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Enhanced lindane degradation in the presence of root exudates of *Acorus calamus* and plant growth promoting rhizobacteria *Ochrobactrum* sp. strain A15

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Abstract

Lindane is a toxic and bioaccumulative organochlorine pesticide. Its extensive use resulted in environmental contamination and need for bioremediation. In the present study bacterium identified as *Ochrobactrum* sp. strain A15 exhibited highest percentage degradation (83.7% of 10 mg L^{-1} lindane) within 15 days. Lindane biodegradation efficiency of this strain increased by 10.7% in the presence of root exudates of *Acorus calamus*. Also, the average lindane biodegradation rate increased and its half-life decreased, when supplemented with root exudates. *Ochrobactrum* sp. A15 showed indole acetic acid production, hydrogen cyanide production, ammonia production, phosphate solubilization and 1-aminocyclopropane-1-carboxylate deaminase activity. Presence of pesticide had a negative effect on hydrogen cyanide production ability and decrease in indole acetic acid production and phosphorus production. No effect on 1-aminocyclopropane-1-carboxylate deaminase activity and ammonia production activity by bacteria was observed in the presence of lindane. Therefore, the combination of *A. calamus* and *Ochrobactrum* sp. strain A15 has the intrinsic capability to remove lindane and to support plant growth in the existence of lindane and this combination can be exploited for bioremediation of lindane.

Key words: *Acorus calamus*, lindane, *Ochrobactrum* sp., root exudates.
Abbreviations: ACC, 1-aminocyclopropane-1-carboxylic acid; HCN, hydrogen cyanide; IAA, indole acetic acid; MSM, Mineral Salt Medium.

Introduction

Lindane (γ-hexachlorocyclohexane) is a toxic and highly bioaccumulative organochlorine pesticide. It has been used since the Second World War and until the 1990-ies extensively on crops of vegetables, fruits, and grains as well as in storage rooms to control insect-borne disease, making this compound a standout amongst the possible identified organochlorine contaminants on the earth (Chang et al. 2011; Dominguez et al. 2018). Lindane has been identified in water in a wide range from ng L^{-1} in regular water to mg L^{-1} in water contaminated by washing of lindane residues from stockpiles or by the suspension of its hydrophobic contaminants (Sarkar 2016). Lindane is ecologically persistent and resistant to degradation, owing to its lipophilicity, high stability and chlorinated nature (Singh, Singh 2017). It presents potential health risks to animals and humans, causing symptoms such as headaches, skin irritation, diarrhea, dizziness, convulsions, nausea, vomiting (Heusinkveld et al. 2010; Mudawal et al. 2018). International Agency for Research on Cancer, World Health Organization and the Environmental Protection Agency have classified lindane as potential carcinogen, neurotoxin and teratogen, bringing about its incorporation to the list of persistent organic pollutants in the Stockholm Convention in 2009 (Khan et al. 2016; Rodríguez et al. 2018). Therefore, the advancement of a suitable technique for the removal of this pollutant has created a need for more research.

Phytoremediation methods are focused on consolidated utilization of plants and microorganisms to improve efficiency of remediation of lindane in contaminated environments (Álvarez et al. 2015; Salam et al. 2017; Simon et al. 2017; Singh, Singh 2017). Plant root exudates contain amino acids, sugars, nucleotides, organic acids, phenolic compounds, flavonones, and certain enzymes (Chaudhry et al. 2005). This combination of substrates offer a nutrient rich environment for degraders of contaminants and is able to potentially improve biodegradation by enhancing pollutant bioavailability, facilitating the co-metabolic transformation of contaminants and/or inducing catabolic enzymes involved in contaminant degradation (Álvarez et al. 2012).

Studies have confirmed that plant species that are most effective in increasing microbial amounts in their rhizosphere as well as in amplifying degradation are those with intense fibrous root systems (Fang et al. 2001; Patel, Patra 2017). *Acorus calamus* (sweet flag) is a wetland plant that possesses a high rate of development, strong

root system, high biomass production and has been very efficient in the degradation of xenobiotics, making it a good candidate for phytoremediation (Marecik et al. 2015; Chandra, Kumar 2015; Singh, Singh 2018a).

