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Arsenic-resistant bacteria isolated from highland soils of northern Thailand and possible application in bioremediation and plant growth promotion

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Abstract

Arsenic contamination in soils causes poisoning in food and finally affects the food chain. Bioremediation by microorganisms is an environmentally friendly method that offers the possibility of detoxifying soil contaminated by arsenic. The present study aimed at isolating and screening bacteria which possess resistance to arsenic from contaminated highland soils. Among 40 isolates obtained, only nine isolates (22.5%) could tolerate a high level of arsenic with the highest MIC of 40 mM. Only four isolates (BAs8, BAs11, BAs19 and BAs29) exhibited promising resistant ability and were selected for further investigations. The selected isolates showed a high potential to solubilize insoluble Ca-phosphate (5.95 to 100.04 mg P L^{-1}), and a moderate potential to produce IAA (9.36 to 20.32 mg IAA L^{-1}). The ability of each isolate to solubilize Ca-phosphate was increased 2 to 6 times when exposed to an arsenite environment. Arsenic-sensitive isolate, BAs7 showed the highest ability to solubilize Ca-phosphate in medium without and with arsenite (55.56 and 100.04 mg L^{-1}, respectively). The results implied that the increment of phosphate solubilization is an important mechanism to exclude arsenic uptake thus less toxicity. The similar results were found with IAA production and might also be another mechanism of bacteria to cope with arsenic toxicity. Our results seemed to be the first report on this clear phenomenon. Isolates BAs29 and BAs11 exhibited high arsenic resistance and established vigorous root growth and dense root hairs of Chinese kale seedlings thus can be used for bioremediation and seedlings growth enhancement.

Keywords: Arsenic contamination, Arsenic-resistant bacteria, IAA production, phosphate solubilization

1. Introduction

Arsenic (As) is a common metalloid and well-known human carcinogen that can harm not only people's health but plants and microorganisms as well. Ongoing applications of arsenical pesticides and chemical fertilizers in Northern Thailand's agricultural highlands have increased the As content of soils and stream sediments. Various farming practices, e.g. the use of animal manures, phosphate fertilizers and As containing agrochemicals may increase As contamination in agricultural soils [1]. The concentration of As in cultivated soils of highland areas of northern Thailand greatly exceed the national environment standard (3.9 mg kg^{-1}) [2]. In these contaminated areas, the extremely high input of agrochemicals is a common practice in farms and seemed to be a primary source of the high As contamination. High As levels in the topsoil, particularly in the root zone, is likely to result in increased concentration in plant and food grains and thus pose a greater risk to human health. Inorganic arsenic has been classified as a class 1 carcinogen by the International Agency for Research on Cancer. It is responsible for bladder, kidney, liver, lung, and skin cancers and is listed as a Class A human carcinogen by the USEPA [3]. Arsenic in the environment comes from natural and anthropogenic sources. Naturally occurring As in the continental crust is present at an average concentration of 1.5 to 5 mg kg^{-1} [4 & 5]. However, As concentrations in soils with human activities vary widely among different locations. In European topsoil, As concentration is estimated at the average of 7.0 mg kg^{-1} [6]. The assumptions of soil As concentration in areas under unrestricted use (e.g., residential) by the USEPA Regional Screening Level (RSL) is 0.39 mg kg^{-1} [7]. This guidance by USEPA is based on a target cancer risk of 1E-06, toxicological guidance values and standard assumptions for exposure assessment and risk assessment.

In the natural environment, the pentavalent arsenate (As(V)) and trivalent arsenite (As(III)) are the most common oxidation states of As [5]. Trivalent arsenic (arsenite) is generally more toxicologically potent than pentavalent arsenic (arsenate) because arsenite can form strong bonds with functional groups such as the thiolates of cysteine residues and the imidazolium nitrogen of histidine residues, of cellular proteins, and thus the bindings inactivated many cellular proteins including enzymes [8]. Various microorganisms including bacteria have evolved many mechanisms to cope with arsenic exposure [2 & 9] thus arsenic-resistant bacteria seemed to have a vital role in the transformation of As, movement of As in soils and the availability of As to plants.

Soil microorganisms play a critical role in As mobility and availability to the plant through various mechanisms, e.g. release of chelating agents, acidification and phosphate solubilization [10 &11]. Several investigations have shown that microorganisms not only affect the mobility and availability of heavy metals but also exhibit plant growth promoting abilities such as producing phytohormone and solubilizing phosphorus and other nutrients [12 & 13]. For these reasons, mechanisms involved in microbial detoxification of arsenic and heavy metals have recently received more attention. In the context of increasing international concern for food and environmental quality, the use of arsenic-resistant bacteria which are also cable of plant growth promotion is of interest.

Accordingly, this paper describes the isolation of bacteria from contaminated highland soils, the screening of bacterial isolates which possess tolerance to various concentrations of As (III) and the evaluation of the minimal inhibitory concentration (MIC) of each bacterial isolate. The high tolerant bacterial isolates were also evaluated for their ability in plant growth promotion, i.e. phosphate solubilization, indole acetic acid (IAA) production, and seed germination enhancement. The promising isolates may be employed as potential bio-inoculants for detoxifying As and improving the growth of plants in As contaminated soils.

2. Materials and Methods

In this study, soil samples contaminated with arsenic (As) were collected from highland soils of northern Thailand. The content of As in soil samples was determined using the mixture of acids. Arsenic-resistant bacteria were isolated and screened for high promising isolates. The high promising isolates were further investigated for plant growth promoting abilities, i.e. phosphate solubilization and indole-3-acetic acid production. Selected isolates were also tested for seedlings growth enhancement.

2.1 Soil sampling and arsenic concentration analysis

Rhizosphere-soil (0 to 20 cm) were collected from seven cultivated soil contaminated with As. The areas were located in highland parts of Chiang Mai Province, northern Thailand (17°30' to 19.5°30' N and 97.7°30' to 99.3°30' E). The soil samples were collected in polyethene bags and kept at 4ºC until analysis.

