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Full Length Research Paper

Bioremediation of petroleum contaminated soils by lipopeptide producing *Bacillus subtilis* SE1

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Hydrocarbons released into ecosystems have led to environmental pollution and generated a serious threat to human health. Bioremediation is an effective method to break down hazardous hydrophobic environmental contaminants with avoiding economic and technical disadvantages. This study aimed to evaluate the efficiency of *Bacillus subtilis* SE1, a lipopeptide biosurfactant producer isolated from a petrochemical contaminated soil, on biodegradation of gasoline, diesel oil, crude oil and used engine oil in soil microcosms. During 35-day incubation, numbers of soil bacteria in petrochemical contaminated soils with *B. subtilis* SE1 addition significantly ($P < 0.05$) increased in comparison with the oil-free soils. Bioaugmentation of SE1 strain also produced a significant ($P < 0.05$) increase in percent reduction of total phenolic content in oil-polluted soils as compared to the control soils at the end of experiment. This study indicates that *B. subtilis* SE1 can be a promising hydrocarbon degrader for *in situ* bioremediation of soil environment polluted with petroleum and petrochemical products.

Key words: *Bacillus subtilis*, biodegradation, bioremediation, gasoline, diesel, crude oil, engine oil.

INTRODUCTION

Hydrocarbon pollution is currently become a critical global issue of increasing concerns regarding environmental, social and health catastrophes. Presence of different types of petroleum and petrochemical products viz. gasoline, diesel, crude oil and used engine oil, released into environments by either accidental spillage or improper disposal practices poses more aggravated problems because most disposal methods have been limited in their applications owing to expensiveness, partial effectiveness and strict environmental conditions. Gasoline constitutes mainly aliphatic hydrocarbons (41-62%) and a mixture of aromatic hydrocarbons e.g. benzene, toluene, ethylbenzene and xylene isomers (10-59%; Speight and Arjoon, 2012). Diesel oil is a refined petrochemical product composed primarily of hydrocarbon combination with carbon numbers ranging from C_9 to C_{20}, iso-alkanes, paraffinic, olefinic, naphtha and aromatic compounds as

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well as trace elements of sulfur, nitrogen, metals, and oxygen (Yakimov et al., 2005). Crude oil contains thousands of different hydrophobic components like *n*-alkanes, cycloalkanes, aromatics, resins, asphaltines and heavy metals (Colwell and Walker, 1977). Polycyclic aromatic hydrocarbons (PAHs) is also a group of recalcitrant compounds found in crude oil at high percentage (Haritash and Kaushik, 2009). Used engine oil is a brown to black waste oil discharged from automobiles when oil is changed. In general, fresh engine oil comprises a higher percentage of lighter hydrocarbon and metal salts. Due to high temperature and mechanical stress during engine combustion, the oil is chemically changed by oxidation, nitration, cracking of polymers and decomposition of organometallic compounds leading to formation of other contaminants such as alkyl benzenes, naphthalenes, methylnaphthalenes and PAHs (Dominguez-Rosado and Pitchell, 2003; Lu and Kaplan, 2008). Exposure of these petroleum and petrochemicals generates a serious health risk because of some compositions known to be mutagenic and carcinogenic agents like benzene (Group 1: Carcinogenic to humans; IARC, 1987) and ethylbenzene (Group 2B: Possibly carcinogenic to humans; IARC, 2000).

In recent years, an increased attention has been paid to bioremediation by means of microbial function in a complex multiphase system, which is proposed to be more effective, environmental-friendly, and cost-effective technology (Adams et al., 2015). However, there is a limitation of biodegradation associated with poor accessibility of microorganisms to hydrophobic compounds due to their low solubility in aqueous systems compatible with microbial life (Millioli et al., 2009). This can be compensated by application of biosurfactants or biosurfactant producing bacteria in oil-polluted sites to increase the solubility and bioavailability of hydrophobic compounds and consequently, accelerate rate of biodegradation.

Among several classes of biosurfactants, lipopeptides are commonly isolated and characterized biosurfactants produced by *Bacillus* genera. This biosurfactant active compound has been reported to have several applications in cosmetics, food industry, household detergents and cleansing industries, petroleum industry, medical health and bioremediation of hydrocarbons in contaminated ecosystems (Marchant and Banat, 2012). In view point of bioremediation, lipopeptides produced by *Bacillus* species has received a great attention due to their degradation efficiency of petroleum hydrocarbon and heavy metals from contaminated soils (Bezza and Chirwa, 2015; Parthipan et al., 2017).

Recently, we isolated a biosurfactant producing strain of *Bacillus subtilis* SE1 from a waste engine oil contaminated soil in Chon Buri Province, Thailand. Biosurfactant produced by *B. subtilis* SE1 was identified as lipopeptide and found to degrade gasoline contaminated in soil biostimulated with nutrients.

Bioaugmentation of biosurfactant-producing microorganisms in hydrocarbon polluted soils is an interesting method that would generate a continuous supply of a non-toxic and biodegradable surfactant and promote the rate of biodegradation (Mnif et al., 2015). Therefore, this study was designed to evaluate *B. subtilis* SE1 ability to enhance biodegradation of gasoline, diesel oil, crude oil and used engine oil in soils under laboratory-scale bioremediation condition.