In plant-microbial associations, the plant provides nutrients and habitat to the associated microorganisms. The microorganisms improve ability to tolerate stress and increase development of plants, and degrades the contaminants, thus detoxifying the environment (Afzal et al. 2014). Making use of this plant-bacterial association usually depends on the bacterial ability to promote growth of plants under stressed conditions and/or to modify contaminant bioavailability or to degrade them. A major aspect for successful phytoremediation is good plant development in conditions of presence of poisonous contaminants (Becerra-Castro et al. 2011).

The present study aimed to isolate and identify root surface bacterium of *Acorus calamus* capable of degrading lindane, to test the effect of root exudates on lindane biodegradation, to assess plant growth promoting activity of isolated bacteria and to study the effect of lindane on these activities. To our knowledge, this is the first study focusing on isolation of plant growth promoting rhizobacteria from *A. calamus*, which has potential in lindane degradation and shows better lindane degradation in the presence of root exudates.

Materials and methods

Chemicals

Lindane (γ-hexachlorocyclohexane; >99% pure) was acquired from Sigma Aldrich (USA). The other chemicals were purchased from customary firms and were of analytical grade. For experiments on bacterial isolation and biodegradation, Mineral Salt Medium (MSM) was used, which contained: $(NH_4)_2HPO4$, 0.5; $MgSO_4$ $7H_2O$, 0.2; K_2HPO_4, 0.1; $Ca(NO_3)_2$, 0.01; $FeSO_4$ $7H_2O$, 0.01 (grams per liter); pH 7.0. Luria Bertani medium was used for growth of isolated bacteria, containing: tryptone, 10; yeast extract, 5; NaCl, 5; glucose, 1 (grams per liter); pH 7.0. Hoagland solution was used to cultivate plants for root exudates collection. It was prepared using Hoagland No. 2 Basal Salt Mixture (Himedia) according to the manufacturer's directions. Media were sterilized by autoclaving at 121 °C for 20 min.

Sampling

Acorus calamus whole plant and roots (excised) were brought from wetland mesocosms sustained at the Indian Agricultural Research Institute, New Delhi (28°38'21.3" N and 77°08'56.5" E). The microcosms were swamped by sewage discharged from sewers receiving domestic and industrial rubbish discharge from the campus and housing units near the region of campus.

For root exudate collection, about one-month old plants were removed from the mesocosms. For isolation of root epiphytic bacteria, root pieces of 12.5 cm were sliced from the plants. These were collected in sterilized plastic pouches, carried to lab in ice boxes maintained at 7 °C and utilized within 6 h.

Isolation and identification of lindane degrading root epiphytic bacteria

For bacterial isolation, roots were shaken in 0.01 M phosphate buffer saline for 1 h. Isolation was done by regular enrichment method, following the protocol described in our previous work (Singh, Singh 2019a). Bacterial suspension (phosphate buffer saline) was inoculated in MSM containing 10 mg L^{-1} lindane and incubated on a shaker at 150 rpm and 30 °C. Every week, 5 mL enriched culture was inoculated to 45 mL new sterilized medium containing lindane (10 mg L^{-1}) and incubated in the above conditions. After five cycles of enrichment, spread plates and streaking technique were utilized to obtain pure isolates. The lindane biodegradation experiment was carried out in MSM with 10 mg L^{-1} lindane. Controls flasks spiked with lindane were not inoculated. The degradation experiment was conducted on a laboratory shaker for 15 days at 30 °C and 150 rpm. To estimate lindane biodegradation, aliqouts were collected at frequent intervals and lindane was extracted by ethyl acetate.

Lindane was determined using an Agilent 7890A gas liquid chromatograph as described in our previous work (Singh, Singh 2019b). The oven temperature was automated for an initial temperature of 150 °C, held for 2 min, ramped at a rate of 5 °C per min till 180 °C, held for 3 min and finally ramped at a rate of 10 °C per min to 210 °C and held at 210 °C for 5 min. The injector and interface temperature were 220 °C and 250 °C, respectively. Nitrogen was used as a carrier gas. A sample was injected in split mode (pulse pressure = 0.84 kg cm^{-2}, split ratio 100:1 and purge flow 3 mL min^{-1}). Peak area and retention time (Rt) were used to determine lindane degradation.