All the samples were analyzed for As content at the Central Laboratory (Thailand) Co. Ltd. In brief, the soil samples were air dried, mixed thoroughly and digested by nitric acid (HNO_3)/ hydrofluoric acid (HF) for 15 min using microwave heating with a suitable microwave system, according to EPA Method 3052. The samples were analyzed by Perkin Elmer Optima 4300 DV Inductively Coupled Plasma Optical Emission Spectrometer (ICP-

OES, USA). The pH (H_2O) of the samples was also determined according to the standard method [14] using pH meter (PHI 34 BECKMAN, USA).

2.2 Isolation and screening of arsenic-resistant bacteria

A serial dilution (10^{-1} to 10^{-5}) and plate count technique was used to isolate bacteria from all the soil samples. An aliquot (0.1 mL) of the soil suspension (10^{-3} to 10^{-5}) of each soil sample was spread on nutrient agar (NA) (HIMEDIA: REF 002) plate (pH 6.5). Colonies which grown on the medium were purified and suspended in 25% glycerol solution (final concentration) for preservation. All isolates preserved in this solution were maintained at -20ºC until use.

In the present study, we focused on bacterial isolation from soils of highland areas which are acidic [2] and with pH values usually below 5.0. Soil pH markedly affects the availability of highly toxic Al^{3+} ion in soil and the toxic of Al^{3+} is predominant below a pH of 4.8. Screening of acid tolerant bacterial isolates using low pH medium supplemented with 50 µM Al have shown that, on the average, very little or no growth was obtained below pH 4.5 and there were differences in bacterial growth between the media at pH 4.5 and pH 4.7 [15-17]. Therefore, in this study, for the first screening, we decided to screen As-resistant bacteria using NA medium supplemented with 50 µM Al at pH 4.5 and pH 4.7. The medium, pH 7.0 was also used for comparison.

For the first screening, the pure bacterial isolates were used to evaluate their resistance under high concentration of As. The culture broth of each isolate was dropped on NA plate containing 50 µM aluminium (Al) and various concentrations of sodium arsenite (($NaAsO_2$: Na-As (III)), i.e. 0, 5, 10, 20 and 40 mM. The initial pH of the medium was adjusted to 4.5, 4.7 and 7.0.

The tolerant isolates were selected for the second screening. In the 2nd screening, the concentration of As was adjusted close to the analyzed level found in the soil. Culture solution (0.01 mL) of each isolate was dropped on NA plate containing 50 µM Al and various levels of Na-As (III); 0, 10, 15, 25, 50, 100, 250 and 650 mg L^{-1} (which equal to 0, 0.08, 0.12, 0.19, 0.38, 0.77, 1.9 and 5.0 mM, respectively). The pH of the media was adjusted to 4.5 and 4.7. Growth observation (the presence or absence of visible growth detected by colony forming on agar plate) was made after seven days of incubation. The minimal inhibitory concentration (MIC) of each bacterial isolate was also determined.

2.3 Phosphates solubilization

High tolerant isolates from the second screening were selected and evaluated for their potential in phosphate solubilization in Pikovskaya's broth medium [18]. The culture broth (0.5 mL) of arsenic-resistant bacteria was inoculated into 50 mL of Pikovskaya's broth (pH 7.0) containing Al (50 µM) and Na-As (III) (15 mg L^{-1}). After five days of incubation, the cultures were centrifuged at 5,000 rpm for 15 minutes. One mL of supernatant was mixed with four mL of color reagent (1:1:1:2 ratio of 6N H_2SO_4, 2.5% ammonium molybdate, 10% ascorbic acid and distilled water). This was then incubated at room temperature in the dark for 30 minutes and observed by measuring optical density at 820 nm using a spectrophotometer (Thermo Scientific, mod. GENESYS 20, USA).

2.4 Indole-3-acetic acid (IAA) production by arsenic-resistant bacteria

The same set of isolates, selected for phosphate solubilizing evaluation was tested for their ability in IAA production. IAA produced by each isolate was quantified by Salkowski method [19]. The culture broth of each isolate (0.50 mL) was inoculated into 50 mL of NB containing tryptophan (0.1 g L^{-1}), Al (50 µM) and Na-As (III) (15 mg L^{-1}). The medium pH was adjusted to 7.0. All the isolates were incubated at room temperature for five days. The cultures were centrifuged at 5,000 rpm for 15 minutes. One mL of supernatant was mixed with two mL of Salkowski's reagent [19], incubated at room temperature in the dark for 30 minutes. The quantity of IAA was measured by a spectrophotometer (Thermo Scientific, mod. GENESYS 20, USA) at 530 nm.

2.5 Seed germination test

Chinese kale is a popular leafy vegetable among Thai consumers and is widely grown in highland areas of northern Thailand. Therefore, it was selected in this study. The Chinese Kale seeds were surface-sterilized by 3% NaClO and shake several times in sterile distilled water. The sterile-seeds (10 seeds per plate) were placed on sterile filter paper in Petri dishes. The bacterial suspension was diluted 50 times with sterile water (1:49; bacterial suspension:sterile water) and then two mL of each isolate, the medium broth and sterile distilled water (control), were added separately to the filter paper. There were three replicates for each treatment. All the plates were placed in the incubator for three days, and the temperature was maintained at 30ºC. Seed germination and root length of Chinese Kale were measured after incubation.

2.6 Statistical Analysis

The data were subjected to analysis of variance using statistical program Statistix7 (SXW). The differences among various treatment means were analyzed by one-way analysis of variance (ANOVA) to determine if they were different from one another. Differences between means were tested by LSD at a significance level of $P < 0.05$.