MATERIALS AND METHODS

Bacterial strain and culture

SE1 strain used in this study was isolated from oil-contaminated soil at a local automobile garage in Chon Buri Province, Thailand. It was proven to produce lipopeptide biosurfactant and degrade gasoline contaminated in soil. It was identified as *B. subtilis* SE1 based on its biochemical and morphological features and 16s rRNA gene sequence analysis and assigned the accession number as MH700588. *B. subtilis* SE1 was inoculated in a 250 ml Erlenmeyer flask containing 100 ml Trypticase Soy Broth (Difco, Sparks, MD, USA) in a shaking incubator (JSR, JSSI-100C, Cheongju, South Korea) at 200 rpm, 30°C for 24 h. Cell pellets were harvested by centrifuging at 8,000 rpm, 4°C for 10 min, washed thrice with sterile phosphate buffer saline (PBS). Cell pellets were re-suspended in PBS and adjusted to 1.5 A.U. at 580 nm using a spectrophotometer (Cintra 40 Double beam, GBC Scientific Equipment, Braeside, Victoria, Australia) to obtain SE1 strain number of 10^9 CFU/ml for subsequent use.

Biodegradation of petroleum in soil microcosm

Gasoline and diesel were purchased from a local gas station operated by Public Company Limited. Crude oil was obtained from Thai Oil Public Company Limited while used engine oil was kindly provided by an owner of local automobile garage. All petroleum products were kept in amber-colored bottles at 4°C until use. Biodegradation of petroleum and petrochemical products was tested as previously described by Abioye et al. (2012) with slight modification. A thirty-five-day long biodegradation study was set up under indoor laboratory condition in independent triplicates. Soil was collected from an automobile garage located in Chon Buri Province. Soil used in this study was loamy and dark brown in color. Soil sample (300 g) was thoroughly mixed (3 ml) with one of these petrochemicals: gasoline, diesel, crude oil and used engine oil. Each petroleum contaminated soil was divided to two batches: 1) petroleum contaminated soils and 2) petroleum contaminated soils plus *B. subtilis* SE1 suspension in a 500 ml Erlenmeyer flask. Cell suspension of *B. subtilis* SE1 (1 ml) was aspirated in the contaminated soil according to the treatment. The soil was tilled every week to maintain the moisture content and allow oxygen transfer. During static incubation at 30 °C in the dark, soils were sampled from each treatment at 2 h, 3, 7, 14, 21, 28 and 35 day post-inoculation to evaluate pH change using a calibrated pH meter (Denver instrument, UB-10, Bangkok, Thailand), viable bacteria count and total phenolic content quantification.

Viable bacterial count

At each sampling interval, total viable cell count was evaluated using standard plating technique. Soil samples were 10-fold diluted in physiological saline prior to spreading in triplicates onto Plate Count Agar (Difco, Sparks, MD, USA). After incubation at 30°C for

24 h, all bacterial colonies were enumerated and calculated as log colony forming unit (CFU)/g.

Quantification of total phenolic content

Total phenolic content in the soils was assayed using Folin - Ciocalteu (FC) reagent (Box, 1983) with minor modification. Briefly, an aliquot (0.1 ml) of sample solution was mixed with 20% (w/v) Na_2CO_3 (1.5 ml), 10% (w/v) FC reagent (0.5 ml) and distilled water (7.9 ml) in a volumetric flask. The mixed solution was allowed to stand at room temperature in the dark. After 3 h incubation, absorbance was measured at 765 nm using a spectrophotometer. A standard curve of gallic acid was prepared and concentration of total phenolic content was quantified as gallic acid equivalent from the standard curve. Total phenolic content was calculated and expressed as percent reduction of total phenolic content (PRP) as equation below:

$$\text{PRP value (\%)} = \frac{(PC_i - PC_x)}{PC_i} \times 100$$

where PC_i = initial concentration of total phenolic content and PC_x = concentration of total phenolic content at day x.

Statistical analysis

Data were expressed as mean ± standard deviation. Data were normalized and transformed when needed. Differences were determined using a student's *t* test to determine difference between the treated and control groups at a significant level of $P < 0.05$. All statistical analyses were performed using SPSS version 19.0, Chicago, Illinois, USA.

RESULTS

At the beginning of experiment, pH values of unbioaugmented soils were significantly ($P < 0.05$) lower than those of bioaugmented soils containing gasoline or diesel while similar pH was observed in soils with crude oil or used engine oil. At the end of experiment, pH values of petroleum contaminated soils with SE1 addition significantly ($P < 0.05$) increased ranging from 5.75 ± 0.01 to 6.03 ± 0.01, compared to those of unbioaugmented soils (5.58 ± 0.02 - 5.63 ± 0.02; Table 1).

In gasoline biodegradation study, PRP values of SE1 added soil and unbioaugmented SE1 soil increased to 14.29±2.30% and 10.44 ± 2.12%, respectively at 3 days post-incubation and remained relatively constant until 14 days post-incubation. Afterwards, PRP level of SE1 added soil significantly increased from 16.12 ± 3.16% to 27.27 ± 8.30% at 35 days post-incubation while no appreciable change occurred in unbioaugmented SE1 soil (Figure 1). A significant ($P < 0.05$) difference in PRP levels between treated and control groups was observed by 7 days post-incubation. The plate count revealed a significant ($P < 0.05$) increase in growth of soil bacteria from 5.09 ± 0.19 log CFU/g at the beginning of experiment to 7.11 ± 0.21 log CFU/g at 35 days post-incubation.

The patterns of bacterial growth and PRP value in diesel oil polluted soil were similar to those in gasoline-contaminated soil (Figure 2). A significant ($P < 0.05$) increase in bacterial count was observed since 7 days post-incubation and PRP value was significantly different ($P < 0.05$) within day 3 of experiment. PRP value of soil polluted with diesel oil with SE1 added increased sharply from 0% at the beginning of experiment to 28.24 ± 5.75% at day 3 of incubation and afterwards slowly increased until reaching 42.92 ± 4.28% at day 35 of incubation. On the contrary, PRP value of the control soil slightly increased during incubation period and reached 19.75 ± 1.35% at day 35 of incubation.

