H_2O$, 0.2; $FeSO_4.H_2O$, 0.01, was used for the biodegradation assays.

2.2. Polycyclic aromatics hydrocarbon degradation in liquid medium

Crude petroleum-degrading *Streptomyces* sp. Hlh1, previously isolated and characterized (Baoune et al., 2018), was cultured in Erlenmeyer flasks containing 30 mL of TSB and incubated for 3 days, at 30 °C in orbital shaker. Biomass was harvested by centrifugation (4000 × *g*, 15 min, 4 °C), washed two times and re-suspended in sterile distilled water.

Flasks with 20 mL of MM were supplemented with anthracene, phenanthrene (36 mg L^{-1}) or pyrene (46 mg L^{-1}) (final concentration). Prior to the microbial inoculation, flasks were left uncapped for 30 min to allow the evaporation of the acetone. Then, *Streptomyces* sp. Hlh1 was inoculated in the flasks at final concentration of 0.01 g L^{-1} and incubated for 7 days at 30 °C in orbital shaker. Non-inoculated flasks were used as control and three replicates of each treatment were done. At the end of the incubation period, centrifuged culture supernatants (9000 × *g*, 10 min, 4 °C) were used to determine contaminant concentrations as described below. Microbial biomass was estimated after centrifugation by washing the pellets with 25 mM Tris-EDTA buffer (pH = 8) and drying to constant weight at 105 °C.

2.3. Soil preparation and contamination

A model loam soil was collected from an urban area free of contamination at 5–15 cm depth. Prior to use, soil was air-dried, lightly ground, and finally sieved through a 2-mm sieve. Glass pots filled with 200 g of soil were sterilized (three successive sterilizations at 100 °C for 60 min each, whit 24 h intervals) (Polti et al., 2014). Sterilized soil facilitates the study of the effect of the *Streptomyces* strain on the hydrocarbons dissipation as well its effect on the growth of the plant, avoiding the possible interference of soil microbiota. Soil moisture was adjusted to 20% using sterile distilled water and then, each glass pot was artificially contaminated with filter-sterilized crude petroleum (20 g kg^{-1}) or pure-PAHs (anthracene, phenanthrene 36 mg kg^{-1}; pyrene 46 mg kg^{-1}) individually. Soil samples were thoroughly mixed and the pots were left uncovered for 6 h to allow evaporation of acetone. Then, pots were kept for 36 h at room temperature, so that water and hydrocarbons equilibrate in the soil.

2.4. Plant-bacteria bioassay

Endophyte-free maize (*Zea mays*) seeds not treated with fungicide were surface-sterilized using 5% (*v/v*) of sodium hypochlorite for 2 min, followed by 2 min immersion in ethanol 70% (*v/v*). Seeds were thoroughly rinsed 5 times with sterile distilled water (Yousaf et al., 2011). The seeds were then placed into sterile Petri dishes with filter paper (Wattman No. 1) moistened with sterile distilled water, until germination, in a climate controlled room (25 °C, 16:8 light:dark, 65% relative humidity).

The rhizosphere zone of each germinated seed (three per pot) was inoculated with a cell suspension of *Streptomyces* sp. Hlh1 (final concentration of 1 g kg^{-1} of wet weight), which had been prepared as mentioned before. Non-inoculated plants and non-planted soils were used as controls in both, contaminated and non-contaminated systems. Three replicates were prepared for each treatment. Pots were incubated in a climate controlled room (25 °C, 16:8 light:dark, 65% relative humidity) for 15 days. Plants were watered with sterile water when needed. At the end of the experiment, soil samples were taken for determining residual petroleum and pure-PAHs.

2.5. Plant analyses

Maize plants were harvested at the end of the assay to determine the length of the shoots and roots (Calvelo Pereira et al., 2010); fresh and dry weight as well as carotenoid and chlorophyll content. For this purpose, the soil attached to the roots was removed by gently washing with distilled water. Afterwards, length of roots and shoots was measured using a millimeter scale and plant biomass was recorded as fresh weight. Chlorophyll content was determined according to the protocol of Arnon (1949). Briefly, 500 mg of fresh leaves were cut into small pieces, homogenized in 10 mL of 80% acetone and then centrifuged at 2400 rpm for 10 min, at 4 °C. The absorbance of the plant extract was measured at 645, 663 and 480 nm. Acetone 80% was used as blank. Detection and quantification limits of the method were 0.08 μg L^{-1} and 0.50 μg L^{-1}, respectively. Chlorophyll content was calculated according to the formula of Arnon (1949).

The carotenoid content in the plant extract was calculated according to the formula given by Kirk and Allen (1965). The remaining parts of plants samples were dried at 105 °C until constant weight to determine dry weight.

Tolerance Index (TI) was calculated as the mean dry weight of a plant grown on the presence of a hydrocarbon, divided by the mean dry weight of a plant grown on non-contaminated control soil (Diwan et al., 2010). TI values greater than 1 reflect a net increase in biomass and suggest that plants have developed tolerance, whereas TI values lower than 1 indicate a net decrease in biomass and a stressed condition by the plants. TI values equal to 1 indicate no difference relative to control treatments.

2.6. Hydrocarbons analysis

2.6.1. Polycyclic aromatic hydrocarbons analysis

Residual pure-PAHs were extracted from liquid and soil samples by adding 10 mL of acetone. The mixture was homogenized and filtered through a 0.22 μm – PTFE membrane (Microclar, Argentina). Filtered solutions were stored at −20 °C until analysis (Bourguignon et al., 2014).

