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EVALUATION OF PLANT GROWTH PROMOTING BACTERIA FOR INDUCING STRESS TOLERANCE IN PLANTS AGAINST PETROLEUM HYDROCARBONS

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Petroleum hydrocarbons are among the most common recalcitrant group of organic pollutants. Use of plants in association with microorganisms for degradation of petroleum hydrocarbons is an innovative and publically accepted green technology. Twenty seven bacterial isolates possessing bioremediation potential and ACC-deaminase activity were assessed for plant growth promotion with alfalfa, maize and canola. Out of 27 bacterial isolates 8 were further used in pot experiment with alfalfa, maize and canola grown on sand bearing 10000 mg kg^{-1} petroleum hydrocarbons contamination. Sixty days after sowing, the plants were harvested and various growth attributes such as plant height, root length and biomass production were recorded. The results revealed that bacterial inoculation induced stress tolerance in plants as compared to un-inoculated control. Bacterial inoculation compensated the reduction in growth by their plant growth promoting activity. It was observed that bacterial isolate PM32Y was the most efficient that caused 63%, 77% and 66% increase in root length of alfalfa, maize and canola, respectively as compared to un-inoculated control. The same bacterial isolate increased plant height by 59%, 63% and 59% of alfalfa, maize and canola, respectively. The study concluded that the bacterial inoculation induced stress tolerance in all three crops as compared to un-inoculated control and thereby converged use of plants and bacteria can be more successful tool for remediation of petroleum contaminated soil.

Keywords: Petroleum hydrocarbon, canola, inoculation, remediation

INTRODUCTION

Soil and water ecosystem are being jeopardized due to petroleum contamination which results from leakage from various above ground and underground sources, transportation of petroleum products and industrialization processes. Traditional physico-chemical remediation techniques are expensive and source of secondary contamination (Zhou and Song, 2004). An increased interest lies in the promotion of such environmental methods in cleaning up of petroleum contaminated sites which are less expensive and ecofriendly. Biological methods are inexpensive and do not require addition of chemicals to the ecosystem as compared to physiochemical methods (Oliveria *et al.*, 2015). Higher plants in combination with selected microorganisms offer a very ecofriendly and aesthetic alternative for cleanup of petroleum contamination. Degradation of petroleum hydrocarbons by biological means is ecofriendly because the oil is modified in such a way that end products are environmentally beneficial and friendly to all living things (Toledo *et al.*, 2006). Use of microorganisms for degradation or detoxification of xenobiotic compounds in the environment is called bioremediation (Bamforth and Singleton, 2005). Microorganisms used for the bioremediation are mostly indigenous or isolated from the contaminated sites and used somewhere else. Bioremediation is perceived by the people as an appealing strategy as it is a natural process (Bamforth and Singleton, 2005). However, the bioremediation is restricted due to some factors such as limitation of compounds that are biodegradable, survival and growth of microorganisms, supply of air and nutrient such as P and N etc. The alternate to bioremediation is phytoremediation that is *in situ* application of plants in association with naturally occurring microorganism for detoxification of pollutants. Phytoremediation is generally applied to sites contaminated with organic, nutrient or metal pollutants. Phytoremediation has earned splendid acceptance in the last decade as viable remediation technology (Arthur *et al.*, 2005). The phytoremediation has multifold benefits such as restriction of wind erosion, reinforcement of soil and above all is its aesthetic value (Smith *et al.*, 2006). Some considerations in the process of phytoremediation to be operative are tolerance of plants to elevated concentration of pollutants (Germida *et al.*, 2002), impaired plant growth due to stress phytohormone ethylene (Arshad *et al.*, 2007), less uptake by plants due to hydrophobic and lipophilic nature and higher molecular weight.

Bioremediation assisted by plants is possible strategy to overcome such constraints and thereby degradation of

recalcitrant soil contaminant like polyaromatic hydrocarbons may be enhanced (Huang *et al.*, 2004). This co-existence of plants and microorganisms is based on symbiotic relationship. Roots of higher plants provide molecular oxygen, inorganic nutrients through sloughed off cells, soluble exudates and lysates (Anderson *et al.*, 1993; Banks *et al.*, 2000). Improvement of soil aeration is caused by plant roots by directly giving off oxygen to the root zone as well as allowing improved entry of oxygen into the soil by diffusion along old root channels (Nye and Tinker, 1977). If microbes don't have degradative pathways for petroleum hydrocarbons and cannot use them as carbon source, even then petroleum hydrocarbon carbons can be mineralized through the process of co-metabolism (Dalton and Stirling, 1982). Plant enzymes degrade the compounds and microbes then end degradation by converting compounds into carbon dioxide and water. Some structural analogs of polyaromatic hydrocarbons such as phenols, terpernes and flavonoids are released by some plant roots as exudates and thus promote the growth of petroleum hydrocarbon degrading bacteria (Fletcher and Hedge, 1995) and can act as trigger of polycyclic aromatic hydrocarbons degradation-pathway (Singer *et al.*, 2003). Plant growth promoting bacteria (PGPB) with ACC-deaminase enzyme and bioremediation potential benefit plants by reducing biosynthesis of ethylene and by reducing concentration of petroleum hydrocarbon through degradation. The reduction of biosynthesis of ethylene carried out by ACC-deaminase containing bacteria by hydrolyzing immediate precursor of ethylene i.e 1-aminocyclopropane-1-carboxylate into α-ketobutyric acid and ammonia (Shaharoona *et al.*, 2006). These microorganisms with ACC-deaminase activity can compensate reduction in plant growth due to stress by improving plant growth. But, unfortunately, the isolation and subsequent inoculation with bacteria having ACC-deaminase activity does not fulfill the job of remediating the petroleum hydrocarbons contamination unless they are acclimated to petroleum contamination. The success of such bacteria depends on their dual nature of plant growth promotion and bioremediation potential (Glick, 2010). Hence, one of the aims of the study were to isolate bacteria possessing both ACC-deaminase activity and bioremediation potential, so that upon inoculation the survival of the bacteria may not be the limiting factor. Similarly, ability of plants to survive and grow on polluted site is also important factor for plant assisted bioremediation (Wenzel, 2009). The considerations for selection of plant species are tolerance to contaminants and other environmental conditions, production of large biomass, ability to grow on various soil types and native to the area (Germida *et al.*, 2002; Hutchinson *et al.*, 2003). Alfalfa has been used by various researchers in phytoremediation studies due to its perennial nature, fibrous root system and thereby increased surface area for microbiological activity, diffuse distribution all over the world and well adapted to different climatic conditions (Ouvrard *et al.*, 2011; Cook and Hesterberg, 2013; Zhang *et al.*, 2013). Maize on the other hand being annual crop has also been used by various researchers for phytoremediation of petroleum hydrocarbons (Adam and Duncan, 2002). Canola is well known hyperaccumulator and has extensively been used in the phytoremediation of soils contaminated with inorganic pollutants. Being a deep tap rooted crop, canola is suitable candidate for use in phytoremediation when the aim to extract contaminant from deeper zone of soil. Moreover, petroleum hydrocarbons being hydrophobic in nature pose water stress to growing plants in petroleum hydrocarbons contaminated soil. Canola requires less water as compared to maize and alfalfa and therefore may be a suitable candidate for use in phytoremediation of petroleum contaminated soils. This study was aimed at assessing the tolerance of alfalfa, maize and canola to petroleum hydrocarbons contamination under ambient conditions of light and temperature. Another aim of the study was to isolate bacteria possessing both ACC-deaminase activity and bioremediation potential and their compatibility with selected crops.