Viable bacterial counts in crude oil treated soils with/without *B. subtilis* SE1 bioaugmentation were similar in the ranges of 5.40 ± 0.03 0-5.41 ± 0.02 log CFU/g at the beginning of incubation (Figure 3). At the end of experiment, bacterial count in crude oil contaminated soil without *B. subtilis* SE1 was 6.71 ± 0.01 log CFU/g, which was significantly ($P < 0.05$) lower than that of SE1 added soil (7.16 ± 0.04 log CFU/g). Similar to diesel oil biodegradation study, a significant increase in PRP value of SE1 treated soil with crude oil contamination was noticed by 3-day post-incubation. PRP level of crude oil treated soil with *B. subtilis* SE1 addition increased obviously during incubation period until reaching 29.55 ± 4.36% at 35 days of experiment. In contrast, PRP level of crude oil contaminated soil without SE1 strain increased comparatively slowly and reached 15.06 ± 4.33% at the end of incubation.

Value of PRP in the control and treated groups related with used engine oil was relatively similar in the ranges of 0-9.95 ± 2.76% during the first 14 days of incubation. Thereafter, PRP value of soil polluted with used engine oil and added with SE1 significantly increased to 22.62 ± 3.70% at 35 day post-treatment while a slight change in PRP value occurred in soil polluted with used engine oil without SE1 added at the end of experiment (16.39 ± 1.39%; Figure 4). Viable bacterial count in used engine oil contaminated soil with SE1 addition significantly increased ($P < 0.05$) from 5.50 ± 0.05 log CFU/g at the beginning of experiment to 7.34 ± 0.03 log CFU/g at 35 days post-incubation.

DISCUSSION

Bioaugmentation of *B. subtilis* SE1 exhibited an effective option to bioremediate the hydrocarbon polluted soils evident by significant growth of viable bacteria in the soils soaked with one of these petrochemicals: gasoline, diesel oil, crude oil and used engine oil, and increase in PRP values during a 35-day incubation. Growth of petroleum degrading bacteria in polluted soils after inoculation is important factor to facilitate biodegradation success (Das and Mukherjee, 2007). Due to *B. subtilis* SE1 isolated from soil soaked with used engine oil, it survived and adapted to grow well with hydrophobic substrates as sole carbon

Table 1. pH of petroleum polluted soils with or without *Bacillus subtilis* SE1 bioaugmentation for 35 days.

Petroleum products	Beginning of experiment		35 days post-treatment	
	None (Control)	SE1 bioaugmentation	None (Control)	SE1 bioaugmentation
Gasoline	5.63 ± 0.02[a]	5.83 ± 0.03[b]	5.59 ± 0.02[a]	5.84 ± 0.04[b]
Diesel oil	5.55 ± 0.04[a]	5.67 ± 0.06[b]	5.58 ± 0.02[a]	5.75 ± 0.01[b]
Crude oil	5.42 ± 0.05[a]	5.40 ± 0.02[a]	5.63 ± 0.02[a]	6.03 ± 0.01[b]
Used engine oil	5.37 ± 0.04[a]	5.37 ± 0.03[a]	5.61 ± 0.01[a]	6.03 ± 0.01[b]

Letters indicate significant difference ($P < 0.05$) between treatments at each sampling interval.

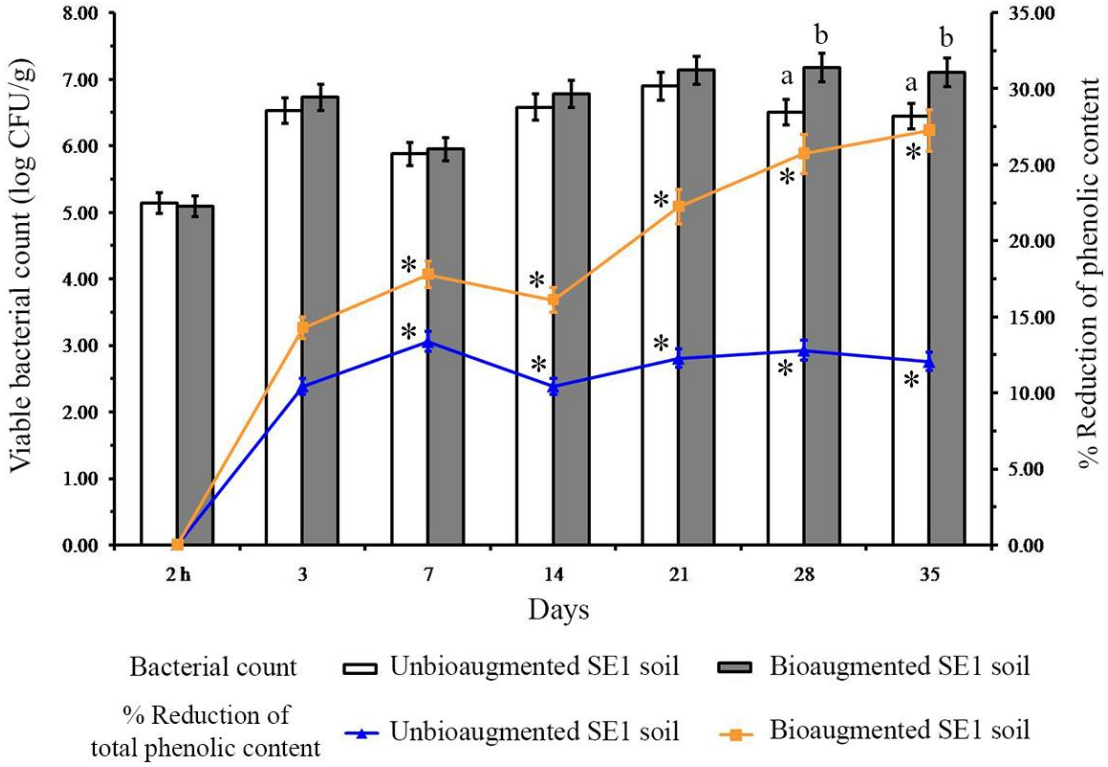

Figure 1. Bacterial growth and percent reduction of total phenolic content in gasoline contaminated soil during 35-day incubation. Asterisks on the lines indicate significant difference (P < 0.05) between treatments at each sampling interval. Letters on the bars indicate significant difference (P < 0.05) between treatments at each sampling interval.

source as reported by other authors (Das and Mukherjee, 2007). A significant increase in viable bacteria over the controls indicated that indigenous microflora of soil microcosms lacked ability to degrade a variety of hydrocarbons in petroleum and petrochemicals used in this study. Therefore, addition of hydrocarbon-utilizing bacteria is needed to accelerate biodegradation rate of a complex hydrocarbon mixture.