3. Results

3.1 Soil analysis and bacterial isolation

The pH values of the soils varied from location to location. The surface soil pH value of the area was low (Table 1) and ranged from very strongly acidic to slightly acidic. The soil pH of Maehae, Angkhang and Vavee was 5.09, 4.80 and 4.67, respectively and could be classified as very strongly acidic [20]. In contrast, the soil pH of Pangda Tungroeng and Sobkhong was slightly acidic. All the soil samples contained higher arsenic level than the standard background level (3.9 mg kg^{-1}) [21]. The soils from Tungroeng and Vavee contained around ten times higher As (39.48 and 30.52 mg kg^{-1}, respectively) than the standard level (Table 1). The rest of the soils samples also contained a high level of As with values ranging from 5.45 to 16.06 mg kg^{-1}. Although all the soil samples contained quite a high level of As, resistant bacterial isolates (47 isolates) (Table 1) could be obtained. The largest number of resistant bacterial isolates was obtained from Angkhang soil (10 isolates; 21.27% of the total isolates). There seemed to be no relationship between As concentration in soils and number of resistant bacterial isolates. Although 47 bacterial isolates were obtained, only 40 isolates were kept and used for further investigations because seven isolates were contaminated and died during preservation.

Table 1 Arsenic concentrations and bacterial isolates obtained from arsenic contaminated soil.

Location	Coordinates	AMSL[1] (m)	pH	Total Arsenic conc. (mg kg^{-1})[2]	No. of isolates	% of total isolates
Prabathhuaytom	17°43'32 N, 98°57'14 E	528	5.63	5.61	6	12.76
Pangda	18°51'20 N, 98°45'38 E	620	6.45	8.83	5	10.63
Tungroeng	18°47'28 N, 98°48'36 E	847	6.62	39.48	7	14.89
Maehae	18°47' 31N, 98°32'14 E	1178	5.09	5.45	8	17.02
Angkhang	19.54°39' N, 99.5°2'41 E	1468	4.80	6.66	10	21.27
Vavee	19°45'54 N, 99.5°33'26 E	1597	4.67	30.52	5	10.63
Sobkhong	17°39' 49N, 98°11'57 E	1765	6.39	16.06	6	12.76
				Total	47	(100%)

[1]Height above mean sea level
[2]The standard background level for arsenic in soil is set at 3.9 mg kg^{-1}, National Environment Board No.8, (1994)

3.2 Arsenic resistance and minimum inhibitory concentration of bacterial isolates

The arsenic tolerant ability of all the isolates (40 isolates) was evaluated. All the isolates could grow well under pH 5.0, 6.0 and 7.0 when Na-As (III) was not added to the medium (data not shown). However, when the medium was supplemented with Na-As (III) most of the isolates could not tolerate high concentration of arsenic at pH 4.5 and 4.7 (Table 2). Only nine isolates (isolate BAs7, BAs8, BAs11, BAs19, BAs20, BAs22, BAs29, BAs30 and BAs36) showed fair or healthy growth at an arsenic concentration of 0, 5, 10 and 20 mM. No isolates could grow on the medium at 40 mM Na-As (III) (data not shown).

The minimum inhibitory concentration (MIC) of arsenite against bacteria isolated from contaminated soils of highland areas was evaluated. The MIC was defined as the lowest concentration that completely inhibited bacterial growth [22]. It was observed that most of the isolates had low MIC (<5 to 5 mM arsenite) regardless of the pH

value (Table 2). At pH 4.5 and 4.7, the highest MIC was observed in BAs20, BAs29 and BAs30 (MIC 40 mM arsenite). When the pH was raised to 7.0, these three isolates still had the MIC of 40 mM arsenite, and only a few more isolates could tolerate to this concentration of As (BAs7, BAs8, BAs19, BAs22, BAs27 and BAs36) (Table 2).

The number of arsenic-resistant isolates at 10 and 20 mM arsenite under pH 4.5 and 4.7 were the same (four and three isolates which equal to 10 and 7.5% of the total isolates (40 isolates), respectively) (Figure 1). At these two As concentrations under neutral pH (7.0), the number of resistant isolates was more than double increased (10 (25%) and 8 (20%) isolates, respectively). Thus, an increase in Na-AsIII concentration resulted in a lower resistance of As.

Table 2 Minimum inhibitory concentration (MIC) of arsenic-resistant bacteria under different of $NaAsO_2$ and pH levels in nutrient agar containing 50 µM Al.

Bacterial Isolates	Areas	MIC of isolates exposed to $NaAsO_2$ at 0, 5, 10, 20 and 40 mM			Bacterial Isolates	Areas	MIC of isolates exposed to $NaAsO_2$ at 0, 5, 10, 20 and 40 mM		
		pH 4.5	pH 4.7	pH 7.0			pH 4.5	pH 4.7	pH 7.0
BAs 1	Vavee	NG	NG	NG	BAs 21	Tungroeng	5	10	5
BAs 2		5	5	5	BAs 22	Prabathhuaytom	5	10	40
BAs 3		5	5	5	BAs 23		10	5	10
BAs 4		5	5	10	BAs 24		5	5	5
BAs 5	Pangda	10	10	10	BAs 25		5	5	5
BAs 6		10	10	10	BAs 26		5	5	5
BAs 7		10	10	20	BAs 27		10	5	10
BAs 8	Sobkhong	10	10	40	BAs 28	Angkhang	5	5	5
BAs 9		5	5	5	BAs 29		40	40	40
BAs 10	Maehae	NG	NG	NG	BAs 30		40	40	40
BAs 11		10	10	40	BAs 31		5	5	5
BAs 12		NG	NG	NG	BAs 32		5	5	5
BAs 13		5	5	5	BAs 33		5	5	5
BAs 14		10	10	10	BAs 34		5	5	5
BAs 15	Tungroeng	10	10	10	BAs 35		5	5	5
BAs 16		NG	NG	NG	BAs 36		20	20	40
BAs 17		5	5	5	BAs 37		5	5	5
BAs 18		5	5	5	BAs 38	Maehae	5	5	5
BAs 19		10	10	40	BAs 39		5	5	5
BAs 20		40	40	40	BAs 40		5	10	5