MATERIAL AND METHODS

***Isolation and characterization of bacterial isolates*:** For isolation of bacterial isolates soil samples contaminated with different products of petroleum hydrocarbons were collected from different ecological zones (Rice zone, Central mixed zone, Low rainfall zone, and High rainfall zone) of Punjab Pakistan. The reason for collection of oil contaminated samples from different ecological zone was to isolate bacteria from habitat with different climatic conditions as meteorological parameters such as temperature and moisture have severe effect on growth and survival of the bacteria. The soil samples were collected by using tube augar and depth of sampling was up to 90 cm. The soil samples were sealed in zipped plastic bag and immediately shifted to laboratory to avoid evaporation of oil contents. Soil samples with history of contamination with different petroleum products were collected. The rationale behind the isolation of bacteria from contaminated soil was that upon inoculation in the contaminated site they could better acclimatize. The collected soil samples were stored at $4^{O}C$ in dark prior to use for the isolation. Glucose peptone agar medium with modification by filter-sterilized petroleum hydrocarbons as sole carbon source was used for isolation and purification of bacterial isolates. Bacterial isolates were characterized for bioremediation potential, ACC-deaminase activity and plant growth promoting activity under axenic conditions (Asghar *et al.*, 2011).

***Plant growth promoting activity under axenic conditions*:** Screening on the basis of bioremediation potential and ACC-deaminase activity sorted out 27 bacterial isolates which

possess both high bioremediation potential and ACC-deaminase activity (Asghar *et al.*, 2011). Further step was to assess the plant growth promotion activity of these 27 bacterial isolates. For this purpose, growth promotion experiment under axenic condition was conducted in pre-sterilized growth pouches in growth room. DF salt minimal media (Dworkin and Foster, 1958) was used for preparation of broth culture. DF salt minimal media and flasks were sterilized at 120±1°C for 15 minutes and allowed to cool under laminar flow hood for half an hour. Erlenmeyer flasks each containing 100 mL of sterilized DF salt minimal media were inoculated with these 27 bacterial isolates to get broth culture of each isolate. After inoculation, flasks were incubated for 72 hours at 28±1°C in rotary shaking incubator at 100 rpm. A uniform cell density of 10^8 to 10^9 was maintained by recording optical density of 0.5 at 535 nm wavelength. This broth culture was used to inoculate seeds of alfalfa, maize and canola. Prior to inoculation, surface disinfection of seeds was carried out by dipping them in ethanol for few seconds and subsequently in $HgCl_2$ for two minutes. Four seeds per replicate of each crop were inoculated and placed in pre-sterilized growth pouches. The treatments were triplicated. For nutrition, 10 mL of half strength sterilized Hoagland's solution (Hoagland and Arnon, 1950) per pouch was used two times in a week and in remaining days of the week sterilized distilled water was used to keep the growth pouch moist to avoid dehydration of seedlings. Growth pouches were placed in growth room and temperature was adjusted at 28±1°C. After fifteen days of sowing, data regarding root and shoot length, total fresh biomass and oven-dried biomass were recorded.

***Root length by Delta T-Scanner*:** Roots length of alfalfa, canola and maize plants was measured by using Delta T-Scan (Delta-T Devices Ltd. Cambridge, England). The sand particles of rhizoplane were thoroughly washed to avoid interference in measurement and damage to delicate glass of scanner. Washed roots were placed on scanner and the roots were scanned to get the image of roots. The image was converted to monochrome. This image was then loaded to adobe photo shop of delta T-scanner and the image was made as bitmap image. The image was then loaded to delta T-scan and root length was measured in centimeter. The same procedure was repeated to get the length of root of each plant.

***Germination test*:** Coarse textured and 2 mm sieved sand was spiked with crude oil to attain a concentration of 1%, 2% and 3% (w/w). Ten viable seeds of maize, alfalfa and canola were sown in plastic container of diameter 13×6 cm where 13 cm was length of jar and 6 cm was internal diameter. The germination test was carried out in controlled light and temperature incubation room. Half strength Hoagland (Hoagland and Arnon, 1950) solution was used for nutrition. Germination was assessed by emergence of seedling. Results were recorded as percentage of total seeds sown.