Substantial increases in PRP values were produced when inoculating *B. subtilis* SE1 into oil polluted soils. Similarly, several authors reported bioaugmentation of lipopeptide producing *Bacillus* species for biodegradation of crude oil by *B. subtilis* DM-04 (Das and Mukherjee, 2007), *B. subtilis* TB1 (Barin et al., 2014); gasoline by *B. subtilis* (Darsa et al., 2014) and diesel oil by *B. subtilis* ATCC 21322 (Whang et al., 2008). Many *Bacillus* strains were also isolated and reported to produce biosurfactant simultaneously with biodegradation of hydrocarbons in used engine oil e.g. *B. subtilis* CN2 (Bezza and Chirwa, 2015) and *Bacillus salmalaya* 139SI (Dadrasnia and Ismail, 2015). Differences in PRP values were observed when *B. subtilis* SE1 bioaugmented in soils contaminated with each type of petrochemicals. In general, biodegradation rate of petroleum hydrocarbons is dependent mainly on hydrocarbon compositions in petroleum and physicochemical characteristics of polluted systems.

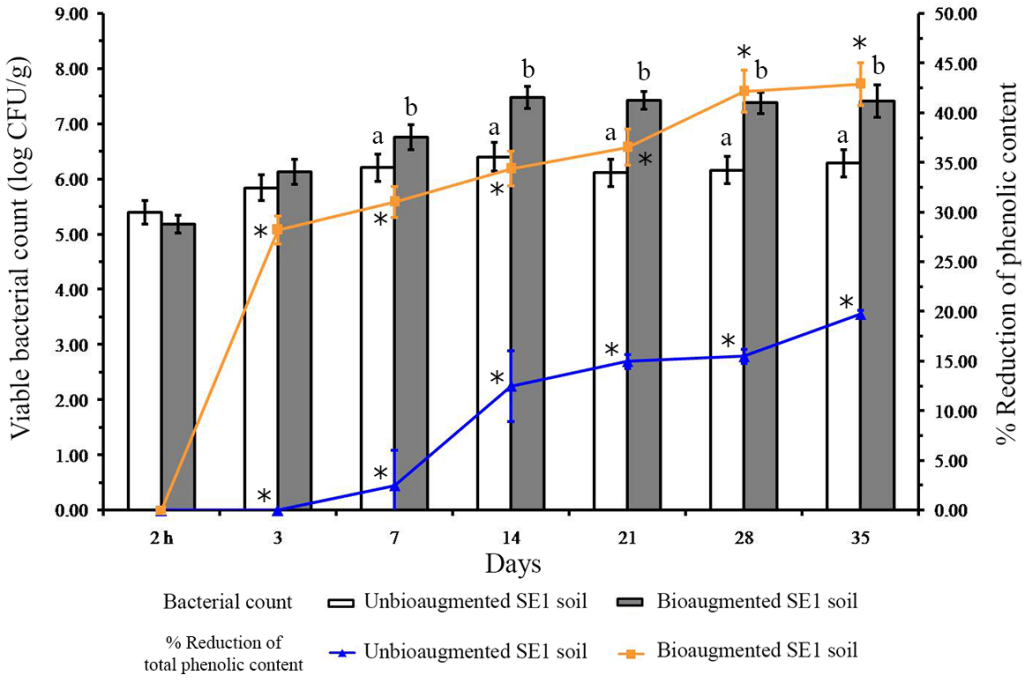

Figure 2. Bacterial growth and percent reduction of total phenolic content in diesel contaminated soil during a 35-day incubation. Asterisks on the lines indicate significant difference ($P < 0.05$) between treatments at each sampling interval. Letters on the bars indicate significant difference ($P < 0.05$) between treatments at each sampling interval.