16S rRNA gene amplification was performed for the bacterial strain exhibiting the highest percentage of lindane degradation and sequencing was outsourced from SciGenome Labs, India. The obtained sequence was submitted to GenBank under accession number MF109973. Further, to identify the bacterial strain, BLAST was performed (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Collection and analysis of root exudates

To collect root exudates, *A. calamus* was grown in Hoagland solution. For this, plants obtained from mesocosms were placed in Erlenmeyer flasks covered by aluminum foil from the sides and bottom to avoid sunlight. Hydroponic containers were filled with Hoagland's solution (half strength). The solution was substituted every day with sterile water in morning and again new Hoagland solution in the evening, for 15 days. The water (exudates in water)

collected in the evening was utilized as a source of root exudates. It was collected every day and kept at 4 °C. The solution was lyophilized, redissolved in distilled water, filter sterilized and used for further analysis.

pH of root exudate solution was measured using pH tester. Total carbohydrate concentration in root exudates was estimated using the sulfuric acid-UV method (Albalasmeh et al. 2013). Determination of protein concentration in root exudates was done by Bradford assay (Bradford 1976).

To determine concentration of phenolic compounds in root exudates, a 1 mL sample was mixed with 9 mL distilled water in a test tube. To this 0.5 mL of Folin–Ciocalteu reagent (Singleton et al. 1999) was added. The test tube was shaken and kept for 5 min at room temperature, and then 5 mL of 0.5% Na_2CO_3 and 9.5 mL of distilled water were added, mixed well and incubated for 1 h at room temperature. UV absorbance was read at 750 nm. The standard curve was plotted using gallic acid.

Lindane biodegradation in the presence of root exudates

Study of lindane biodegradation with addition of root exudates was conducted as described above. Two sets, treatment and control, were carried out. Each contained MSM with root exudates at concentration 1 g L^{-1} and 10 mg L^{-1} lindane. Experimental flasks were inoculated with bacteria. The control was not inoculated. Lindane residual analysis was performed using gas liquid chromatography.

Degradation kinetics

Average rate of lindane biodegradation in the presence and absence of root exudates was estimated by equation:

$$r_{av}(d^{-1}) = C_0 - C_t / (\Delta t),$$

where r_{av} is an average rate of degradation, C_0 and C_t represent lindane concentration at time 0 and time t, respectively, Δt is a length of biodegradation period.

Lindane degradation kinetics was fitted using the first-order kinetic equation:

$$C_t = C_0 e^{-kt}.$$

Lindane biodegradation half-life ($t_{1/2}$) was computed by:

$$t_{1/2} = \ln 2 / k.$$

Plant growth promoting activity of lindane degrading bacteria

The ability of bacteria to generate indole-acetic acid (IAA) was determined by the method described by Glickmann, Dessaux (1995) and Pattern, Glick (2002). Nutrient broth with tryptophan (1 mg mL^{-1}) was inoculated in culture of bacteria grown overnight and kept at 30 °C for 1 day. The obtained cultures were subjected to centrifugation, mixed with Salkowski's reagent and kept at room temperature (Gordon, Weber 1951). The optical density was calculated at 535 nm. IAA was estimated using a standard graph for pure IAA. To determine the effect of lindane on IAA by *Ochrobactrum* sp. A15, nutrient broth (tryptophan 1 mg mL^{-1}) was added to lindane (10 mg L^{-1}).

Ammonia (NH_3) production was determined in peptone water using the protocol given by Cappuccino, Sherman (1992). A positive result was indicated by the development of yellow to dark brown colour. To determine the effect of lindane on ammonia production by bacterium, peptone water was added to lindane (10 mg L^{-1}).