***Plant performance in petroleum contaminated soil*:** Screening of 27 bacterial isolates on the basis of their plant growth promotion activity, bioremediation potential and ACC-deaminase activity yielded eight bacterial isolates coded as PM32Y, SFD2S2, WZ3S1, MZT72, JM44, SP104Y, SM73 and WZ3S3. These bacterial isolates were used further to observe effect of bacterial inoculation on the growth attributes of maize, alfalfa and canola in petroleum contaminated soil. For this purpose, a pot experiment was conducted under ambient conditions of light and temperature. The growth medium for plant was sand and nutrients were artificially supplied by the application of sterilized Hoagland solution (Hoagland and Arnon, 1950) two times in a week. The sand was contaminated with petroleum hydrocarbons to attain a concentration of 1% (10000 mg kg^{-1} w/w). Prior to contamination, the sand was sterilized to kill indigenous microorganisms by autoclaving three times at 120°C.

Seeds were inoculated with bacterial isolates prior to sowing. For inoculation, broth culture was prepared separately for each isolate. This broth was incubated for 72 hours in shaking incubator at 100 rpm at 28°C. The uniform cell density of 10^{-8} to 10^{-9} CFU g^{-1} was attained and used for inoculation of seeds. Seeds were surface sterilized by dipping them in 95% ethanol for few seconds and subsequently immersion in 0.2% $HgCl_2$ for two minutes. This was followed by several washings with sterilized distilled water to remove the disinfectants from seed surface. For inoculation of seeds, slurry was prepared by mixing sterilized peat, 5% sugar solution and broth culture. Seeds were dressed with this slurry and dried under laminar flow. Seeds were sown 2 cm deep and layered with non-contaminated standard sand to ensure maximum germination.

***Experimental design and layout*:** Completely randomized design was used with three replications of each treatment. The experiment comprised of ten treatments; two control and eight bacterial isolates. In treatment plan two controls were kept; non-contaminated and non-inoculated control and Phyto control, where only plants were grown without contamination and without inoculation. First control was kept to ensure that there was no growth suppression factor for plant other than petroleum hydrocarbons contamination and the Phyto control was set to segregate the response of plants with inoculation and without inoculation. Eight bacterial isolates were used to inoculate each crop with three replications to assess the effect of bacterial inoculation on growth attributes of plants in contaminated soil under ambient conditions of light and temperature.

RESULTS

***Plant growth performance under stress free axenic conditions*:** Bacterial isolates previously screened on the

basis of bioremediation potential, ACC deaminase activity (data not shown) were used to assess plant growth promotion of alfalfa, maize and canola grown in growth pouches under stress free axenic conditions in controlled light and temperature. The results regarding various growth attributes are described below.

***Root length*:** Bacterial inoculation significantly improved the growth of roots of all three inoculated crops alfalfa, maize and canola. Bacterial isolates PM32Y, SFD2S2, SFD5S3, WZ3S1, JM44, SFK2S2, WZ3S3 and WZR32S significantly increased root length of alfalfa (Table 1). Percent increase in root length as compared to Phyto control was 70%, 67%, 67% and 66% caused by WZR32S, PM32Y, WZ3S3 and SFD5S3, respectively. As shown in (Table 1) the effect of inoculation on maize was more pronounced as compared to alfalfa when grown under stress free conditions. Root elongation of maize was prominent in treatments receiving PM32Y, WZ3S1, WZ3S3, SFD2S2, SM112 and WZR32S inoculation which caused percent increase of 90%, 78%, 84%, 74%, 73% and 70%, respectively as compared to Phyto control. Bacterial isolates coded as MZT72, SP62W, JM44, SFK1S2, and SM73 caused significant increase in root length. The percent increase by these bacterial isolates was 68%, 66%, 65%, 62% and 61%, respectively. Canola roots showed maximum response to bacterial inoculation as compared to alfalfa and maize. As indicated by the data (Table 1), the most promising increase in root length of canola was caused by the inoculation of PM32Y which increase root of canola by 116% as compared to Phyto control revealing that the PM32Y was consistent in improving root length of all three crops. Other considerable increase of 82%, 81%, 79%, 68%, 63%, 63% and 62% in root length of canola was caused by bacterial isolates SFD2S2, WZ3S3, SFK2S2, WZR32S, SFK1S2, SM112 and SFD5S3, respectively. Compatible with other two crop alfalfa and maize, the bacterial isolate WZ3S1 failed to maintain its consistency in increasing root length of canola.

***Shoot length*:** Shoot elongation of alfalfa was greatly improved by bacterial isolate PM32Y which increased maximum shoot length among all the bacterial isolates. The PM32Y increased shoot length of alfalfa by 67% followed by an increase of 66% caused by SFD5S3 inoculation (Table 1). The PM32Y was statistically non-significant with WZ3S1, SFD2S2 and SFD9S4 which increased shoot length

Table 1. Plant growth promotion as affected by bacterial inoculation in growth pouches under axenic conditions