PAHs determination was carried out by RP-HPLC using an HPLC equipment (Alliance e2695, Waters Co., MA, USA) coupled to a PDA 2998-detector operating at a fixed wavelength (λ = 254 nm). Samples were automatically injected into C18 μBondapak HPLC column (4.6 × 250 mm, 50 Å pore size, 5 μm particle size). The mobile phase was methanol:water (9:1) at a flow rate of 1 mL min^{-1} for 30 min (Manohar et al., 2001). The limits of detection (LOD) and quantification (LOQ) for the PAHs were determined from free samples (n = 5) as the lowest concentrations yielding signal-to-noise ratios of 3:1 and 10:1 or higher calculated for average baseline noise, respectively. The LOQ was subsequently determined by analysis of five spiked samples prepared at their respective concentrations. Stock solutions of phenanthrene, anthracene and pyrene (Sigma-Aldrich) were prepared by dissolving 1 mg of each in 100 mL of acetone. PAHs standards solutions were kept in the dark at 4 °C until analysis. Mixed working solutions of PAHs ranging from 0.5 to 2.0 ng L^{-1} were prepared by the appropriate dilution of aliquots of the stock solutions in acetone to prepare the calibration curves and calculate the LOD for all compounds. The LOD was: phenantrene (0.010 ng), anthracene (0.0009 ng) and pyrene (0.071 ng). The LOQ (10 S/N) was: phenanthrene (0.039 ng), anthracene (0.002 ng) and pyrene (0.209 ng). Both LOD and LOQ are expressed as ng per injection. The mean recovery from 10 mL of samples was in the range of 73–84%.

2.6.2. Petroleum crude hydrocarbons analysis

Ten grams of each individual soil sample were weighed into 20 mL glass vials, and 10 mL of pentane were added. The vials were shaken on a horizontal shaker at 120 oscillations per min for 1 h. The extracts were decanted overnight and the organic phase separated. For the determination of semi-volatile hydrocarbons, 1 μL of the extracts was injected, in the splitless mode, into an Agilent 7890 A gas chromatography equipped with a flame ionization detector (FID) and a HP5 capillary column (25 m, long; 0.25 mm, ID; 0.25 μm, film thickness) with nitrogen as carrier gas (3 mL min^{-1}). Temperatures of the injection port and detector were 285 °C and 325 °C, respectively. The oven temperature program was as follows: initial temperature of 30 °C for 3 min, increasing at 15 °C min^{-1} to 300 °C which was kept for 5 min, and then increasing at 15 °C min^{-1} to the final temperature of 325 °C. The OpenLab software was used for integrating chromatogram area to encompass straight chain hydrocarbons from C6 to C35. Quantification of the components was performed with the standards proposed by the methods EPA 8015 and TNRCC 1005. The ratios *n*-C17/pristane and *n*-C18/phytane were calculated. The quantification limit was 20 mg kg^{-1} and the detection limit was 5 mg kg^{-1}.

2.7. Statistical analysis

Statistical analysis was conducted using R software, version **3.5.3.** All data reported in this study were the means values of three independent replicates. Data were evaluated statistically by one-way analysis of variance (ANOVA), and significant differences between mean values were determined by Fisher post-test with a probability level of $p < 0.05$. The data normality was checked by using the Shapiro test.

3. Results

3.1. PAHs biodegradation in liquid medium

Growth and removal of pure-PAHs by *Streptomyces* sp. Hlh1 were evaluated in artificially contaminated liquid system. At the end of the assays, microbial growth ranged from 0.27 to 0.28 g L^{-1}, showing that *Streptomyces* sp. Hlh1 was able to use these pollutants as a sole source of carbon and energy, because no other carbon compound had been added to the culture medium. Consequently, no growth was observed in the absence of PAHs (Supp. Fig.1). Results of the quantitative assessment of the degradation of pure PAHs, showed degradation percentages of 64% (23 mg L^{-1}), 85% (30 mg L^{-1}) and 94% (44 mg L^{-1}) for phenanthrene, anthracene and pyrene, respectively.

3.2. Petroleum hydrocarbons removal in soil

The synergistic potential of *Streptomyces* sp. Hlh1 with maize plants to remove petroleum hydrocarbons was evaluated in artificially

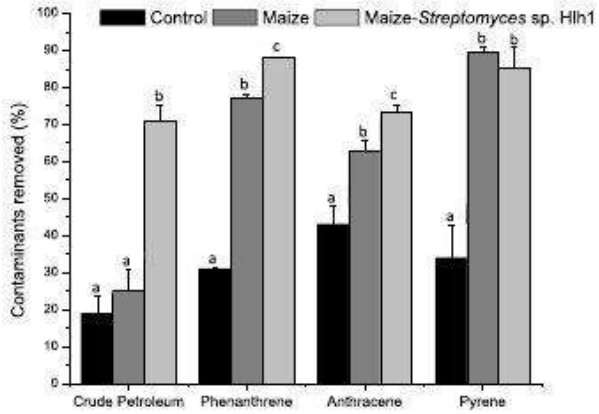

Fig. 1. Crude petroleum and pure PAHs dissipation from artificially contaminated soil after 14 days of treatment. Bars showing different letters indicate they were significantly different ($p < 0.05$, Fisher's post-test).

contaminated soils. Crude petroleum, phenanthrene, pyrene or anthracene dissipation was recorded after 14 days for soils assayed with the following treatments: Maize-*Streptomyces* Hlh1; Maize (non-inoculated) and Controls (non-inoculated and non-planted soil). In a previous work, *Streptomyces* sp. Hlh1 had been grown on petroleum hydrocarbon contaminated soil, so that its ability to degrade the contaminant from soils had been demonstrated (Baoune et al., 2019).