A bacterial strain was tested for production of hydrogen cyanide (HCN). Culture was inoculated on nutrient agar plates (supplemented by 4.4 g L^{-1} glycine; Lorck 1948). Whatman No. 1 filter paper was soaked in 2% Na_2CO_3 in 0.5% picric acid (picrate solution) and positioned in the lid of a Petri plate. Cultures were incubated at 30 °C for 4 to 5 days. A positive result was confirmed by change of colour of paper from yellow to reddish brown. To study the consequence of lindane on HCN production, lindane (10 mg L^{-1}) was added to Nutrient Agar plates.

A bacterial strain was tested for phosphorus solubilization activity. An aliquot of 10 µL overnight cultured bacterium was inoculated as a spot on Pikovskaya agar medium plates (Pikovskaya 1948). Development of a clear region sorrounding the bacteria showed the solubilization of organic/inorganic phosphates. Quantitative analysis of phosphorus solubilization was performed according to the protocol of King (1932) using a standard graph for KH_2PO_4. To determine the effect of lindane on phosphorus solubilization, lindane (10 mg L^{-1}) was added to Pikovskaya agar medium.

The 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase assay was performed for *Ochrobactrum* sp. A15 as described by Penrose, Glick (2003). To determine the effect of lindane on ACC deaminase activity, lindane (10 mg L^{-1}) was added to Dworkin and Foster minimal medium.

The siderophore production test was performed following the protocol of Alexander, Zuberer (1991). Presence of orange halos surrounding the colonies indicated a positive result.

Statistical analysis

Analysis of variance (ANOVA) was used to evaluate significant differences between mean of triplicates, using Graphpad Prism 7 software. *P* values less than 0.05 were regarded as significant.

Results

Lindane biodegradation and identification of bacterium

Among all the isolated strains, A15 showed the highest percentage degradation, 83.7 ± 1.11% of 10 mg L^{-1} lindane within 15 days. In non-inoculated flasks (control), pesticide degradation was 5.09 ± 0.16% (Fig. 1). The bacterium was identified as *Ochrobactrum* sp. strain A15 by the BLAST outcome of the 16S rRNA gene sequence (GenBank accession number MF109973).

Lindane biodegradation by *Ochrobactrum* sp. A15 was simultaneous with the bacterial growth in culture media

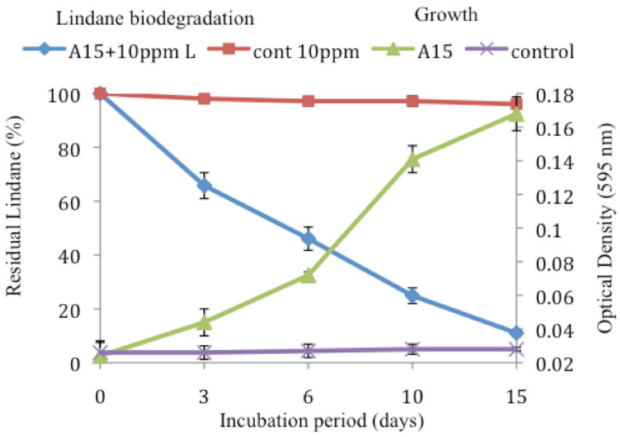

Fig. 1. Lindane (10 mg L^{-1}) degradation and *Ochrobactrum* sp. A15 growth in Minimal Salt medium during 15 days of incubation.

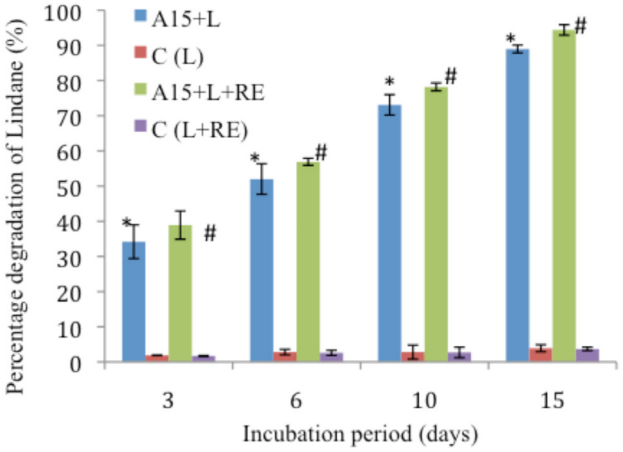

Fig. 2. Effect of root exudates of *Acorus calamus* on lindane degradation by *Ochrobactrum* sp. strain A15. * shows significant difference in lindane degradation in the presence and absence of root exudates. L: lindane, RE: root exudates, C: control.