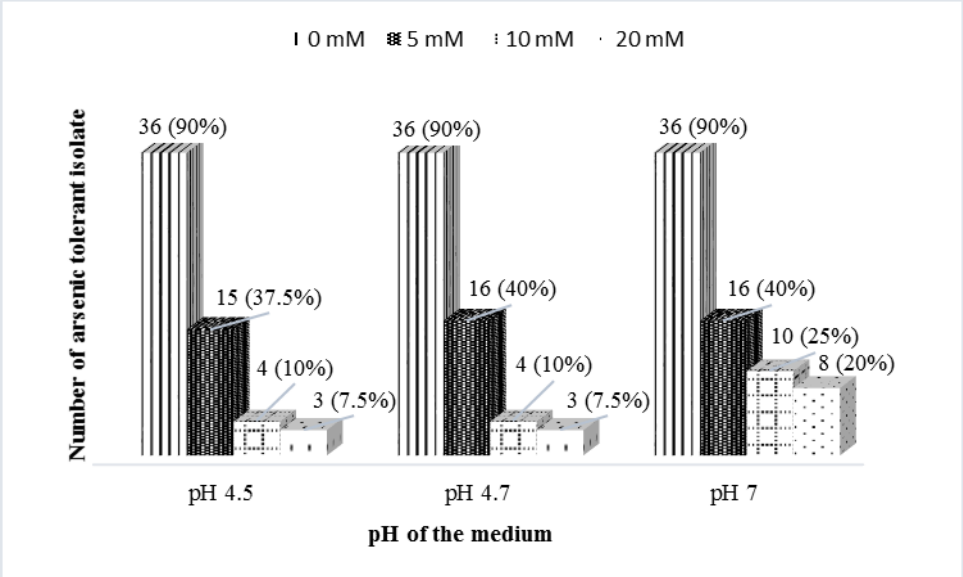

Figure 1 Number and the percentage (% of total isolate) of arsenic-resistant bacteria under different levels of $NaAsO_2$ and pH.

After the 1st screening, nine isolates (isolate BAs7, BAs8, BAs11, BAs19, BAs20, BAs22, BAs29, BAs30 and BAs36) were selected for the 2nd screening in another set of $NaAsO_2$, i.e. 0, 10, 15, 25, 50, 100, 250 and 650 mg L^{-1}. Our results indicated that without As addition, healthy growth of all isolates was obtained at all pH levels (0 mg L^{-1}) (data not shown). The highest MIC at pH 4.5 was achieved with isolate BAs8, BAs11, BAs19 and BAs29 (250 mg L^{-1}) while only isolate BAs11 exhibited the highest MIC at pH 4.7 (650 mg L^{-1}) (Table 3). At pH 5.5 and 6.0, isolates BAs8, BAs11, BAs19, BAs22 and BAs29 showed the same highest MIC of >650 mg L^{-1}. Among all the screened isolates, BAs7 seemed to be sensitive to AsIII at all pH levels. Its MIC was ranged from <10 to 15 mg L^{-1}. From the results of the 2nd screening, sensitive isolate, medium and high tolerant isolates, i.e. isolate BAs7, BAs8, BAs11, BAs19 and BAs 29, were selected for further investigations.

Table 3 The minimum inhibitory concentration (MIC) of $NaAsO_2$ against selected isolates.

Bacterial isolates	MIC of isolates exposed to $NaAsO_2$ at 0, 10, 15, 25, 50, 100, 250 and 650 mg L^{-1}			
	pH 4.5	pH 4.7	pH 5.5	pH 6
BAs 7	15	25	25	25
BAs 8	250	250	>650	>650
BAs 11	250	650	>650	>650
BAs 19	250	250	>650	>650
BAs 20	15	25	250	650
BAs 22	15	250	>650	>650
BAs 29	250	250	>650	>650
BAs 30	15	15	100	250
BAs 36	50	100	250	650

3.3 Phosphate solubilizing ability

Five selected isolates were evaluated for their phosphate (P) solubilizing ability in Pikovskaya's broth medium (PKVb) and PKVb plus Na-As (III) (15 mg L^{-1}) and Al (50 µM) (PKVbp). All the selected five arsenic-resistant isolates showed a certain ability to solubilize P in both PKVb and PKVbp. It was interesting to note that all the isolates solubilized more P under stress conditions in PKVbp. Isolate BAs7 showed the highest ability to solubilize P and released about twice the amount of solubilized P (100.04 mg L^{-1}) in PKVbp greater than that in PKVb (55.56 mg L^{-1}) (Table 4). The same phenomenon was observed in the rest of the isolates. Isolates BAs11 and BAs19 solubilized around 3 to 4.5 times of P in PKVbp (27.27 and 32.80 mg L^{-1}, respectively) higher than those in PKVB (5.95 and 10.66 mg L^{-1}, respectively. The pH of both PKVb and PKVbp was decreased by all the selected isolates when compared to the control treatment. The pH of PKVb and PKVbp inoculated with BAs7 was lowest (4.53 and 4.54, respectively) (Table 4).

Table 4 Phosphate solubilizing ability of arsenic-resistant bacteria.

Treatment	Pikovskaya's broth		Pikovskaya's broth supplemented with Na-As (III) and Al	
	pH	Solubilized P (mg L^{-1})	pH	Solubilized P (mg L^{-1})
Control	5.62	-	5.70	-
BAs7	4.53	55.56 ± 4.80a	4.54	100.04 ± 8.35a
BAs8	4.98	13.12 ± 2.82c	4.77	21.32 ± 4.78d
BAs11	4.79	5.95 ± 1.55d	4.76	27.27 ± 5.25cd
BAs19	5.05	10.66 ± 3.74cd	4.95	32.80 ± 3.08c
BAs29	5.00	37.93 ± 4.97b	4.85	48.79 ± 2.46b

Values are means of three replications ± SE
Means with the same letter are not significantly different (P< 0.05)

3.4 Indole-3-acetic acid (IAA) producing ability

All the isolates showed ability in IAA production (Table 5) . The highest IAA producing isolate (20.32 mg L^{-1}) was obtained from BAs19 in NB medium supplemented Na-As (III) and Al, followed by BAs29 (12.65 mg L^{-1}), BAs7 (12.49 mg L^{-1}), BAs8 (12.39 mg L^{-1}) and BAs11 (9.86 mg L^{-1}). In the NA medium, BAs11 performed highest IAA production (18.41 mg L^{-1}), followed by isolate 7 (10.22 mg L^{-1}), isolate 8 (9.53 mg L^{-1}), isolate 19 (9.12 mg L^{-1}) and isolate 29 (9.06 mg L^{-1}). All isolates increased the pH of the medium.