Treatment/ Parameter	Plant growth attributes											
	Maize				Alfalfa				Canola			
	Root Length	Shoot Length	Total Fresh weight	Total Dry weight	Root Length	Shoot Length	Total Fresh weight	Total Dry weight	Root Length	Shoot Length	Total Fresh weight	Total Dry weight
CONTROL	6.08L	9.13j	0.92j	0.34k	7.29k	4.90k	0.60L	0.27L	7.13L	8.20r	0.72m	0.30j
PM32Y	11.55a	16.40a	1.99a	0.84a	12.20a	8.20a	1.52a	0.73a	15.43a	16.00a	1.22a	0.60a
SFD2S2	10.58ad	16.00ab	1.86ab	0.85a	11.69ab	7.79ab	1.02de	0.59bc	13.00b	14.93b	1.10b	0.54b
SFD5S3	9.38ei	10.87gi	1.14eh	0.40fi	12.07a	8.15a	1.18b	0.61b	11.53cd	14.47bc	1.00c	0.48cd
WZ3S1	11.83ac	13.88c	1.72b	0.62b	11.75ab	7.82ab	0.93fh	0.55cd	9.23fj	12.33ei	1.06b	0.48cd
SFD1S1	8.20jk	11.38fg	1.02gj	0.34jk	10.53df	6.50eg	0.86hi	0.53df	9.83fh	10.93jm	0.95cd	0.44e
JM44	10.03cf	12.67d	1.38cd	0.48de	11.09bd	6.90de	0.86hi	0.52dg	9.96fg	12.70eg	1.00c	0.46de
SM14	8.68hk	10.70gi	1.03gj	0.34jk	10.24df	7.19be	0.88gi	0.54de	10.13ef	12.07fj	0.90de	0.40f
SFD9S4	8.24jk	10.11hj	1.16eg	0.39gi	10.29df	7.67ac	0.82ij	0.48fh	9.60fh	11.67gk	0.80il	0.38fg
MZT72	10.20bf	12.32df	1.35cd	0.49cd	10.31df	6.84de	1.01df	0.48fh	8.33ik	11.50hl	0.83fi	0.39f
GC23Y	7.70k	10.83gi	1.10ei	0.36ik	10.23df	5.81gj	0.84hi	0.44hi	8.50ik	11.23im	0.79il	0.36gh
SM31	9.57di	12.53de	1.14eh	0.44ef	10.21df	6.12fh	1.00df	0.56cd	12.40bc	14.70b	1.11b	0.56b
SST6S1	6.03L	10.07hj	0.96ij	0.34jk	9.10 gi	7.17be	0.73jk	0.29kl	8.360ik	10.93jm	0.75lm	0.38fg
FSD1S1	7.60k	11.43fg	1.18ef	0.36ik	9.04 hi	5.35ik	0.65kl	0.32k	11.03de	12.53eh	0.85eh	0.36gh
SFK2S2	9.60di	12.75d	1.36cd	0.62b	11.59ac	5.76hj	0.87hi	0.47gh	12.73b	11.43hl	0.86eg	0.36gh
SP54	7.77k	9.90hj	1.03fj	0.38hk	8.67 ij	5.15jk	0.64l	0.33k	8.230jk	10.47lo	0.77km	0.33hi
SM112	10.50ad	13.25cd	1.36cd	0.49cd	9.71 fh	6.74df	1.16bc	0.56cd	11.60cd	13.17df	1.06b	0.49c
SFK1S2	9.87cg	12.20df	1.24de	0.41fh	8.78 ij	6.72df	1.08cd	0.55cd	11.63cd	12.00fj	0.77jm	0.34hi
GCDS11	9.40ei	10.05hj	1.04fj	0.34jk	8.11jk	7.16be	0.68kl	0.33k	8.10kl	10.33lp	0.84fi	0.31ij
SP112	8.18jk	10.92gh	1.01hj	0.38gj	10.70ce	6.04fi	0.74jk	0.39ij	9.30fl	8.87qr	0.82gj	0.35gh
SP104Y	9.13fj	10.67gi	1.15eh	0.41fh	8.77ij	5.80gj	0.81ij	0.39j	9.23fj	10.13mp	0.74eh	0.39f
SST4S3	8.93gj	10.57gi	1.01hj	0.36ik	8.05jk	5.44hk	0.67kl	0.31kl	8.50ik	9.23pr	0.86eh	0.36gh
SP62W	10.06cf	13.10cd	1.15eg	0.42fg	8.62ij	5.85hk	0.82ij	0.33k	9.93fg	10.7kn	0.88ef	0.34hi
SM73	9.77ch	11.46ef	1.35cd	0.51cd	9.97eg	7.06ce	0.88hi	0.49eh	9.06gk	9.50oq	0.73de	0.49c
SST1S3	7.67k	9.83ij	0.97ij	0.36ik	8.78ij	6.10fh	0.67kl	0.29kl	8.20jk	9.20pr	0.98c	0.33hi
WZ3S3	11.20ab	16.02ab	1.49c	0.62b	12.18a	7.41bd	1.03de	0.48fh	12.87b	14.27bd	0.81hk	0.49c
SST5S2	8.63ik	11.48ef	1.06fj	0.34jk	8.97 hj	5.93gi	0.68kl	0.31kl	8.80hk	9.53nq	0.90e	0.33hj
WZR32S	10.33be	15.17b	1.36cd	0.52c	12.37 a	7.26bd	0.97eg	0.47h	12.00bd	13.47ce	0.98c	0.48cd

Means sharing the same letter (s) are statistically non-significant according to Duncan's multiple range test ($p<0.05$)

of alfalfa by 60%, 59% and 56%, respectively as compared to Phyto control. In case of maize, bacterial isolate PM32Y caused a maximum increase of 80% in shoot length. Bacterial isolates SFD2S2 and WZ3S3 also showed their compatibility with maize as each caused 75% increase in shoot length in growth pouch assay under axenic conditions. Likewise root length, shoot length of canola was most significantly increased by bacterial inoculation grown under stress free condition in growth pouches. Phyto control. Bacterial isolate PM32Y caused increase in shoot length of canola by 107% and showed consistency in improving plant growth with all three crops. This maximum increase was followed by other noticeable increase of 86%, 83%, 81%, 78% and 68% in shoot length caused by bacterial isolates SFD2S2, SM31, SFD5S3, WZ3S3 and WZR32S, respectively.

***Fresh biomass*:** In growth pouch assay under axenic conditions, alfalfa produced maximum fresh biomass in response to PM32Y inoculation (Table 1). This maximum increase in fresh biomass of alfalfa was 154% as compared to Phyto control. Bacterial isolates SFD5S3, SM112, SFK1S2, WZ3S3, SFD2S2, MZT72, SM31 and WZR32S also significantly increased biomass of alfalfa by 97%, 94%, 80%, 71%, 70%, 69%, 67% and 62%, respectively. Response of maize to bacterial inoculation regarding increase in biomass was also pronounced. The bacterial isolate PM32Y and SFD2S2 were the best among all the bacterial isolates in increasing biomass of maize as these increased 116% and 102% as compared to Phyto control (Table 1). Bacterial isolates WZ3S1, WZ3S3, JM44, SFK2S2, SM112, WZR32S, MZT72, and SM73 also caused noticeable increase of 87%, 62%, 50%, 48%, 48%, 48%, 47% and 46%, respectively in fresh biomass of maize. Bacterial isolate PM32Y maintained its consistancy in plant growth promotion with all three crops alfalfa, maize and canola as it caused maximun increase in fresh biomass of canola like alfalfa and maize. The PM32Y caused an increase of 69% in fresh biomass of canola followed by 54% increase resulted from the inoculation of SM31 as second best bacterial isolate regarding increase in fresh biomass of canola.