(Fig. 1). There was a significant difference ($P < 0.05$) in bacterial growth and percentage degradation of lindane by strain A15 was observed in comparison to control (Fig. 1).

Analysis of root exudates

Root exudate pH in water was slight alkaline (7.9 ± 0.06). Total protein content of exudates was 146.8 ± 0.56 µg mL^{-1}, total carbohydrate content was 161.4 ± 1.92 µg mL^{-1} and concentration of phenolic compounds was 36 ± 0.06 µg mL^{-1}.

Lindane biodegradation in the presence of root exudates

Ochrobactrum sp. strain A15 degraded 94.4 ± 1.5% lindane in the presence of root exudates during 15 days of incubation period. Lindane biodegradation efficiency of this strain increased by 10.7% when supplemented with root exudates, compared to that in the absence of root exudates (83.7 ± 1.11%; Fig 2).

Degradation kinetics

Average rate of lindane biodegradation increased and half-life decreased when supplemented with root exudates, as compared to lindane biodegradation without root exudates (Table 1). Significant increase ($P < 0.05$) in average rate of biodegradation and significant decrease ($P < 0.05$) in half life was observed.

Plant growth promoting activity of Ochrobactrum *sp. strain A15*

The results of tested plant growth promoting traits is shown in Table 2. *Ochrobactrum* sp. A15 demonstrated production of IAA, ammonia, HCN, phosphate solubilization and ACC deaminase activity but did not exhibit siderophore production. *Ochrobactrum* sp. strain A15 was observed to produce 233.6 ± 0.2 µg mL^{-1} IAA. In the presence of lindane, the bacteria produced 173.2 ± 0.3 µg mL^{-1} IAA. No decrease in ammonia production by bacteria was observed in the presence of lindane, but the bacterial strain *Ochrobactrum* sp. strain A15 could not produce HCN in the presence of lindane.

Ochrobactrum sp. strain A15 was estimated to solubilise 266.0 ± 1.6 µg mL^{-1} phosphorus. *Ochrobactrum* sp. strain A15 showed a decrease in phosphorus solubilization in the presence of lindane. The bacteria solubilised 226.2 ± 1.6 µg mL^{-1} phosphorus in the presence of lindane. Decrease in ACC deaminase activity by bacteria was observed in the presence of lindane.

Discussion

Nine morphologically different bacterial colonies showing excellent growth on media plates were selected and subjected to degradation study. Among the isolated strains, A15 had the highest percentage degradation and the bacterium was recognized as *Ochrobactrum* sp. strain A15. In a previous study, isolation of *Ochrobactrum anthropi* from lindane-polluted river sediment has been also reported (Pesce, Wunderlin 2004).

Lindane biodegradation by *Ochrobactrum* sp. A15 was simultaneous with bacterial growth in culture media (Fig. 1), thereby demonstrating exploitation of lindane as the only source of carbon. Bacterial growth with lindane as only

Table 1. Chemical properties of cow manure vermicompost used in the present experiments

Parameter	Without root exudates	With root exudates
Average rate of degradation (mg L^{-1} day^{-1})	0.59 ± 0.006*	0.63 ± 0.001*
$T_{1/2}$ (days)	4.78 ± 0.04#	3.75 ± 0.03#

Table 2. Plant growth promoting activities of *Ochrobactrum* sp. strain A15

Plant growth promoting activity	Absence of lindane	Presence of lindane
IAA production (μg mL^{-1})	233.6 ± 0.2	173.2 ± 0.25
Ammonia production	+	+
HCN production	+	+
Phosphorus solubilization (μg mL^{-1})	266 ± 1.56	226.24 ± 1.56
ACC deaminase activity	+	+
Siderophore production	–	–

supply of carbon was also observed in other studies (Sagar, Singh 2011; Singh, Singh 2018b; Singh, Singh 2019a).