Table 5 Indole 3-acetic acid (IAA) produced by arsenic-resistant bacteria

Treatment	Nutrient Broth		Nutrient broth supplemented with Na-As (III) and 50 µM Al	
	pH	IAA (mg L^{-1})	pH	IAA (mg L^{-1})
Control	7.0	-	7.0	-
BAs7	8.50	10.22 ± 1.61b	8.09	12.49 ± 0.36b
BAs8	8.43	9.53 ± 0.26b	8.08	12.39 ± 1.12b
BAs11	8.34	18.41 ± 1.05a	7.98	9.86 ± 0.07c
BAs19	8.46	9.12 ± 0.13b	8.14	20.32 ± 1.32a
BAs29	8.46	9.06 ± 0.02b	8.01	12.65 ± 0.47b

Values are means of three replications ± SE
Means with the same letter are not significantly different (P< 0.05)

3.5 Seedlings growth enhancement by arsenic-resistant isolates

The five selected isolates were tested for their effects on Chinese Kale seed germination and root growth. The control without bacterial inoculation gave 80% seed germination while the inoculation of arsenic resistance bacterial isolates could enhance the seed germination by up to 86.67 to 96.67% (Table 6). Isolate BAs29 exhibited the highest percentage of seed germination (96.67%). Therefore, inoculation of arsenic resistance bacterial isolates increased the seed germination by 6.67 to 16.67% over the control.

All the arsenic-resistant isolates increased the root length of the seeds compared to the control except for BAs8 (Table 6, Figure. 2). The highest root length was found with BAs7 (1.98 cm).

Table 6 Effects of arsenic-resistant isolates on seed germination and root length of Chinese Kale.

Treatment	OD (600 nm)	pH	Diluted culture solution (1:50)	
			Seed germination (%)	Root length (cm)
Control	0	6.91	80.00	1.55 ± 0.35
BAs7	0.963	8.66	93.33	1.98 ± 0.37
BAs8	0.932	8.72	86.67	1.45 ± 0.15
BAs11	0.613	7.56	90.00	1.74 ± 0.26
BAs19	1.214	8.72	86.67	1.85 ± 0.29
BAs29	0.910	8.74	96.67	1.72 ± 0.39

Values are means of three replications ± SE
Means are not significantly different (P< 0.05)

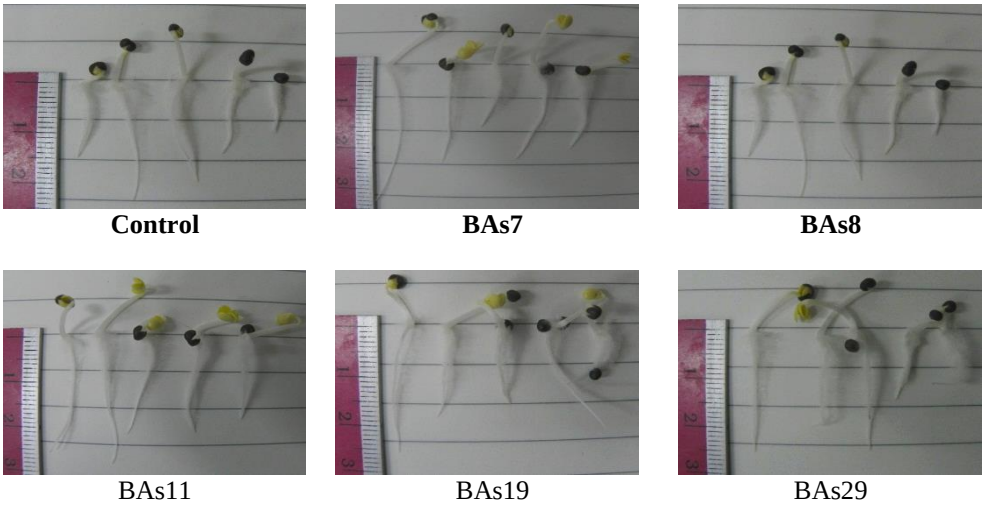

Figure 2 Effect of arsenic-resistant bacterial inoculation; BAs7, BAs8, BAs11, BAs19 and BAs29, on Chinese kale root growth promotion.

Among four arsenic-resistant isolates (BAs8, BAs11, BAs19 and BAs29), BAs8 gave the lowest phosphate solubilizing ability and root growth promotion while BAs29 gave the highest phosphate solubilizing ability and the similar root length (1.72 cm) to those of BAs11 and BAs19 (1.74 and 1.85 cm, respectively (Figure. 3). The arsenic-sensitive isolate, BAs7, gave the highest phosphate solubilizing activity and root length promotion although this isolate did not show high IAA production (Figure. 3 & 4). However, on the average (30 seedlings/treatment), it was observed that BAs29 gave the highest seed germination (Table 6), the greatest root vigor and dense root hairs compared with BAs7, BAs8 and BAs19. It was also observed that BAs11 also gave vigorous root growth and long and dense root hairs similar to BAs29 (data not shown).

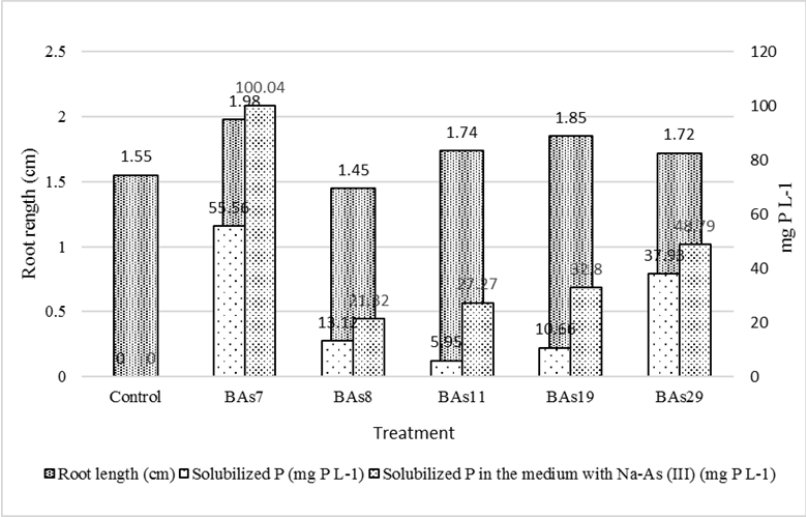

Figure 3 Relationship between phosphate solubilizing ability and Chinese kale root growth promotion of arsenic-resistant bacteria.