There was evidence for crude petroleum and PAHs removal since contaminants decreased at the end of the exposure period in all the biological treatments (Fig. 1). PAHs showed higher removal values (~50%) from soils implanted and non-inoculated, in comparison with control soil, strongly suggesting that maize seemed to be functional for the treatment of these PAHs. Moreover, statistically significant differences in the contaminants removal were found between biological treatments and controls ($p < 0.05$), except for the treatment with maize in the presence of crude petroleum. The presence of *Streptomyces* sp. Hlh1 accelerates the dissipation of petroleum hydrocarbonsfrom soils, under the tested conditions. Crude petroleum, phenanthrene and anthracene were dissipated more efficiently from soil implanted and inoculated (70%, 88% and 73%, respectively); (14,176, 31.4, 26.1 mg kg^{-1}, respectively) in comparison to soils implanted and non-inoculated (26%, 77% and 62%, respectively) (5217, 27.5, 22.3 mg kg^{-1}, respectively) (Fig. 1). Pyrene was removed up to 89% (41.5 mg kg^{-1}) and 85% (39.5 mg kg^{-1}) from soil with inoculated and non-inoculated plants, respectively, without statistically significant difference.

Fractions of crude petroleum hydrocarbons dissipated were also evaluated. Residual concentrations of crude petroleum components after the treatments are shown in Table 1. It was observed that hydrocarbons removal increased in the presence of inoculated plants, in comparison to non-inoculated plants and control pots (Table 1).

In the inoculated and planted soil, hydrocarbons dissipations were up to 77% (C8–C24) and 69% (C24–C30), while in the non-inoculated planted system were up to 34% (C8–C24) and 40% (< C24–C30).

All experiments were done using sterile soil samples, so the losses of petroleum hydrocarbons and PAHs observed in controls could be attributed to abiotic factors such as volatilization, evaporation, photo-oxidation and irreversible sorption, among others (Soleimani et al., 2010).

In general terms, soils planted improved significantly ($p < 0.05$) PAHs removal comparing to the non-planted soils. This effect was not observed by crude petroleum, which could be due to the toxic effects of low molecular weight petroleum compounds (Iqbal et al., 2019).

The ratio *n*-C17/pristane and *n*-C18/phytane are considered as indicators of hydrocarbons biodegradation. In this study, both ratios were

Table 1
Residual concentrations (mg kg^{-1}) of crude petroleum components obtained from the soil after the treatments. Values sharing the same letter were not significantly different, among the treatments ($p < 0.05$, Fisher's post-test).

Hydrocarbons	Control	Maize	Maize-*Streptomyces* sp. Hlh1
Phenanthrene	25 ± 0 ^{a}	6 ± 0 ^{b}	2 ± 1 ^{c}
Anthracene	20 ± 2 ^{a}	8 ± 1 ^{b}	5 ± 0 ^{c}
Pyrene	31 ± 4 ^{a}	3 ± 1 ^{b}	2 ± 1 ^{b}
< C8	217 ± 54 ^{a}	141 ± 49 ^{a}	50 ± 24 ^{b}
C8 to < C10	2151 ± 146 ^{a}	1813 ± 87 ^{b}	641 ± 88 ^{c}
C10 to < C12	2680 ± 195 ^{a}	2728 ± 137 ^{a}	1038 ± 107 ^{b}
C12 to < C14	3086 ± 201 ^{a}	2780 ± 268 ^{a}	1172 ± 156 ^{b}
C14 to < C16	3175 ± 170 ^{a}	3000 ± 223 ^{a}	1221 ± 127 ^{b}
C16 to < C18	1753 ± 68 ^{a}	1566 ± 148 ^{a}	649 ± 47 ^{b}
C18 to < C20	1060.65 ± 63 ^{a}	996 ± 96 ^{a}	385 ± 27 ^{b}
C20 to < C22	848 ± 51 ^{a}	808 ± 83 ^{a}	306 ± 14 ^{b}
C22 to < C24	344 ± 10 ^{a}	368 ± 38 ^{a}	135 ± 43 ^{b}
C24 to < C26	350 ± 22 ^{a}	315 ± 32 ^{a}	121 ± 15 ^{b}
C26 to < C28	238 ± 23 ^{a}	200 ± 30 ^{a}	72 ± 4 ^{b}
C28 to < C30	116 ± 5 ^{a}	70 ± 19 ^{b}	35 ± 5 ^{c}
C30 to < C32	0	0	0
> C32	0	0	0
Total Hydrocarbons	16,019 ± 800 ^{a}	14,783 ± 969 ^{a}	5824 ± 541 ^{b}

Data are means with standard deviation presented after the observed value.

Table 2
Crude petroleum removal from soils after the biological treatment and *n*-C17/pristane and *n*-C18/phytane ratios calculated. Values sharing the same letter were not significantly different ($p < 0.05$, Fisher's post-test).

	Control soil	*Z. mays*	*Z. mays* -*Streptomyces* sp. Hlh1
Crude petroleum removal (%)	19.9 ± 4.0 ^{a}	26.1 ± 4.8 ^{a}	70.9 ± 2.7 ^{b}
Ratio *n*-C17/pristane	1.65 ± 0.21 ^{a}	1.48 ± 0.33 ^{a}	0.97 ± 0.09 ^{b}
Ratio *n*-C18/phytane	1.74 ± 0.24 ^{a}	1.57 ± 0.32 ^{a}	1.03 ± 0.16 ^{b}
n*-C17	168 ± 24 ^{a}	122 ± 26 ^{a}	36 ± 5 ^{b}
Pristane*	102 ± 2 ^{a}	83 ± 7 ^{b}	38 ± 8 ^{c}
n*-C18	177 ± 26 ^{a}	130 ± 22 ^{a}	38 ± 11 ^{b}
Phytane*	102 ± 2 ^{a}	84 ± 14 ^{a}	36 ± 6 ^{b}

Data are means with standard deviation presented after the observed value.

significantly lower ($p < 0.05$) in the presence of plants inoculated with *Streptomyces* sp. Hlh1, in comparison to soil without plants or implanted and non-inoculated (Table 2).