Root exudates offer a nutrient rich environment for contaminant degraders and are able to potentially improve biodegradation. This can be by enhancing pollutant bioavailability, facilitating the co-metabolic transformation of contaminants and/or inducing catabolic enzymes concerned with contaminant degradation (Álvarez et al. 2012). In our study, lindane degrading activity of bacteria increased in the presence of root exudates, confirming that the released root exudates were a source of carbon and energy to the bacterial strain. Our results are in agreement with earlier studies where proteins, carbohydrates, and phenols were identified in root exudates of maize plants and they represented possible source of carbon and energy for *Streptomyces* strains (Álvarez et al. 2015). Also, lindane degradation by bacterium was promoted by root exudates of chilli, corn and coriander (Nagpal, Paknikar 2006). Plant exudates from *Elymus dauricus* have been observed to reduce chlorobenzoic acid levels (Siciliano, Germida 1998). Root exudates also changed the bacterial degradation specificity or led to additional degradation of chlorobenzoic acids (Vrchotová et al. 2013).

Our results show that plant root exudates noticeably influenced lindane degradation by the isolated bacterial strain, which supported the suggestion that co-metabolism could be plant-microorganism communication vital for bioremediation, which is in agreement with results published by Alvarez et al. (2012).

Root inhabiting bacteria promote host plant growth by direct or indirect means are named plant growth promoting rhizobacteria. These plant bacterial interactions are known to improve plant health (Juanda 2005). IAA is a necessary phytohormone and is an important signaling molecule in plant development regulation (Kumar et al. 2012). In our study, *Ochrobactrum* sp. strain A15 produced IAA. Production of IAA by root bacteria helps in stimulation of cell division, thus promoting growth of roots. Lindane had a negative effect on the ability of bacteria to produce IAA. This could be due to alteration of the IAA production pathway by the pesticide or an inhibiting effect of pesticide on IAA production. Similar reduction in IAA production was observed in *Mesorhizobium* sp. (Ahemad, Khan 2016) and in *Burkholderia* sp. strain L2 (Tripti et al. 2015). Ammonia production is another attribute important for plant growth promotion, which indirectly influences plant growth. In our study, no decrease in ammonia production by bacteria was observed in the presence of lindane. HCN production by rhizobacteria is hypothesized to participate significantly in the biological control of pathogens, thereby inducing plant resistance (Kumar et al. 2012). It has been reported that ammonia and HCN produced by rhizobacteria play a vital function in biocontrol (Brimecombe et al. 2000). In the present study the bacterial strain *Ochrobactrum* sp. strain A15 could not produce HCN in the presence of lindane. Phosphorus plays a significant function in plant growth and development. In our study, *Ochrobactrum* sp. strain A15 could solubilise phosphorus. A previous study reports the presence of high concentration of phosphate-solubilizing bacteria in the rhizosphere as compared to the non-rhizospheric zone (Reyes et al. 2007). ACC is the precursor of ethylene, a phytohormone related with the stress. ACC deaminase activity reduces the symptoms of stress in plants and enhances root growth (Toklikishvili et al. 2010). This is one of the major bacterial activities that decrease plant stress due to contaminants and improve its development during phytoremediation (Glick, Stearns 2011). No decrease in ACC deaminase activity by bacteria was observed in the presence of lindane.

Previous studies have also reported *Ochrobactrum* sp. to be efficient in IAA production, phosphorus solubilization and ammonia production (Chakraborty et al. 2009; Saini et al. 2017).

Conclusions

The present study showed that the combination of plant *Acorus calamus* and its root epiphyte bacteria *Ochrobactrum* sp. strain A15 had an intrinsic ability to remove lindane. Also, this bacterium can support plant growth even with lindane. To the best of our knowledge, this is the first report of plant growth promoting and lindane degrading root epiphytic bacteria from *A. calamus*. Therefore, this combination can be exploited for the bioremediation of lindane and thereby in in controlling the environmental contamination problem.

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