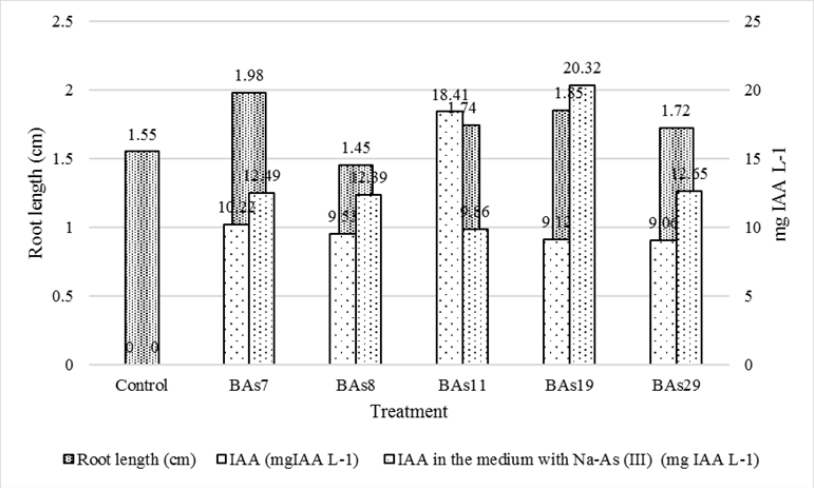

Figure 4 Relationship between indole-3-acetic acid producing ability and Chinese kale root growth promotion of arsenic-resistant bacteria.

4. Discussion

High concentrations of As (5.45 to 39.48 mg kg^{-1}) were found in cultivated highland soil in northern Thailand and are likely to have an adverse impact on upstream, downstream, groundwater and thus food chains. Office of National Environment Board of Thailand set an arsenic maximum concentration limits (MCL) in agricultural soil at 3.90 mg As kg^{-1}[23]. Chitpirom et al. (2009) [24] reported that arsenic concentration of agricultural soil samples from central Thailand were 4.11 to 4.35 mg kg^{-1}. Majumder et al. (2013) [25] found that arsenic concentrations in soils of West Bengal, India varied from 7.4 to 13.4 mg kg^{-1}. The levels of soil arsenic vary widely in different countries (0.1 to 40 mg kg^{-1}). Anthropogenic sources exceed natural sources by 3 to 1 in the environment [26]. In the present study, soluble arsenic in the contaminated highland soils may be readily available for uptake by plant root leading to elevated levels of arsenic in soils. This can result in increased concentration of arsenic in plants. Eating crops grown on contaminated soils may increase the health risks. A few recent studies report 85 to 95% inorganic arsenic in rice and vegetables, which suggest more studies for standardization [26].

In this study, under low pH (4.5 and 4.7) only a few isolates could tolerate high concentrations of arsenite with the highest MIC of 40 mM. The number of tolerant isolates at 40 mM MIC was doubled when the pH was raised to 7.0. Majumder et al. (2013) [25] concluded that some isolates were hyper-resistant to arsenite at 16 to 47 mM which was similar to our results. Aksornchu et al. (2008) [27] obtained high arsenic tolerant bacteria which were able to grow in medium containing 40 mM sodium arsenite from soils contaminated with 40 to 1,000 mg As kg^{-1}. Thus, the few isolates (BAs20, BAs29 and BAs30: MIC 40 mM arsenite) obtained from this study could be considered as hyper arsenic tolerant bacteria. The tolerant isolates obtained in this study could withstand higher arsenite concentration under slightly acidic and/or neutral pH than extreme acidic environment suggesting pH effect on resistant ability. The results of the present study suggested that arsenic has low solubility under neutral or slightly acidic pH. The solubility seemed to be increased considerably in strongly acidic conditions thus lower the MIC of bacteria. Arsenic concentration and species are influenced by pH and redox potential of soil [26]. Arsenate is often the dominant species in the aerobic environment or acidic soil. The mobility of arsenite and arsenate is a function of their adsorption, which in turn is controlled primarily by pH [28-30]. However, some sensitive isolates, e.g. BAs7 did not seem to tolerate higher As(III) concentration although the pH was increased.

Our results indicated that arsenic tolerant isolates could solubilize Ca-phosphate into available P form by lowering the pH of the medium suggesting organic acid(s) might be produced to increase phosphate availability under P deficiency. The principal mechanism in the soil for mineral phosphate solubilization is lowering of soil pH by the microbial production of organic acids and mineralization of organic P by acid phosphatases [31]. Arsenate is taken up via phosphate transport system in both prokaryotes and eukaryotes since phosphate ion is similar to arsenate ion [32 & 33]. Substitutions for phosphate and subsequent inhibition of oxidative phosphorylation is the major toxicity of As(V). In the plant, it was observed that increasing phosphate supply decreased As uptake markedly in As hyperaccumulation *Pteris vittata* [34]. It was seen in this study that As tolerant bacterial isolates solubilized much higher P (2 to 6 times) in medium with Na-As(III) than in medium without Na-As(III). There seemed to be no report on the relation between soluble phosphate and As uptake by bacteria. However, the results of this study implied that the isolates needed more available phosphate to compete

with arsenate uptake. The isolates solubilized more phosphate for lowering arsenate absorption and thus less toxicity in their cells. Our results seemed to be the first report on As detoxify phenomenon outside the bacterial cell by enhancing phosphate-solubilizing ability when exposed to As.