***Dry biomass*:** Maximum increase in oven dried biomass of alfalfa was observed in response to PM32Y bacterial inoculation. This increase was 171% over Phyto control (Table 1) Bacterial isolate SFD2S2 increased 151% oven dried biomass as compared to Phyto control in case of maize which was followed by increase of 148% over control caused by PM32Y. Bacterial isolates WZ3S1, WZ3S3, SFK2S2, WZR32S, SM73, SM112, MZT72 and JM44 were also effective as these bacterial isolates increased oven dried biomass of maize by 82%, 76%, 71%, 54%, 51%, 45%, 44%, 41%, respectively, as compared to Phyto control. Canola also showed great response to bacterial inoculation as out of 27 bacterial isolates, 11 bacterial isolates prominently increased oven dried biomass of canola comparative to Phyto control. These prominent bacterial isolates were PM32Y, SM31, SFD2S2, SM112 , SM73, WZ3S3, WZ3S1, WZR32S, SFD5S3, JM44 and SFD1S1 which increased oven dried biomass of canola over Phyto control by 101%, 87%, 81%, 64%, 64%, 64%, 62%, 62%, 53% and 47%, respectively. The bacterial isolate PM32Y was found to be the most efficient in increasing oven dried biomass. In short, bacterial isolates PM32Y, SFD2S2, WZ3S1, SM112, JM44, WZ3S3 and WZR32S were all consistent with all three crops alfalfa, maize and canola in increasing oven dried biomass comparative to Phyto control, however, the percent increase by these consistent bacterial isolates over Phyto control varied from crop to crop.

***Germination*:** Viable seeds of each crop alfalfa, maize and canola were sown in sand spiked with crude oil concentration of 0%, 0.5%, 1%, and 3% to assess germination potential of each crop in freshly spiked petroleum contaminated soil. At 0% contamination, there was 100% germination of all three crops indicating that there was no suppression factor other than petroleum hydrocarbon contamination. Crude oil concentration at level 0.5% did not severely affect germination of all three crops; however, at 1% and 3% concentration germination of canola was affected significantly as compared to maize and alfalfa. Germination of maize and alfalfa was statistically non-significant with each other at all three (0.5%, 1% and 3%) concentration. Emergence of alfalfa and maize seedling was considerable up to 3% concentration of crude oil. Both in alfalfa and maize seeds germination percentage was 90% (Fig. 1). However, germination of canola was severely affected at 3% crude oil concentration as less than 50% seed germination was observed.

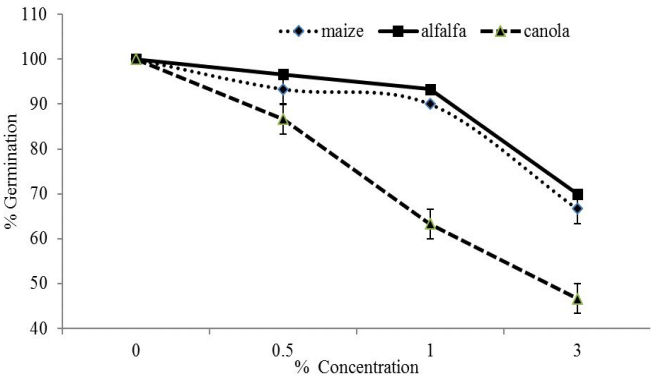

Figure 1. Germination percentage at different concentration of crude oil.

Plant growth performances in contaminated sand under ambient conditions of light and temperature:

***Root length*:** Bacterial isolate PM32Y maintained its consistency in increasing root length of alfalfa in wire house (ambient light and temperature) experiment as indicated by maximum increase of 63% over Phyto control. Other

noticeable increase in root length of alfalfa was observed in treatments receiving inoculation of bacterial isolates WZ3S3, SM73 and WZ3S1 which caused root elongation by 53%, 46% and 41%, respectively; as compared to Phyto control (Fig. 2a). As for as the root elongation of maize by the effect of bacterial inoculation in TPH contaminated soil under ambient light and temperature is concerned, bacterial isolates showed significant effect in improving root length of maize. Maximum increase of 77% in root length was observed in the treatment receiving inoculation of PM32Y (Fig. 2c) followed by 74% increase caused by the isolate JM44. However, increase in root length by SFD2S2, WZ3S1, MZT72, SM73 and WZ3S3 could not be neglected as these isolates caused considerable increase of 59%, 50%, 42%, 41% and 38%, respectively, as compared to Phyto control. Similarly, root length of canola was also increased by bacterial inoculation significantly in wire house experiment. Maximum 66% increase over Phyto control was observed in response to inoculation of bacterial isolate PM32Y followed by 57% increase caused by bacterial inoculation WZ3S3 (Fig. 2b).

Shoot length: Bacterial isolates PM32Y, WZ3S3, SM73 and WZ3S1 were prominent with respect to increase in shoot length of alfalfa under ambient light and temperature. Inoculation with these bacterial isolates caused 59%, 52%, 41% and 37% increase, respectively. Bacterial isolates SFD2S2, WZ3S1, JM44 and MZT72 also significantly increased shoot length of alfalfa as compared to Phyto control (Fig. 3a). Response of maize with respect to increase in shoot length to bacterial inoculation was significant. The PM32Y was most efficient as it increased maximum shoot length (Fig. 3c). Maximum 63% increase in shoot length caused by inoculation with PM32Y which was followed by 33% increase with inoculation of SFD2S2 and JM44. In case of canola, maximum 59% increase was observed in treatment receiving inoculation of PM32Y (Fig. 3b). Bacterial isolate WZ3S3 increased 51% shoot length of canola as second highest increase.