3.3. Effects of petroleum hydrocarbons on plant development

Maize plants were harvested at the end of the assay. Parameters such as roots and shoots length, plant biomass, and carotenoid and chlorophyll content were analyzed.

All plants survived and no mortality was noticed in either contaminated or non-contaminated soils. According to the tested contaminants, the length of roots and shoots were variable (Fig. 2) showing a strong tendency to reduction in comparison to root and shoots development of control plants grown on non-contaminated soil. Especially, crude petroleum seems to deeply influence plant development, due to roots length of plants grown on this contaminant was shorter in comparison to roots length of plants grown in pure-PAHs.

For most of the contaminants tested, plants inoculated with *Streptomyces* sp. Hlh1 exhibited higher roots and shoots length, as well as higher contents of chlorophyll and carotenoids in comparison to non-inoculated plants growing in contaminated soil (Fig. 3). Only for pyrene, contents of chlorophyll and carotenoids were similar for both inoculated and non-inoculated plants.

Moreover, a significant increase in chlorophyll and carotenoids contents was registered in plants inoculated with *Streptomyces* sp. Hlh1,

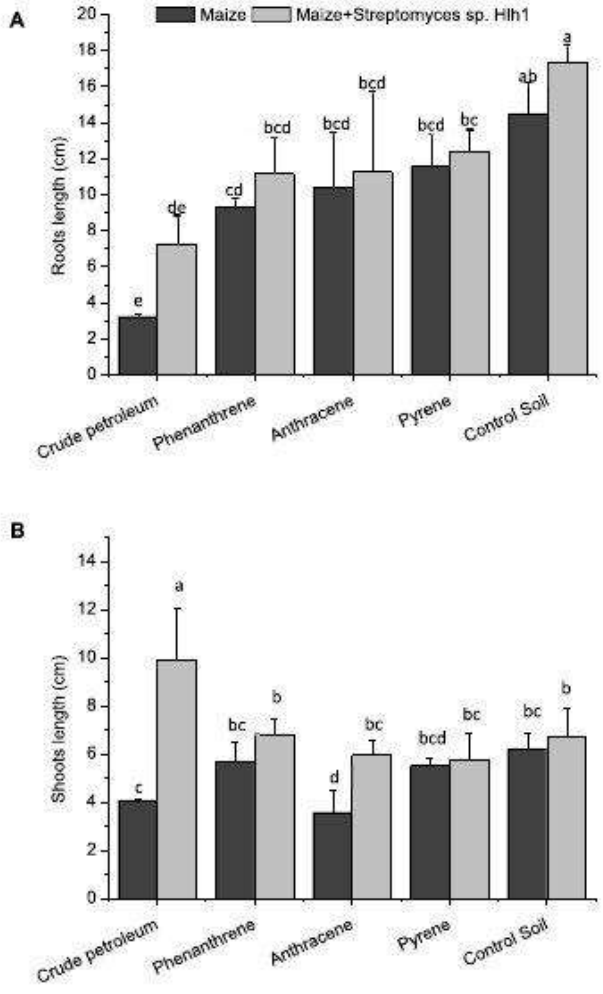

Fig. 2. Seedling development of *Zea mays* grown on soils artificially contaminated with petroleum hydrocarbons. (A) Root length; (B) Shoot length. Bars showing different letters indicate they were significantly different ($p < 0.05$ Fisher post-test).

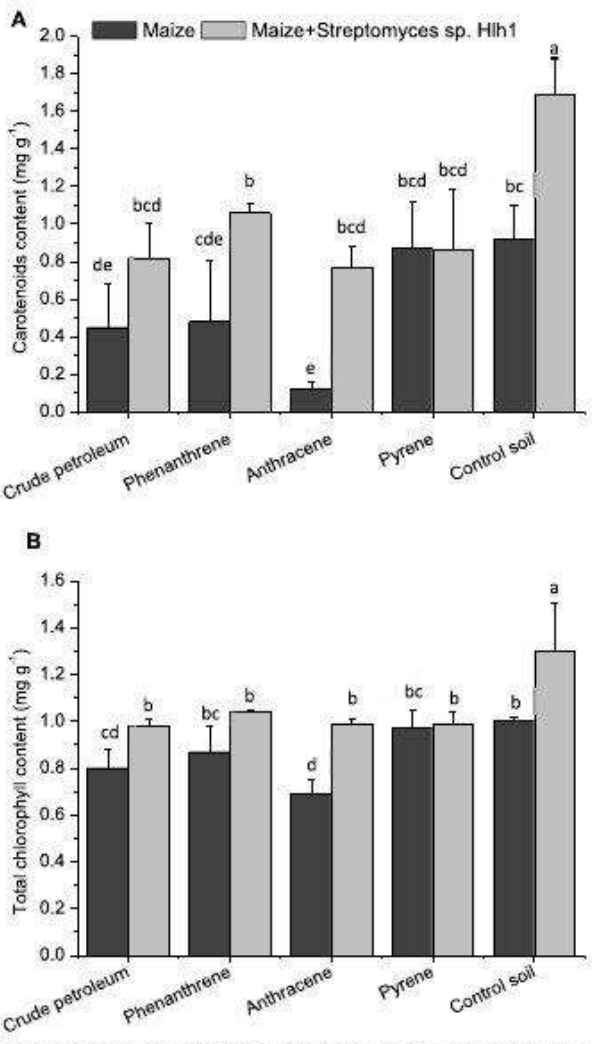

Fig. 3. (A) Carotenoids and **(B)** Chlorophyll content of *Zea mays* plants grown on soils artificially contaminated with petroleum hydrocarbons. Bars showing different letters indicate they were significantly different ($p < 0.05$, Fisher's post-test).

in absence of pollutants ($p < 0.05$).