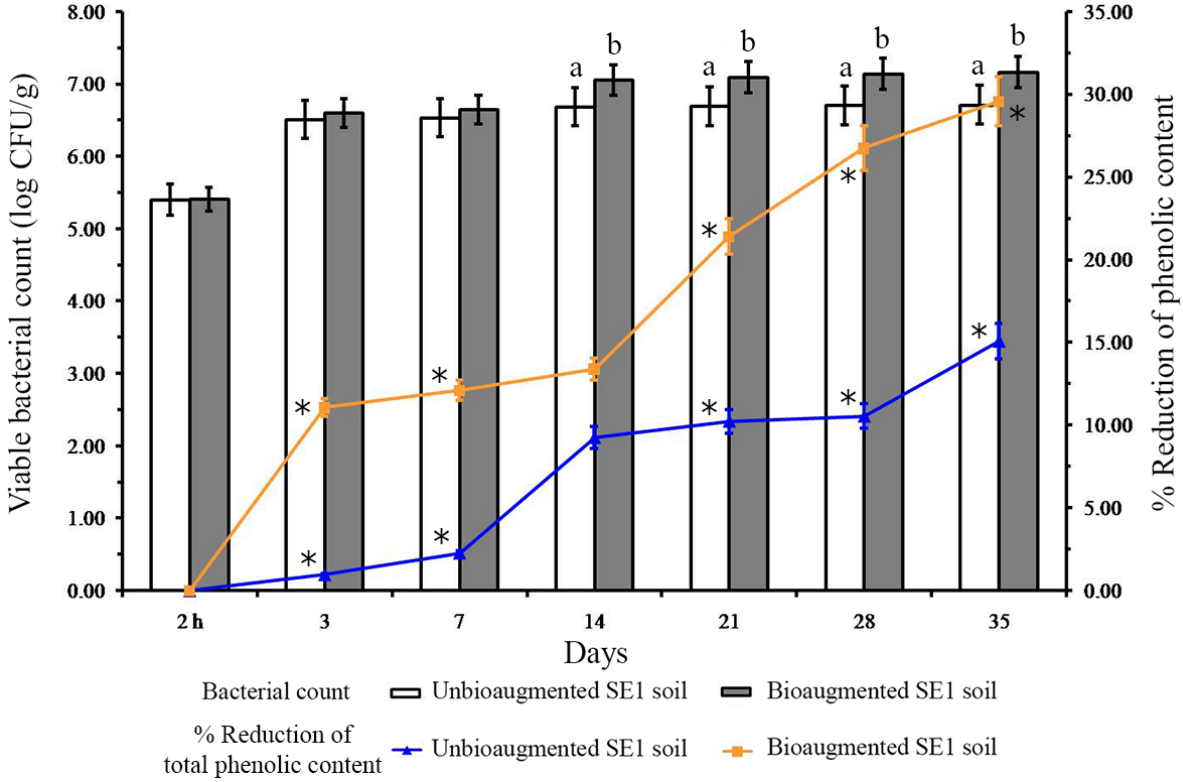

Figure 3. Bacterial growth and percent reduction of total phenolic content in crude oil contaminated soil during 35-day incubation. Asterisks on the lines indicate significant difference ($P < 0.05$) between treatments at each sampling interval. Letters on the bars indicate significant difference ($P < 0.05$) between treatments at each sampling interval.

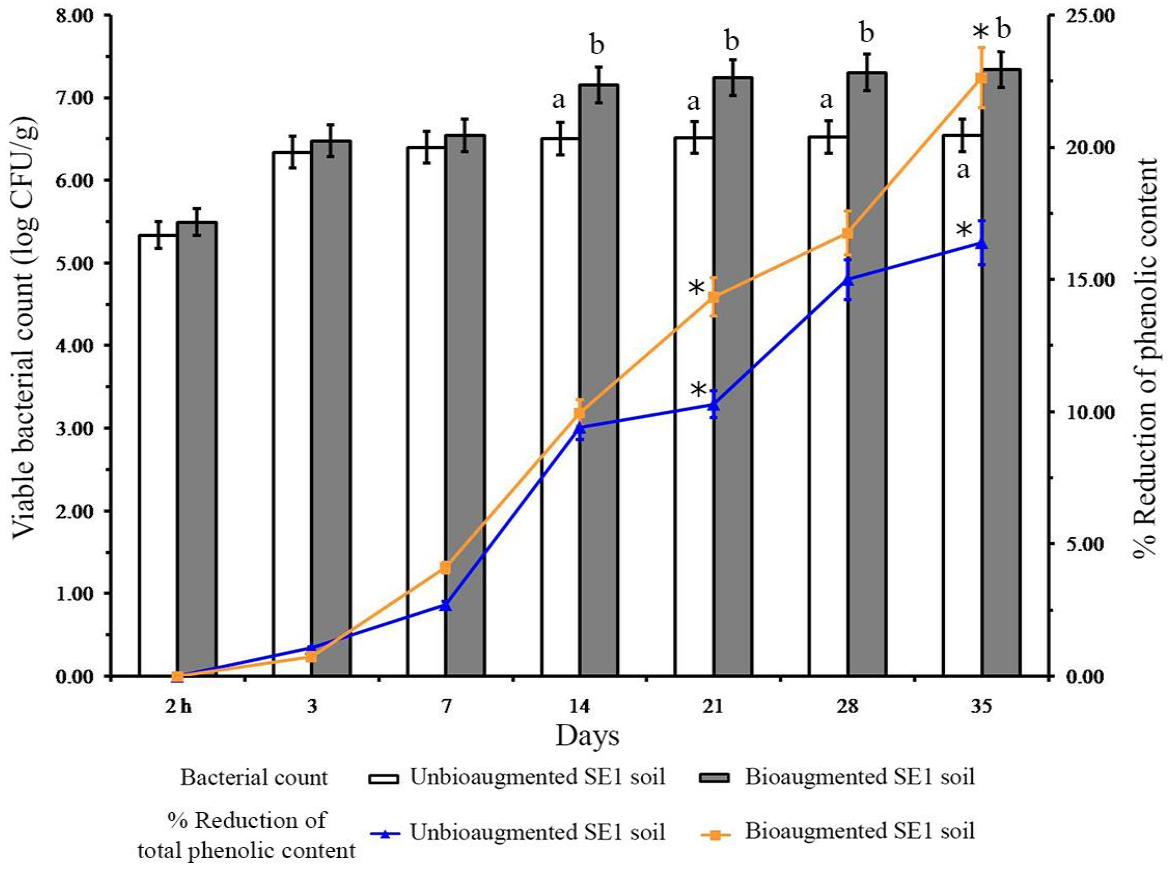

Figure 4. Bacterial growth and percent reduction of total phenolic content in used engine oil contaminated soil during 35-day incubation. Asterisks on the lines indicate significant difference ($P < 0.05$) between treatments at each sampling interval. Letters on the bars indicate significant difference ($P < 0.05$) between treatments at each sampling interval.