***Fresh biomass*:** Maximum 67% increase in fresh biomass of alfalfa was noticed in response to inoculation of bacterial isolate PM32Y as compared to Phyto control. Bacterial isolates WZ3S3, SM73 and WZ3S1 were also prominent and caused 58%, 44% and 39% increase in fresh biomass of alfalfa as compared to Phyto control (Fig. 4a). Maize also showed positive response to bacterial inoculation as maximum 64% increase in fresh biomass as compared to Phyto control was observed in response to inoculation of bacterial isolate PM32Y (Fig. 4c).

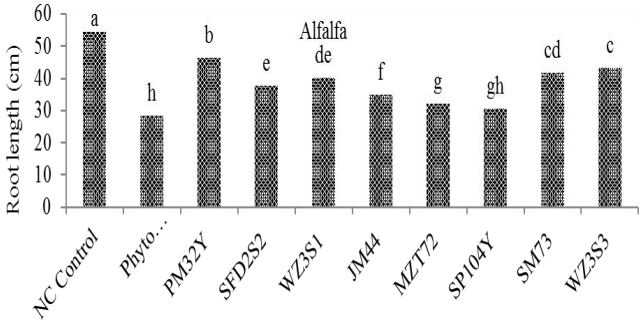

Figure 2a. Effect of bacterial inoculation on root length of alfalfa grown in petroleum contaminated soil under ambient conditions of light and temperature. Bars sharing the same letter (s) are statistically non-significant according to Duncan's multiple range test ($p<0.05$).

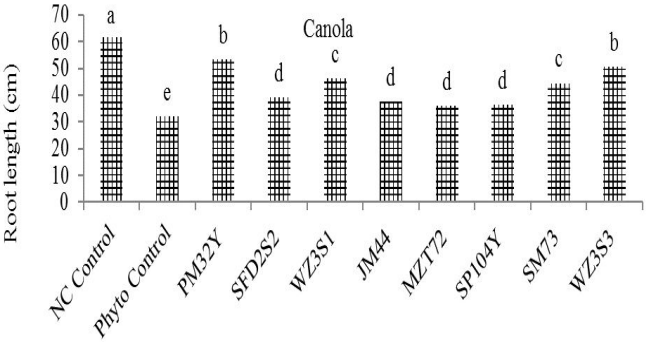

Figure 2b. Effect of bacterial inoculation on root length of canola grown in petroleum contaminated soil under ambient conditions of light and temperature. Bars sharing the same letter (s) are statistically non-significant according to Duncan's multiple range test ($p<0.05$).

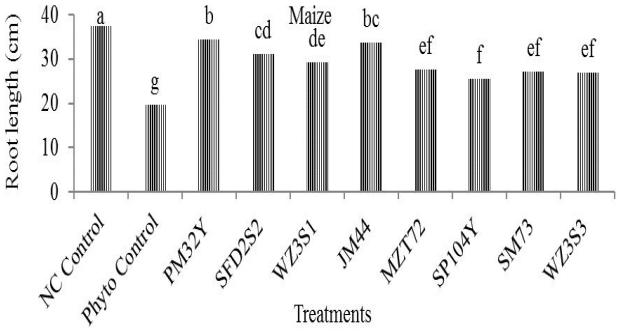

Figure 2c. Effect of bacterial inoculation on root length of maize grown in petroleum contaminated soil under ambient conditions of light and temperature. Bars sharing the same letter (s) are statistically non-significant according to Duncan's multiple range test ($p<0.05$).

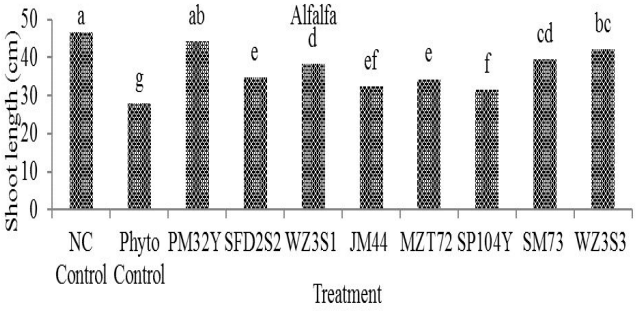

Figure 3a. Effect of bacterial inoculation on shoot length of alfalfa grown in petroleum contaminated soil under ambient conditions of light and temperature. Bars sharing the same letter (s) are statistically non-significant according to Duncan's multiple range test ($p<0.05$).

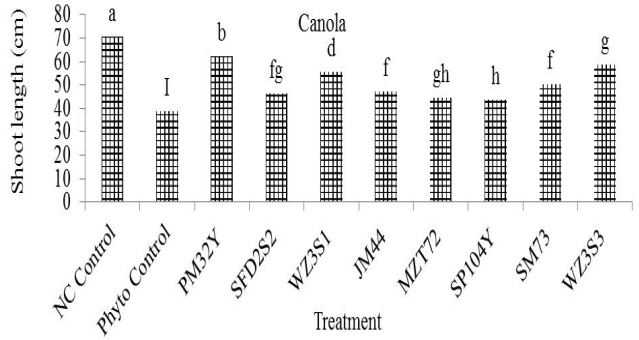

Figure 3b. Effect of bacterial inoculation on shoot length of canola grown in petroleum contaminated soil under ambient conditions of light and temperature. Bars sharing the same letter (s) are statistically non-significant according to Duncan's multiple range test ($p<0.05$).