Fresh and dry weight of plants grown in different experimental conditions did not show significant differences, while inoculated plants showed slightly higher fresh weights in comparison to non-inoculated plants. Such differences were not detected when dry weights of the plants were obtained (Supplementary Table 1).

4. Discussion

Plants-endophytes associations are recognized as synergistic partnerships useful for cleaning contaminated soils (Newman and Reynolds, 2005; Ryan et al., 2008). Several aspects related to the implementation of this technology such as pollutants nature, soil type, plant species, inoculum density and inoculation methods have been intensively researched and deeply debated (Afzal et al., 2011, 2013; Khan et al., 2013; Zheng et al., 2018). In any case, there is a general consensus that plant-microbe association is a highly desirable component of any phyto-management program (Glick et al., 2007). Within Bacteria domain, Actinobacteria have received special attention to be active for bio/phyto-remediation (Alvarez et al., 2017). Particularly, the *Streptomyces* genus, whose members are distributed extensively in soil, water, and in association with plants (Balachandran et al., 2012; Baoune et al., 2018; Polti et al., 2007), have been reported to be able to degrade petroleum hydrocarbons (Bourguignon et al., 2016). In addition to their metabolic diversity, strains of the *Streptomyces* genus may be well suited for soil inoculation because of their production of spores that can persist and disperse. In this context, the present work is shedding light on the role of a petroleum-degrading endophytic *Streptomyces* strain to improve phytoremediation of petroleum hydrocarbons contaminated soil.

An intensive review about the success of several actinobacteria strains to bioremediate contaminated systems was compiled and analyzed by our research group (Alvarez et al., 2017). Nevertheless, no study has been reported about plant-endophytic *Streptomyces* as partnerships useful for petroleum hydrocarbons dissipation from soil.

Previously, Baoune et al. (2018) determined the ability of *Streptomyces* sp. Hlh1 for degrading crude petroleum in liquid culture medium. The degradation of phenanthrene, pyrene and anthracene from crude petroleum observed in that study, was lower in comparison to the degradation obtained in the present study using the pure-PAHs added individually into the culture medium. It seems quite evident that

hydrocarbons degradation was not only highly dependent on the type of petroleum hydrocarbons but also depends on their presence in a mixture or individually. Crude petroleum contains an extreme range of organic compounds of different molecular size. In general terms, crude petroleum oil has a complex composition of hydrocarbons, nitrogen, oxygen and sulfur compounds, and metallic constituents (Speight, 2014) which could interfere in the bioavailability of each compound. Interestingly, although pyrene (four-ringed compound) has been used as a model of high molecular weight PAH, *Streptomyces* sp. Hlh1 achieved the highest removal for pure-pyrene, which is considered more persistent and difficult to biodegrade than phenanthrene and anthracene (Ghosal et al., 2016). In addition, microbial growth was not inhibited by the contaminants. This ability to grow in the presence of petroleum hydrocarbons could response to selective evolutionary pressure that would have been exerted by the environment in which the microorganisms were isolated, leading to the acquisition of metabolic capabilities to survive and grow in polluted environments. Most studies have demonstrated that the use of microorganisms isolated from contaminated systems tend to function more effectively than using those not adapted to contaminants (Pingas, 2011). The capacity of *Streptomyces* strains to grow and degrade hydrocarbons was described in the literature as a common feature of the genus, although the studies were usually carried out on the determination of the degradation potential of *n*-alkanes or PAHs (Balachandran et al., 2012; Baoune et al., 2018; Barabás et al., 2001; Ferradji et al., 2014). To date, some actinobacteria including genera *Rhodococcus, Gordonia, Streptomyces* and *Amycolatopsis* have been shown to be predominantly PAHs degraders (Bourguignon et al., 2014; Isaac et al., 2013). Based on the chemical composition and the concentration of crude petroleum, the degrading effectiveness of *Streptomyces* strains has been reported from 50% to 99% (Balachandran et al., 2012; Baoune et al., 2018; Ferradji et al., 2014).

Microbial inoculation promotes soil enzyme activity, PAHs removal and plant growth. Endophytic bacteria may be of particular interest as bioinoculant since they have the advantage of proliferating within plant tissue thus facing less competition for nutrients and being protected from the high-stress environment of polluted soils (Sturz et al., 2000). Through roots and leaves, hydrocarbons can achieve vascular system and intercellular spaces, reducing plant growth or inducing plant mortality (Arellano et al., 2017). Despite the toxicity of hydrocarbons is a limiting factor for plants that cannot easily degrade them, some plants have tolerance mechanisms by which tolerate, immobilize and/or accumulate hydrocarbons in their different parts, and even degrade and eliminate them (Khan et al., 2013; Li et al., 2012). This response depends not only on the plants type but also on the concentration of hydrocarbons and the exposure time (Arellano et al., 2017). In the present study, maize plants were able to tolerate the concentrations of hydrocarbons used, and also contributed to dissipation of crude petroleum and PAHs. Moreover, significant degradation of crude petroleum and PAHs was observed in soil by inoculated plants since hydrocarbons dissipation was higher compared to non-inoculated plants and non-planted soils. It would be possible to hypothesize that the effect of *Streptomyces* sp. Hlh1 could be related to stimulation mechanisms to the plants, which excreted root exudates that attract rhizospheric microbes. Several works have demonstrated enhanced removal of organic contaminants in the rhizosphere because of the increase of microbial density and/or activity due to the release of plant root exudates (Becerra-Castro et al., 2013; Ying et al., 2011). For instance, Simón Solá et al. (2019)found that the addition of maize root exudates to the culture medium led to an important increase of *Streptomyces* strain biomass as well as a higher Cr(VI) and lindane dissipation.