Petroleum and petrochemical products consist generally of different hydrophobic compositions. For example, diesel oil constitutes a complex mixture of hundreds of aromatic hydrocarbons, predominantly iso-alkanes, paraffinic, olefinic, naphtha and aromatic compounds (Yakimov et al., 2005) while used engine oil contains a variety of recalcitrant compounds, like alkyl benzenes, naphthalenes, methylnaphthalenes, PAHs and metals (Dominguez-Rosado and Pitchell, 2003; Lu and Kaplan, 2008). Marchut-Mikolajczyk et al. (2018) reported that *B. pumilus* 2A was an effective hydrocarbon degrader of both diesel oil and waste engine oil but exhibited different degree of diesel oil and engine oil degradation. In addition, 2A strain showed different degradation efficiency of each hydrophobic component found in the two petrochemical products.

Our recent study confirmed that bioaugmentation of *B. subtilis* SE1 together with nutrient biostimulation enhanced substantially biodegradation of gasoline contaminated in soil. In fact, supplementation of biosurfactant producing bacteria into polluted sites may provide more practical than addition of exogenous biosurfactant produced in fermentation reactors because of avoiding high cost arising from production and preparation of purified biosurfactants (Mnif et al., 2015). However, *in situ* biodegradation success of hydrocarbons depends on the selection of biosurfactant producing bacteria. In our study, all four petroleum and petrochemical products seemed to not have a harmful effect on bacterial growth. The growth of bacteria together with increase in PRP levels is possibly due to enhancing bioavailability and solubility of hydrocarbons. Biosurfactant can increase solubilization of hydrophobic compounds and enhance rate of biodegradation by two distinct ways. First, biosurfactants increase substrate bioavailability by increasing the surface area of immiscible hydrophobic substances leading to increased solubility and enhanced direct contact between bacteria and water-insoluble hydrocarbon, thereby increasing bacterial growth and rate of bioremediation. Another mechanism is associated with increased hydrophobicity of bacterial cell surfaces allowing hydrocarbon substrates to easily pass through bacterial cells (Bezza and Chirwa, 2015). In our recent study, we observed that lipopeptide produced by *B. subtilis* SE1 had high emulsifying activity and markedly reduced surface tension from 72.27 to 25.95 mN/m. Therefore, addition of *B. subtilis* SE1 with high surface tension reduction and emulsification index into hydrocarbon contaminated soils could be enough to promote the bacterial access to

hydrophobic substrates and eventually increase biodegradation of all four tested petroleum in soil systems.

Apart from increase in bioavailability of hydrophobic substrates, *B. subtilis* SE1 may produce hydrocarbon degrading enzymes resulting in improved biodegradation success in this study. It is widely known that enzymes responsible for hydrocarbon biodegradation pathways are low substrate-specific and can react with more than one hydrocarbon substrates. For example, cycloalkanes are structurally changed to their corresponding cycloalhohols or cycloketones, easily degraded by a number of bacteria in soil microcosms, by initially induced by the alkane monooxygenases (Barin et al., 2014). Therefore, presence of contributed enzymes in hydrocarbon biodegradation pathways of *B. subtilis* SE1 should be further studied.

This study encourages the application of biosurfactant-producing bacteria for *in situ* bioremediation of petroleum-contaminated environments because bioremediation process is cost-effective and environmental-friendly. Our results provided evidence that a stain of lipopeptide producer, *B. subtilis* SE1, isolated from oil-impacted soil and accustomed to environmental conditions in Thailand, enhanced significantly PRP values and promoted the growth of soil bacteria in petroleum and petrochemical polluted soils. Therefore, *B. subtilis* SE1 has a potential use for bioremediation of soil environment polluted with petroleum and petrochemical products in Thailand and may have possible applications in microbial enhanced oil recovery and related technologies.

Conclusion

This study showed that *B. subtilis* SE1 was useful in bioremediation of soils polluted with petroleum and petrochemicals (gasoline, diesel oil, crude oil and used engine oil) because growth of soil bacteria markedly increased in petroleum contaminated soils along with significant increase in PRP levels during 35-day incubation.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Nano Selenium: Reduction of severe hazards of Atrazine and promotion of changes in growth and gene expression patterns *on Vicia faba seedlings*

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Atrazine is a widely used herbicide on many crops and is considered one of water pollutant with approved biological hazards on plants, animals and human. *Vicia faba* seedlings as a biological system, is used to investigate the genotoxicity of Atrazine. Also, Nano selenium (N-Se), a Nano particle with reactive oxygen species scavenging activity was applied to reduce the genotoxicity of Atrazine. Atrazine treatment at concentration of 35 mg/L is applied. Two concentrations of N–Se (10 and 20 ppm) were used alone and in combination with Atrazine (35 mg/L) in addition to control treatment. Changes in germination percentage, shoot and root length, hydrogen peroxide (H_2O_2) content, lipid peroxidation product malondialdehyde (MDA), chromosomal aberrations, and mitotic index were determined. Semi quantitative RT-PCR analysis (sqRT-PCR) was applied to investigate associated changes in expression pattern of some stress related genes such as antioxidant enzymes, heat shock proteins (*HSP17.9*, *HSP70.1*), photosystem II (PSII) and Metalothioniene (MT). Atrazine treatment recorded the lowest germination percentage and caused a reduction in shoot and root length. Significant increase in H_2O_2 value, MDA contents, and chromosomal abnormalities percent resulted in Atrazine treatment. Noticeable suppression in expression level of all studied genes was accompanied with Atrazine treatment. N–Se, in its two concentrations with Atrazine causing a reduction in all severe effects of Atrazine and improving seedlings performance. Treatments with N–Se induced the expression of *MT* gene with increase in expression level alongside increase in the concentration of N-Se. This is one of rare studies that investigate the biological effects of N–Se *in vivo* anti-mutagenesis of Atrazine as well as a first record of Nano metalic particles N–Se as inducer for MT genes.