Indole-3-acetic acid (IAA) is a naturally occurring auxin with broad physiological effects and is known to enhance plant growth. Many arsenic-resistant bacteria exhibited the ability to produce IAA with the value range from 3.28 to 36.5 mg L^{-1} [35]. In our study, all the selected isolates could produce IAA (9.53 to 20.32 mg L^{-1}) indicating that the IAA levels found were slightly lower than the literature. It was interesting to note that IAA production by all the isolates (except for BAs11) was increased when As(III) was added to the medium. This might be another mechanism of bacteria to cope with arsenic toxicity. It is possible that the increase of plant growth promoting the ability of highly tolerant and sensitive isolates is related to its heavy metal resistance.

On the average, inoculation with the tested isolates could enhance seed germination and root growth. It appeared that the root length showed a correlation with phosphate solubilizing ability of bacterial isolates. Isolate BAs7 gave the highest phosphate solubilizing activity and root length while BAs8 gave the lowest phosphate solubilizing activity and root length. The correlation between IAA and root growth depends on IAA concentration. The IAA concentration of 0 to 5.0 mg L^{-1}, and 2.5 mg L^{-1} gave a positive effect on inducing the formation of root hair. However, the IAA higher than 5.0 mg L^{-1} did not show significant increase in the root hair formation and thus the primary root growth was severely inhibited [36]. In the present study, although the effect of IAA was not obvious, it was obsered that BAs11 and BAs29 gave vigorous root growth and long and dense root hairs as compared to other isolates. This phenomenon implied that BAs29 and BAs11 established compatible interactions with the seedlings thus ensured later shoot growth promotion of Chinese kale. Wang et al. (2016) [37] concluded that vigorous root system including dense root hairs ensured efficient acquisition of the plant nutrients during early growth and is assumed to be important for later plant development.

Various physicochemical techniques, e.g., oxidation and reduction, chemical precipitation and filtration have been applied for the removal of toxic heavy metals. However high input cost with some disadvantages associated with such techniques resulting in ineffective output and secondary environmental pollution. Using high tolerant bacterial isolates could be a realistic and desirable strategy for maintaining crop production in As-contaminated soils as well as reduction of As uptake by crops. The tolerant isolates obtained in this study are excellent candidates for the bioremediation process of the As polluted areas. Nevertheless, additional research would be necessary to identify effects of these arsenic-tolerant and plant growth-promoting bacterial isolates on detoxification of soil arsenic and enhancement of plant growth and yield under controlled and field conditions.

5. Conclusion

Out of 40 isolates, only four isolates, i.e. BAs8, BAs11, BAs19 and BAs29 performed promising tolerant ability under various pH values. Phosphate solubilizing ability of these isolates was increased markedly when they were exposed to arsenite, particularly sensitive isolate (BAs7), suggesting less arsenic uptake thus higher tolerant ability. It was interesting to note that IAA production by all the isolates (except for BAs11) was also increased when As(III) was added to the medium. The clear phenomenon of increment in P solubilization and IAA production by the arsenic-resistant isolates in this study seemed to be the first report on simple mechanisms to cope with high arsenic environments. In addition to high arsenic resistance, BAs29 and BAs11 also gave a vigorous and dense root hairs of the seedlings thus ensured later shoot growth promotion of Chinese kale. The use of these two promising isolates in crop production would lead to a less uptake of arsenic by plant signify their potential application for sustainable bioremediation of As in the environment.

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7. References

[1] Li, Y.X., Chen, T.B., 2005. Concentrations of additive arsenic in Beijing pig feed and the residues in pig manure. Resources Conservation and Recycling 45, 356-367. doi.org/10.1016/j.resconrec.2005.03.002

[2] Shutsrirung, A., 2012. Selection of microorganism in highland for soil quality improvement in acid and high arsenic soils. Final report, Highland Research and Development Institute (Public Organization). Chiang Mai, p. 52.

[3] U.S. EPA., 2005 Supplemental guidance for assessing cancer susceptibility from early-life exposure to carcinogens, Risk Assessment Forum U.S. Environmental Protection Agency, Washington.

[4] Ritchie, A.R., 1980. Handbook of geochemistry. Earth-Science Reviews 16,59-60.