According to Fatima et al. (2016), the persistence and the action of endophytes in the plant environment play a critical role to improve crude petroleum dissipation. Regarding this approach, maximum dissipation of hydrocarbons was achieved in inoculated and planted soil contaminated with 20 gkg^{-1} of crude petroleum. Similar results were informed by Andria et al. (2009), since the authors reported the use of 20 gkg^{-1} of diesel was the most efficient concentration for the microbial colonization of plants and degradation of hydrocarbons.

Streptomyces sp. Hlh1 was able to improve hydrocarbons degradation from crude petroleum in soil samples. The highest removal was observed in shorter chain hydrocarbons. This is in agreement with previous studies that showed that the shorter and intermediate hydrocarbons (C_{10}–C_{20}) are the most easily degradable, even if their higher solubility in water makes them more toxic (Liu et al., 2018). Moreover, the inoculated maize plants showed greater hydrocarbons degradation than the non-inoculated plants, indicating that *Streptomyces* sp. Hlh1 could be responsible of improving contaminants dissipation since the assays presented here were carried out under sterile conditions.

It is well known that in petroleum polluted soils, the reduction of plants development is due to toxic nature of hydrocarbons which reduce water and nutrient uptake (Kirk et al., 2005). A similar impact of other environmental pollutants such as heavy metals has been observed on maize plants (Polti et al., 2011). Crude petroleum and its by-products can reduce shoots and roots development which could be refer to the delay in cell expansion (Athar et al., 2016). According to Calvelo Pereira et al. (2010), the biomass distribution of plants grown in contaminated soil and also the physiological activity is determined not only by the level of soil contamination but also by the type of contaminant.

The results presented in this work strongly suggest that the inoculation of maize plants with the endophyte *Streptomyces* sp. Hlh1 reduced crude petroleum and PAHs by-products toxicity. In fact, *Streptomyces* sp. Hlh1 improve shoot and root length of maize plants, This could be refer to the production of PGP metabolites by *Streptomyces* sp. Hlh1 such as IAA and ACC deaminase activities, previously demonstrated for this strain (Baoune et al., 2018). It is well known that IAA and ACC deaminase contribute to the growth and development of plants under stress conditions. The ACC deaminase is a key enzyme responsible for reducing ethylene levels in plants and, therefore, promoting the growth and development of roots (Glick, 2014; Khan et al., 2013). According to Sheng et al. (2008), the endophytic *Enterobacter* sp. 12J1 could effectively remove pyrene and improve maize and wheat growth in pyrene contaminated soils. However, no significant differences were found in the fresh weight between inoculated and non-inoculated plants grown in presence of petroleum hydrocarbons.

Chlorophyll content of leaves is used as indicator of plants stress (Huang et al., 2004). In accordance with other studies, our results showed petroleum hydrocarbons caused significant reduction of chlorophyll and carotenoids contents in non-inoculated plants (Das and Kumar, 2016; Shabir et al., 2016). In opposite, plants inoculated with *Streptomyces* sp. Hlh1 presented higher levels of chlorophyll and carotenoids, make evident a better performance compared to non-inoculated plants.

The ratios *n*-C17/pristane and *n*-C18/phytane are usually used as index for the estimation of hydrocarbons biodegradation. Since *n*-C17 and *n*-C18 are easily degradable compounds and pristane and phytane are relatively less degradable compounds, these ratios are higher in the case of fresh inputs, whereas low ratios indicate significant degradation of petroleum hydrocarbons (Bajt, 2017; Rostami et al., 2019). In the present work, the ratios were lower in presence of *Streptomyces* sp. Hlh1, indicating higher biodegradation of crude petroleum.

To our knowledge, this study is the first to demonstrate the ability of an endophytic *Streptomyces* strain to remove petroleum hydrocarbons and improve plant growth in contaminated soils.

5. Concluding remarks

As summarized in this article, the data presents the evidence that *Zea mays* is able to remediate soil contaminated with petroleum hydrocarbons and its remediation ability is improved by the inoculation of the endophytic *Streptomyces* sp. Hlh1. Higher degradation level of both pure-PAHs and crude petroleum were observed in the inoculated plants

compared to non-inoculated ones. Similarly, better plant growth was demonstrated in inoculated plants grown in contaminated soils.

The data presented in this work provide evidence about the outstanding possibility to use maize plants inoculated with *Streptomyces* sp. Hlh1 as an important component of a phyto-management program of hydrocarbons contaminated soils. Further studies are needed to understand the microbial mechanisms involved for improve the plant performance and contaminants dissipation. In this context, the study of physico-chemical variations on soils along the phytoremediation process will be a complementary tool to better understand such mechanisms.

Conflicts of interest

The authors declare that they have no conflict of interest

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ecoenv.2019.109591.

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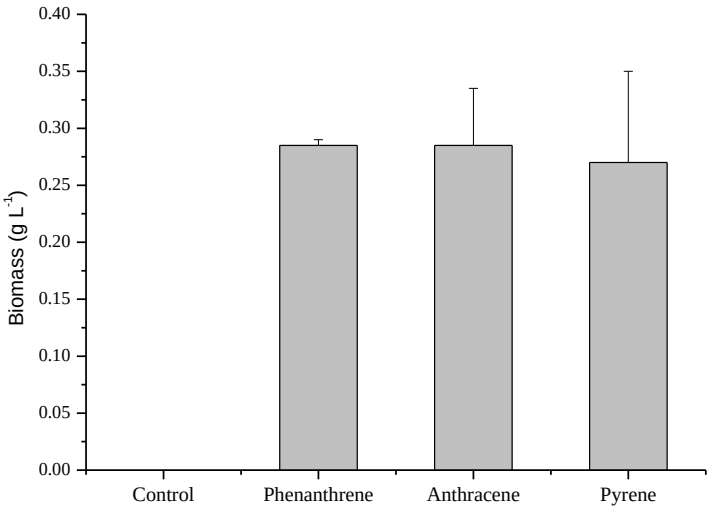
Chapitre III: "Effectiveness of the *Zea mays*-*Streptomyces* association for the phytoremediation of petroleum hydrocarbons impacted soils"



Supplementary Figure 1. Growth of *Streptomyces* sp. Hlh1 in liquid medium supplemented with pure PAHs, after 72 h at 30 ° (Baoune et *al*., 2019b).