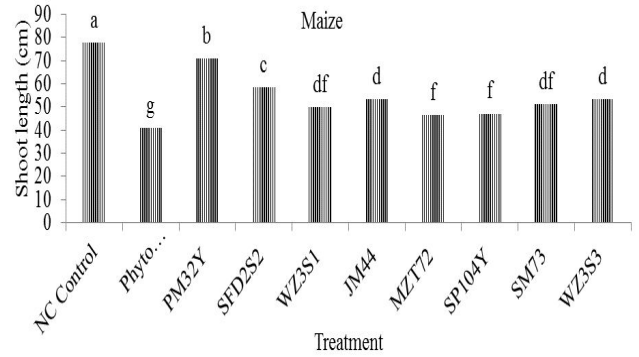

Figure 3c. Effect of bacterial inoculation on shoot length of maize grown in petroleum contaminated soil under ambient conditions of light and temperature. Bars sharing the same letter (s) are statistically non-significant according to Duncan's multiple range test ($p<0.05$).

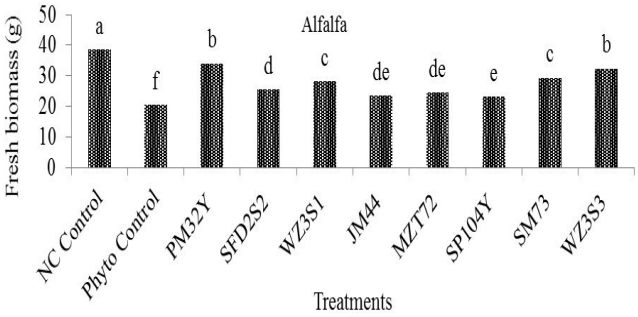

Figure 4a. Effect of bacterial inoculation on fresh biomass of alfalfa grown in petroleum contaminated soil under ambient conditions of light and temperature. Bars sharing the same letter (s) are statistically non-significant according to Duncan's multiple range test ($p<0.05$).

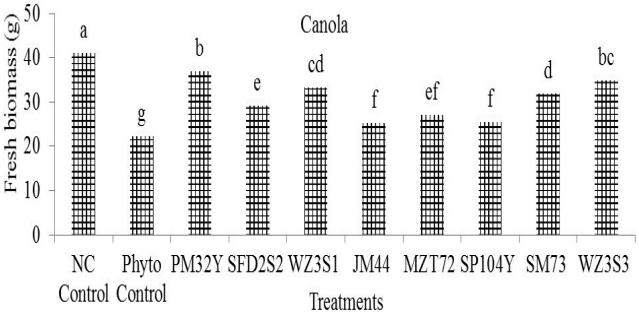

Figure 4b. Effect of bacterial inoculation on fresh biomass of canola grown in petroleum contaminated soil under ambient conditions of light and temperature. Bars sharing the same letter (s) are statistically non-significant according to Duncan's multiple range test ($p<0.05$).

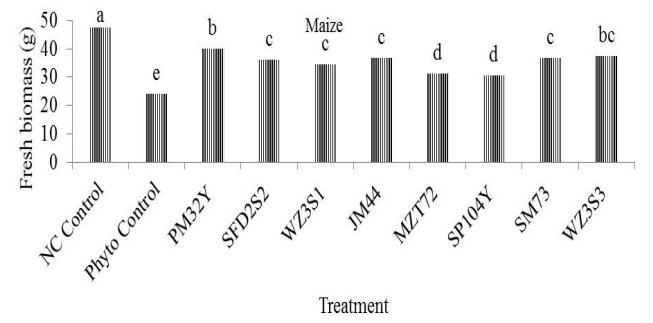

Figure 4c. Effect of bacterial inoculation on fresh biomass of maize grown in petroleum contaminated soil under ambient conditions of light and temperature. Bars sharing the same letter (s) are statistically non-significant according to Duncan's multiple range test ($p<0.05$).

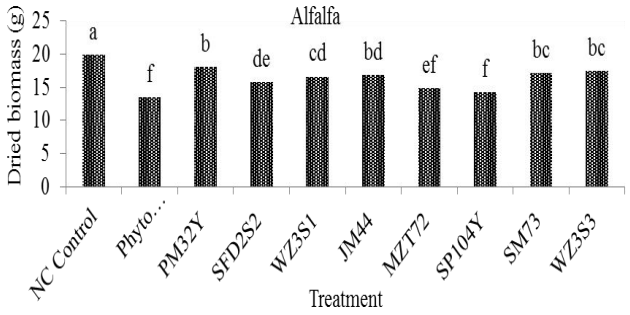

Figure 5a. Effect of bacterial inoculation on dried biomass of alfalfa grown in petroleum contaminated soil under ambient conditions of light and temperature. Bars sharing the same letter (s) are statistically non-significant according to Duncan's multiple range test ($p<0.05$).

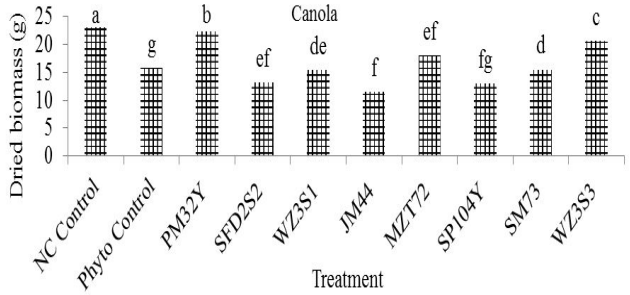

Figure 5b. Effect of bacterial inoculation on dried biomass of canola grown in petroleum contaminated soil under ambient conditions of light and temperature. Bars sharing the same letter (s) are statistically non-significant according to Duncan's multiple range test ($p<0.05$).

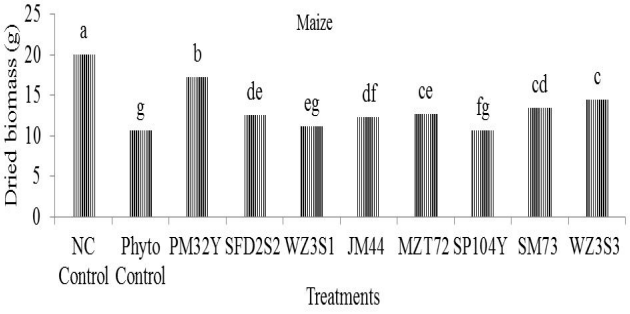

Figure 5c. Effect of bacterial inoculation on dried biomass of canola grown in petroleum contaminated soil under ambient conditions of light and temperature. Bars sharing the same letter (s) are statistically non-significant according to Duncan's multiple range test ($p<0.05$).