Key words: Nano Selenium, Atrazine, chromosomal aberrations, gene expression, *Vicia faba*.

INTRODUCTION

In agriculture, increasing attention is paid to beneficial impacts of nanoparticles (NPs) which applied in low doses on various crops (Jampílek and Kráľová, 2017). Using of NPs can enhance plant growth, guarantee food quality and minimize the waste (García et al., 2010; Sonkaria et al., 2012; Prasad, 2014; Sekhon, 2014).

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Nano–Selenium (N–Se) is recently used in the field as Nano fertilizer (Gao et al., 2002). A few studies have been published concerning the comparison between N–Se and other inorganic Se forms in higher plants (Domokos-Szabolcsy, 2011; Domokos-Szabolcsy et al., 2012). Also, less information are documented about biological effects of N–Se and its application (Chau et al., 2007; Cushen et al., 2012; El-Ramady et al., 2016). The suggested role of N-Se as reactive oxygen species (ROS) scavenger (Bhattacharjee et al., 2014; Sarkar et al., 2015) pointed to the possible application of this promising Nano material to remove the deleterious effects of different stresses.

Indiscriminate use of pesticides and herbicides in agriculture causes many disorders in human and animal health and do pose a potential risk to humans and unwanted side effects to the environment (Aktar et al., 2009). Atrazine (2–chloro–4–ethylamino–6–isopropylamino – 1,3,5 –triazine) is one of triazine class herbicide that is widely used to prevent emergence broadleaf and grassy weeds in variety of crops such as sorghum, pineapple, maize, sugarcane (Kumar and Srivastava, 2015). It is considered as one of the most common contaminants in ground and surface waters (Ribaudo and Bouzaher, 1994; Ali et al., 2016).

Several studies indicated the genotoxicity of Atrazine (Srivastava and Mishra, 2009). Significant increases in DNA strand breaks and frequencies of micronuclei occurred in erythrocytes of *C. auratus* exposed to Atrazine (Cavas, 2011). It is widely separated where it transports from the site of use to areas as far as 1,000 km via atmospheric transport and deposition through precipitation (Mast et al., 2007; Thurman and Cromwell, 2000). Atrazine ecotoxicology effects have been indicated by several studies (Song et al., 2009; Bolle et al., 2004); the European Union banned the use of Atrazine in 2004 because of its contamination of water sources (Commission, 2004)

The present study was designed to investigate the protective role of synthesized N–Se on *V. faba* seedlings treated with Atrazine. Our investigation tracked the associated changes in oxidative stress that occurred in the plant tissue and examining the cytological effects of the herbicide Atrazine with respect to mitotic index, chromosome aberrations and determination of changes in expression pattern using semi quantitative analysis of some stress related genes.

MATERIALS AND METHODS

Experimental method and growth environments

Fifty seeds of *V. faba* in equal size were used for each treatment. Seeds were surface sterilized with 2.5% sodium hypochlorite for 2 min. Sterilized seeds were washed with three changes of sterile distilled water and dried using sterile filter paper. The experiment was divided into six groups. One was left as a negative control where distilled water was used, while the second was considered as a positive control where 35 mg/L of Atrazine were added. The remaining four treatments received 10, 20 ppm of N–Se alone, and in combination with Atrazine.

Seeds at control and all other treatment were soaked for 24 h, and then were recovered in distilled water for one hour as recorded by Pandey and Upadhyay (2007). Germination and seedling development were carried out in five replicates of 10 seeds in a 15-cm diameter Petri dish. Petri dishes were lined with filter paper (Whatman No. 1) moistened with sterilized distilled water and incubated in dark at 25°C. The germination percentage was calculated after 24, 48 and 72 h by this formula:

$$\text{Germination percentage} = \frac{\text{Number of germinated seeds}}{\text{Total number of seeds}} \times 100$$

Seedlings growth was measured in terms of shoot and root length (cm) after seven days of germination.

Cytological analysis

Root tips (1.5-2 cm) of germinated seeds were cut and fixed in Carnoy's fixative solution (ethyl alcohol absolute and glacial acetic acid in the ratio of 3:1) for 24 h. Root tips are kept in 70% ethyl alcohol at 4°C until it is used for cytological analysis.

Aceto–carmine stain in concentration of 2% was used for cytological preparations as described by Darlington (1976). Mitotic index, numbers and types of abnormalities were scored in at least 3000 examined cells per treatment (1000 cell/replicate) using light microscope. Mitotic index (MI) and percentage of abnormal cells were calculated using the following formulas:

$$\text{Mitotic index (MI)} = \frac{\text{Total dividing cells}}{\text{Total dividing and non dividing cells}} \times 100$$

$$\text{Percentage of abnormal cells} = \frac{\text{Total abnormal cells}}{\text{Total dividing cells}} \times 100$$

Data analysis

Statistical package for social sciences (SPSS) software for windows version 20 were applied on obtained data for One-Way Analysis of Variance followed by Duncan test and the results were considered significant at $P < 0.05$.

Estimation of hydrogen peroxide (H_2O_2) content

Half gram of plant sample was homogenized in 5 mM of 0.1% (*w*/*v*) TCA and centrifuged at 12,000 rpm for 15 min to extract hydrogen peroxide as described by Velikova et al. (2000). Ten mM potassium phosphate buffer (pH 7.0) and 1 M potassium iodide were added to the supernatant. The absorbance of the supernatant was recorded at 390 nm. Pre-prepared standard curve was used for calculation of H_2O_2 content.

Evaluation of lipid peroxidation product

The concentration of TBA (thiobarbituric) -reactive products equated with malondialdehyde (MDA) was used to evaluate the level of lipid peroxidation as originally described by Heath and Packer (1968), with slight modifications as in Hendry (1993).

Table 1. The studied genes and actin as housekeeping gene: names, accession no (NCBI) and primers sequences.