Supplementary Table 1. Fresh, dry weight, and tolerance index of *Zea mays* plants grown in soils artificially contaminated with petroleum hydrocarbons. Values sharing the same letter were not significantly different (p<0.05, Fisher's post-test) (Baoune et *al*., 2019b).

	Plant fresh weight (g ± SD)		**Plant dry weight (g ± SD) and Tolerance Index (TI)**			
Contaminants	**Non-inoculated plants**	**Inoculated plants**	**Non-inoculated plants**	**TI**	**Inoculated plants**	**TI**
Crude petroleum	2.56 ± 0.31[a]	2.74 ± 0.23[a]	0.17 ± 0.04[a]	0.71	0.17 ± 0.03[a]	0.71
Phenanthrene	2.79 ± 0.34[a]	3.39 ± 0.83[a]	0.21 ± 0.00[a]	0.88	0.22 ± 0.04[a]	0.92
Pyrene	3.31 ± 1.37[a]	3.65± 0.95[a]	0.22 ± 0.06[a]	0.92	0.20 ± 0.08[a]	0.83
Anthracene	2.48 ± 0.90[a]	2.87 ± 2.09[a]	0.24 ± 0.14[a]	1	0.24 ± 0.14[a]	1
Control (non-contaminated soil)	2.98 ± 0.24[a]	3.31 ± 0.74[a]	0.24 ± 0.06[a]	-	0.25 ± 0.04[a]	-

Data of fresh and dry weight are means with standard deviation presented after the observed value.

Conclusion Générale et Perspectives

Conclusion Générale

À mesure que la population humaine continue à augmenter, il en sera de même pour la demande de nourriture, de carburants à base de plantes et de l'utilisation de stratégies de bioremédiation pour nettoyer l'environnement. L'association étroite entre les bactéries endophytes et leurs plantes hôtes, ainsi que les connaissances sur le fonctionnement des bactéries endophytes dans des environnements contaminés est supposée être explorée pour améliorer la phytoremédiation des sols contaminés par les hydrocarbures pétroliers. Par conséquent, cette étude est entreprise pour examiner le potentiel des actinobactéries endophytes pour leurs activités de dégradation des hydrocarbures et leur capacité à améliorer la croissance des plantes par le moyen de PGP dans des environnements pollués par les hydrocarbures pétroliers (pétrole brut léger et HAP).

Une première expérience préliminaire est réalisée pour évaluer la capacité des isolats endophytes à croître en présence des hydrocarbures volatils en les exposant au pétrole brut léger. Ces derniers ayant montré leur tolérance, sont testés pour leur pouvoir à dégrader le pétrole brut et à produire des métabolites favorisant la croissance des plantes (PGP). Les résultats obtenus ont montré que quatre isolats sont capables d'utiliser le pétrole comme source de carbone et d'énergie.

Le séquençage de l'ARNr 16S des souches hydrocarbonoclastes montre qu'elles appartiennent au genre *Streptomyces*, le genre le plus abondant dans le sol confirmant ainsi l'origine des endophytes présents dans les plantes. De plus, ces souches ont montré un résultat positif pour la production, d'au moins, un des métabolites promoteurs de la croissance des plantes (PGP).

Par ailleurs, la souche ayant présenté un bon profil de dégradation des hydrocarbures et de production des PGP est sélectionnée pour étudier sa capacité à remédier des sols contaminés artificiellement par le pétrole brut.

Parmi les souches hydrocarbonoclastes sélectionnées, *Streptomyces* sp. Hlh1 a montré une bonne dégradation des *n*-alcanes et des HAP à la fois, ainsi qu'un bon niveau de production de métabolites PGP.

L'inoculation de sols stériles ou non stériles contaminés par le pétrole brut a révélé une bonne dégradation des hydrocarbures pétroliers totaux par *Streptomyces* sp. Hlh1. Néanmoins, leur élimination des sols non stériles contaminés à faible concentration de pétrole brut a révélé une forte dégradation. Ceci pourrait être expliqué par l'activité synergique, à la fois de la souche inoculée et de la microflore naturelle du sol.

La laitue est utilisée comme un bio-modèle pour étudier la toxicité des métabolites de biodégradation. Elle s'est avérée être un bon indicateur pour l'étude de la toxicité des métabolites finaux.

Une phytoremédiation assistée biologiquement est réalisée en inoculant les graines de maïs (*Zea mays*) par *Streptomyces* sp. Hlh1 et en les semant dans des sols contaminés avec du pétrole léger ou des HAP. Le modèle choisi (*Zea mays*) s'est avéré efficace dans l'élimination des hydrocarbures étudiés même sans être inoculé. Cette élimination est d'autant plus importante lorsque les plantes de maïs sont associées à *Streptomyces* sp. Hlh1.Ces plantes voient leur croissance améliorée grâce à la présence de cette souche hydrocarbonoclaste. Ces résultats fournissent des preuves supplémentaires pour soutenir le fait que l'action combinée des partenaires appropriés plantes-bactéries peut être efficace pour traiter les sols pollués par les hydrocarbures.