Among rest of the bacterial isolates, WZ3S3, JM44, WZ3S1 and SM73 were also found efficient in enhancing biomass of maize in contaminated soil. Fresh biomass of canola was maximally increased with inoculation of bacterial isolate PM32Y which caused 66% increase as compared to Phyto control (Fig. 4b). The bacterial isolate WZ3S3, WZ3S1 and SM73 were also efficient because they caused 57%, 49% and 44% increase in fresh biomass of canola, respectively, as compared to Phyto control.

***Dry biomass*:** Inoculation with PM32Y most significantly improved oven dried biomass of alfalfa under ambient light and temperature. This increase was 63% over Phyto control (Fig. 5a). Other bacterial isolate which effectively increased oven dried biomass of alfalfa were WZ3S3 and SM73 with 51% and 32% increase, respectively. Bacterial isolate PM32Y increased 93% oven dried biomass as compared to Phyto control in case of maize which was followed by 61% increase caused by inoculation with WZ3S3 as compared to Phyto control (Fig. 5c). Bacterial isolates SM73, MZT72 and SFD2S2 were also effective in increasing oven dried biomass of maize as they caused 51%, 43% and 42% increase, respectively, over Phyto control. Canola also showed great response to bacterial inoculation as out of 8 bacterial isolates, 4 bacterial isolates PM32Y, WZ3S3, SM73 and SFD2S2 significantly increased oven dried biomass of canola as compared to Phyto control (Fig. 5b) These bacterial isolates caused 85%, 63%, 63% and 43% increase, respectively.

DISCUSSION

The most important issue in plant assisted bioremediation is the isolation of petroleum degrading bacteria and selection of plants that are able to grow and survive in petroleum contaminated soil. Bacteria with bioremediation potential and ACC-deaminase activity were supposed to be ideal candidates because of their role in reducing stress induced ethylene and consequently help plant to survive and grow under stressed conditions (Arshad *et al.*, 2007). Tolerance of plants to specific contaminant is the most important step for establishing plant assisted bioremediation (Bona *et al.*, 2011) Once a suitable plant candidate is established in contaminated soil, then degradation process is enhanced by microorganisms that are benefited by plants by provision of nutrients (Hong *et al.*, 2011). Bacteria with high ACC metabolism (OD> 0.75) were assessed for their growth promotion activity for alfalfa, maize and canola to screen out the best strains (ACC metabolism data not shown). Among 27 bacterial isolates, PM32Y proved best strains as this bacterial isolate was consistent with all three crops (alfalfa, maize and canola). The plant growth promotion under stress free axenic conditions (growth pouch assay) may be attributed to ACC-deaminase activity of inoculated bacteria as this trait of bacteria modulate plant growth promotion by altering level of plant hormones such as ethylene, auxin and cytokinin

(Glick, 2012). Similarly, Sessitsch *et al.* (2012) reported that bacteria with ACC-deaminase activity possess plant growth promoting characteristics. Many researchers reported increased growth of plants under stress free conditions by bacteria containing ACC deaminase activity (Belimov *et al.*, 2002 and Ghosh *et al.*, 2003). Growth and development of plant is modified if any factor that result in the change of endogenous level of ethylene of plant (Arshad and Frankenberger, 2002). Hence, inoculation of seeds with ACC-deaminase containing bacteria may have suppressed endogenous level of ethylene and thus resulted in physiological response. Germination of seeds in petroleum contaminated soil is very important and exposition of seeds to petroleum contamination surely reduces the germination of seeds. Sharifi *et al.* (2007) proposed that reduction in germination of seed in petroleum contaminated soil may be due to the formation of biofilm which results in change of physiological process inside seed. The germination, growth performance in contaminated sand revealed that canola is not suitable candidate to be used in phytoremediation of organic contaminant like petroleum hydrocarbons as compared to maize and alfalfa. The reason for this unsuitability may be that canola is tap rooted crop and its surface area of root is less as compared to that of maize and alfalfa which means that less microbiological activity (White *et al.*, 2006). Being hydrophobic in nature, phytoextraction and hyperaccumulation of petroleum hydrocarbons by plants is very difficult and main driving force for the degradation of petroleum hydrocarbon is microorganism living in the rhizosphere or within the roots as endophytic bacteria. Plant growth was significantly improved as compared to Phyto control of each plant revealing that the petroleum contamination suppressed plant growth both under natural conditions of light and temperature. This suppression in plant growth may be overcome in inoculated plants due to alleviation or reducing of stress ethylene. Hong *et al.* (2011) inoculated maize (*Zea mays* L.) with bacteria possessing ACC-deaminase activity and observed significant improvement in growth attributes of maize such as stem, number of leaves, fresh biomass and root biomass as compared to Phyto control. Authors concluded that improved plant growth was due to the effect of inoculation with bacteria containing ACC-deaminase activity and resultantly increased biodegradation of diesel. Similarly, Bisht *et al.* (2014) studied the plant assisted bioremediation of PAHs by *Bacillus* sp. SBER3 possessing IAA, siderophore production and ACC-deaminase activity and observed 83% reduction of PAHs and authors found increased shoot length, root length and biomass of inoculated plants.

***Conclusion*:** The conclusion from the study can be drawn that bacteria isolated from petroleum hydrocarbon contaminated sites having ACC-deaminase activity and bioremediation potential can be beneficial to plants in improving plant growth promotion and can increase tolerance of plants to contaminants. Alfalfa is the most suitable crop as compared to maize and canola to be used in phytoremediation of organic pollutant such as petroleum hydrocarbons contaminated soils.

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