[5] Cullen, W.R., Reimer, K.J., 1989. Arsenic speciation in the environment. Chemical Reviews 89, 713-764. doi: 10.1021/cr00094a002
[6] Stafilov, T., Aliu, M., Sajn, R., 2010. Arsenic in surface soils affected by mining and metallurgical processing in K. Mitrovica Region, Kosovo. International Journal of Environmental Research and Public Health 7, 4050-4061. doi:10.3390/ijerph7114050
[7] USEPA. The United States Environmental Protection Agency. 2010. Regional Screening Levels for Chemical Contaminants at Superfund Sites. [WWW Doccument]. URL http://www.epa.gov/reg3hwmd/risk (accessed 2. 9. 18).
[8] National Research Council. Arsenic in Drinking Water: 2001 Update. Washington, DC: The National Academies Press. 2001. doi.org/10.17226/10194
[9] Jackson, C.R., Jackson, E.F., Dugas, S.L., Gamble, K., Williams, S.E., 2003. Microbial transformation of arsenite and arsenate in the natural environment. Recent Research Developments in Microbiology 7, 103-118.
[10] Smith, S.E., Read, D.J., 1997. Mycorrhizal Symbiosis. San Diego: Academic Press Inc; Science Society of America Journal 48, 758-762.
[11] Abou-Shanab, R.A., Angle, J.S., Delorme, T.A., Chaney, R.L., van Berkum, P., Moawad, H., Ghanem, K., Ghozlan, H.A., 2003. Rhizobacterial effects on nickel extraction from soil and uptake by Alyssum murale. New Phytology 158 (1), 219-224. doi: 10.1046/j.1469-8137.2003.00721.x
[12] Bano, N., Masarrat, J., 2003. Characterization of a new Pseudomonas aerunginosa strain NJ-15 as a potential biocontrol agent. Current Microbiology 46, 324-328. doi: 10.1007/s00284-002-3857-8
[13] Passardi, F., Penel, C., Dunand, C., 2004. Performing the paradoxical: how plant peroxidases modify the cell wall. Trends in Plant Science 9, 534-540. doi.org/10.1016/ j.tplants.2004.09.002
[14] Land Development Department. 2010. Practical manual of soil chemical analysis. Section of soil chemistry research. Division of Agricultural Chemistry. Land Development Department. Ministry of Agriculture and Cooperatives. Bangkok, p. 51.
[15] Wood, M., Cooper, J.E., 1988. Acidity, aluminum and multiplication of Rhizobium trifolii: Effect of initial inoculum density and growth phase. Soil Biology Biochemistry 20, 83-87.
[16] Ozawa, T., Imai, Y., Sukiman, H.I., Karsono, H., Ariani, D., Saono, S., 1999. Low pH and Aluminum tolerance of Bradyrhizobium strains isolated from acid soils in Indonesia. Soil Science and Plant Nutrition 45, 987-992.
[17] Shutsrirung, A., 2003. Characterization of Native Bradyrhizobia in Soybean-Growing Areas of Northern Thailand. Graduate School of Bioresources, Mie University, Japan, p. 253.
[18] Gaur, A.C., 1990. Physiological function of phosphate solubilizing micro-organisms. Omega Scientific Publishers: New Delhi p. 16-72.
[19] Gordon, S.A., Weber, R.P., 1951. The colorimetric estimation of indole acetic acid. Plant Physiology 26, 192-195. doi: https://doi.org/10.1104/pp.26.1.192
[20] Havlin, J.L., Tisdale, S.L., Nelson, W.L., Beaton, J.D., 2014. Soil fertility and fertilizers 8th: An introduction to nutrient management. Pearson Education, Inc., Prentice Hall, p. 515.
[21] Notification of the National Environmental Board, No. 8, B.E. 2537. 1994. Enhancement and Conservation of National Environmental Quality Act B.E. 2535 (1992), Royal Government Gazette 111, Part 16.
[22] Muller, D., Lièvremont, D., Simeonova, D.D., Hubert, J.C., Lett, M.C., 2003. Arsenite oxidase aox genes from a metal-resistant β-proteobacterium. Journal of Bacteriology185, 135-141. doi: 10.1128/JB.185.1.135-141.2003
[23] Weerasiri, T., Wirojanagud, W., Srisatit, T., 2012. Arsenic contamination in soils, water and plants surrounding gold mine at Wangsaphung, Loei province, Thailand. Journal of Environmental Research and Development 6, 381-388.
[24] Chitpirom, K., Akaracharanya, A., Tanasupawat, S., Leepipatpiboon, N., Kim, K., 2009. Isolation and characterization of arsenic-resistant bacteria from tannery wastes and agricultural soils in Thailand. Annals of Microbiology 59, 649-656.
[25] Majumder, A., Bhattacharyya, K., Bhattacharyya, S., Kole, S.C., 2013. Arsenic-tolerant, arsenic-oxidising bacteria strains in the contaminated soil of West Bengal, India. Science of the Total Environment 463-464, 1006-1014. doi.org/10.1016/ j.scitotenv.2013.06.068
[26] Mandal, B.K., Suzuki, K.T., 2002. Arsenic around the world: a review. Talanta 58, 201-235. doi.org/10.1016/S0039-9140(02)00268-0
[27] Aksornchu, P., Prasertsan, P., Sobhon, V., 2008. Isolation of arsenic-tolerant bacteria from arsenic-contaminated soil. Songklanakarin Journal of Science and Technology 30, 95-102.
[28] Luo, W., Lu, Y., Wang, G., Shi, Y., Wang, T., Giesy, J.P., 2008. Distribution and availability of arsenic in soils from the industrialized urban area of Beijing, China. Chemosphere 72(5) , 797- 802 doi:10.1016/j.chemosphere.2008.03.003

[29] Elkhatib, E.A., Bennet, O.L., Wright, R.J., 1984. Kinetics of arsenite sorption in soils. Soil Science Society of America Journal 48, 758-762. doi:10.2136/sssaj1984.03615995 004800040012x
[30] McBride, M.B., 1994. Environmental Chemistry of Soils. Oxford University Press: New York, p. 406
[31] Khan, A., Jilani, G., Akhtar, M.S., Naqvi, S.M.S., Rasheed, M., 2009. Phosphorus solubilizing bacteria: Occurrence, mechanisms and their role in crop production. Journal of Agricultural and Biological Science 1, 48-58.
[32] Dixon, H.B.F., 1997. The biological action of arsenic acids especially as phosphate analogues. Advances in Inorganic Chemistry 44, 127-191. doi.org/10.1016/S0898-8838(08)60 131-2
[33] Tsai, S.L., Singh, S., Chen, W., 2009. Arsenic metabolism by microbes in nature and the impact on arsenic remediation. Current Opinion in Biotechnology 20, 659-667. doi.org/10.1016/j.copbio.2009.09.013
[34] Wang, J., Zhao, F.J., Andrew, A., Meharg, A.R., Joerg, F., McGrath, S.P., 2002. Mechanisms of arsenic hyperaccumulation in Pteris vittata. uptake kinetics, interactions with phosphate, and arsenic speciation. Plant Physiology 130, 1552-1561. doi: https://doi.org/10.1104/pp.008185
[35] Zhu, L.J., Guan, D.X., Luo, J., Rathinasabapathi, B., Ma, L.Q., 2014. Characterization of arsenic-resistant endophytic bacteria from hyper accumulators Pteris vittata and Pteris multifidi. Chemosphere 113, 9-16. doi: 10.1016/j.chemosphere.2014.03.081
[36] Mo, Y.W., Li, X.,Wang, H., 2015. Effect of Auxin Treatment on Root Hair Formation and Aquaporins Genes Expression in Root Hair of Rice. Scientia Agricultura Sinica 48, 4227-4239.
[37] Wang, Y., Thorup-Kristensen, K., Jensen, L.S., Magid, J., 2016. Vigorous Root Growth Is a Better Indicator of Early Nutrient Uptake than Root Hair Traits in Spring Wheat Grown under Low Fertility. Frontiers in Plant Science 7, 1-8. doi:10.3389/fpls.2016.00865