Sur la base de nos résultats, on peut conclure que les plantes poussant dans des sols contaminés par les hydrocarbures présentent un réservoir de bactéries ayant la capacité de dégrader les hydrocarbures et favorisant la croissance des plantes. De plus, l'inoculation des espèces végétales par ces bactéries représente une alternative prometteuse pour éliminer et détoxifier les milieux contaminés. Par conséquent, l'interaction entre les plantes et les bactéries endophytes est un enjeu important à ne pas négliger pour améliorer les stratégies de phytoremédiation.

Perspectives

Les résultats présentés dans cette thèse, à partir des études réalisées à l'échelle du laboratoire, ont permis de mieux comprendre les processus de phytoremédiation dans les sols contaminés par les hydrocarbures. Cependant, plus de recherches sont nécessaires pour combler les lacunes de connaissances restantes. Plusieurs aspects doivent encore être étudiés pour assurer le succès de cette technologie.

Concernant l'isolement des actinobécteries endophytes avec les activités appropriées, des études explorant des techniques rapides sont à envisager pour isoler uniquement celles avec les activités ciblées sans passer à travers les essais de screening classiques et lents.

Quant à la bioremédiation des sols, il serait intéressant d'identifier et de quantifier les métabolites produits durant le processus de biodégradation des hydrocarbures. L'interaction entre la microflore native du sol et la souche introduite est aussi un paramètre à prendre en considération. Des travaux supplémentaires doivent être effectués pour établir le traitement *in situ*.

En référence à la phytoremédiation assistée biologiquement, il est recommandé de suivre la survie *Streptomyces* sp. Hlh1 le long de l'expérimentation et d’explorer les mécanismes par lesquels elle favoriserait la croissance du maïs.

General Conclusion and perspectives

General Conclusion

As the human population continues to increase, so the same for the food demand, fuels plant-based and the use of bioremediation strategies to clean-up the environment. The close association between endophytic bacteria and their host plant, as well as, the knowledge on the functioning of endophytic bacteria in contaminated environments were presumed to be explored to improve phytoremediation of petroleum hydrocarbons impacted soils. Therefore, this study was undertaken to investigate the potential of endophytic actinobacteria for their hydrocarbons degrading and plant growth promoting activities in contaminated environments with petroleum hydrocarbons (light crude petroleum and PAHs).

A first preliminary experiment was performed to assess the ability of endophytic isolates to grow in the presence of volatile hydrocarbons, by subjecting them to light crude petroleum oil. These latter shown a tolerance are tested for their ability to degrade crude oil and to produce plant growth promoting metabolites (PGP). The obtained results have shown that four isolates are able to use petroleum as sole carbon and energy source.

The sequence of the16S rRNA showed that they belong to *Streptomyces* genus. The most abundant genus in the soil, which confirm the origin of the endophytes presented in the plants. As well as, those strains showed a positive result toward the production of at least one plant growth promoting metabolites.

Moreover, the strain that showed a good degradation profile of hydrocarbons and PGP production was selected to study its ability to remediate artificially contaminated soil with crude petroleum oil.

Among selected hydrocabonclastic isolates, *Streptomyces* sp. Hlh1 demonstrated a good degradation for both *n*-alkanes and PAH, as well as, a good level of PGP metabolites production.

The inoculation of sterile and non-sterile contaminated soils with petroleum revealed a good degradation of total petroleum hydrocarbons by *Streptomyces* sp. Hlh1. However, their removal in contaminated non sterile soil at low concentration of petroleum revealed a strong degradation. This would be explained with the synergetic activity of both the inoculated strain and the natural microflora of the soil.

Lettuce was used as a bio-model to study the toxicity of the biodegradation metabolites. It seems to be a good indicator for the study of the toxicity of the final metabolites.

Biological assisted phytoremediation was carried out by inoculating maize seeds (*Zea mays*) with *Streptomyces* sp. Hlh1 and sowing them in contaminated soil with light crude oil or PAH. The chosen model (*Zea mays*) has been found to be effective in the removal of studied hydrocarbons even without being inoculated. This removal is as much as important when maize plants are associated with *Streptomyces* sp. Hlh1. The growth of these plants is improved due to the presence of this hydrocarbonclastic strain. These results provide supplementary evidences to support the fact that combined action of appropriate plant-bacteria partners could be efficient to treat hydrocarbon polluted soils.

On the basis of our results, we conclude that plants grown in hydrocarbon contaminated soils present a reservoir for bacteria having the ability to degrade hydrocarbons and to promote plant growth. As well as, the inoculation of plant species by these bacteria would represent a promising alternative to remove and detoxify contaminated environments. Therefore, the interaction between plants and endophytic bacteria is an important issue not to be neglected for improving phytoremediation strategies.

Perspectives

The results presented in this thesis, from the performed studies at laboratory scale have allowed to understand better the phytoremediation processes of hydrocarbons contaminated soils. However, more research is needed to fill the remaining knowledge gaps. Several sides still need to be studied to ensure the success of technology.

Concerning the isolation of endophytic actinobacteria with the appropriate activities, studies investigating the fast techniques to isolate only the targeted activities, without going through the classical and slow screening assays.

Regarding the bioremediation of soils, it would be of interest identifying and quantifying metabolites produced during the biodegradation of hydrocarbons. The interaction between the native soil microbiota and the introduced strain is also a parameter to be taken into consideration. Further work needs to be done to establish the treatment in situ.

With reference to biologically-assisted phytoremediation, it is recommended to monitor the survival of *Streptomyces* sp. Hlh1 throughout the experiment and to explore the mechanisms by which it would promote maize growth.

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Références

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