Primer code	Gene	Accession no.		Primer sequence 5'----------3'
SO–101	*Actin*	JX444700.1	F	TGGAGATGATGCACCTCGTG
			R	CACGCTTAGACTGTGCCTCA
SO–102	*Catalase*	JQ043348.1	F	CGATGCTGTTCGTCATGCAG
			R	CAGGTGCCCAAGTTCGGTAT
SO–104	*Fe–SOD*	EU884308.1	F	TGAAAGAGACTTTGGTTCAGTTTGA
			R	GATTGCAAGCCATGCCCAG
SO–105	*Cu/Zn-SOD*	EU884303.1	F	CCGAGGATGAGACTAGACATGC
			R	CATCAGGATCGGCATGGACA
SO–106	*HSP–17.9*	KC249973.2	F	TCGACATGCCAGGGTTGAAA
			R	CACAGCTGAAACAGCATCGG
SO–107	*HSP–70.1*	EU884304	F	GACCACCGGTCAGAAGAACA
			R	ACCCGCATTATCCTCAGACT
SO–109	*MT*	X91077.1	F	TCTGGCTGTGGTTGTGGAAG
			R	GGACCGAAGCCTAGAACCAC
SO–110	*PSII–D1*	KF042344.1	F	CGCCGAATACACCAGCTACA
			R	ATATTCAGCTCCCGTCGCAG

Extraction buffer of 50 mM phosphate buffer (pH 7.0) containing 1 mM EDTA, 0.05% Triton X-100, 2% (w/v) poly vinyl pyroolidone PVP and 1 mM AsA was used with plant sample. Two hundred microliters of supernatant were added to 0.5% TBA in 10% TCA. This solution was incubated in a 95°C water bath for 20 min followed by rapid cooling in an ice-water bath to stop the reaction. The products were quantified from the second derivative spectrum against standards prepared from 1,1,3,3–tetraethoxypropane. The amount of MDA was measured calorimetrically using spectrophotometer (UV190IPC) at 532 nm. The TBA-reactive products (MDA) were expressed as $nmol.g^{-1}DW$.

Total RNA extraction and cDNA synthesis

Total RNA was extracted from seedlings of control and all treated seedlings using Simply P Total RNA Extraction kit (BioFlux Cat#BSC52S1) according to manufacturer's procedure. RNA was analysed in 1.2% agarose gel with using RNase–free devices to assess RNA integrity. RNA extracts were diluted 1:10 in DEPC–treated water and RNA concentration was determined using NanoDrop spectrophotometer (BioDrop µLITE.UK). RNA purity values higher than 1.8 was considered acceptable. First–strand cDNA was synthesized using 5 µg of total extracted RNA for each sample according to the protocol supported by GoScript™ reverse transcription Kit (Promega USA) using Oligo $(dT)^{15}$ primer.

Semi–quantitative reverse transcriptase polymerase chain reaction (sqRT–PCR)

Normal PCR was used to amplify the number of copies of specific cDNA sequences *in vitro*. All primers used for sqRT–PCR is listed in Table 1. They were designed based on sequencing data of expressed sequence tags (ESTs) from *V. faba*'s database of selected genes on the website of National Center for Biotechnology Information (NCBI). Primers were designed using the Primer Primer 5 software following the manufacturer's guideline for primer design. Primers were ordered from Oligo Company. Samples of cDNA were standardized on actin transcript amount. Actin cDNA (accession no. JX444700.1) was used as an internal constitutively expressed control (reference gene) using gene specific primer in PCR (Table 1). For typical PCR reaction, 1 µL cDNA was used as template in 25 µL reaction volume according to instruction supported with MyTaq™ Red Mix 2x (BIOLINE). PCR program for sqRT–PCR was optimized for each gene to yield optimal contrast between samples in the fluorogrammes of subsequently performed EtBr-agarose gel electrophoresis. The general program was; 94°C for 5 min, followed by cycle of 94°C for 30 s, 55 to 57°C (Table 1) for 30 s, 72°C for 1 min, and last extension step of 72°C for 5 min. Amplification products (15 µL) were electrophoresed on 1.8% agarose gel stained with EB. For assessment of the changes in gene expression of different genes, integrative density values (IDV) was determined using Totallab© v13.2 soft wear.

RESULTS AND DISCUSSION

Effect of treatments on seed germination and seedlings growth

Data presented in Table 1 revealed that there was no significant differences in the germination percentage, the highest germination percentage of *V. faba* was detected after 72 h in N–Se 10 ppm, N–Se 20 ppm and Atrazine+N–Se20 compared with Atrazine which record the lowest germination percentage.

With respect to root and shoot lengths, results in Table 1 indicated that N-Se had significant effects in both 10 and 20 ppm for root length, but this effect did not differ

Table 2. Effect of Atrazine and N–Se treatments on germination (%) and root and shoot lengths (cm) of *V. faba*.

	Germination (%) 24 h	Germination (%) 48 h	Germination (%) 72 h	Root length (cm)	Shoot length (cm)
C	79.56± 12.98	90.35±11.22	93.05±8.86	5.83±2.70^{ab}	3.38±0.98
AT	70.91±11.78	85.45±11.21	89.58±9.40	4.25±1.23^{b}	2.84±0.60
N–Se10	73.97±25.38	92.85±12.19	93.75±10.45	7.20±3.09^{a}	3.10±0.73
N–Se20	78.57±18.70	91.07±9.44	93.75±10.45	6.93±2.55^{a}	2.74±0.69
AT+10	67.85±17.46	85.71±11.24	91.66±6.45	5.90±1.95^{ab}	2.93±0.68
AT+20	86.72±11.00	90.55±10.50	93.15±11.77	6.02±1.41^{ab}	2.65±0.47
	0.35	0.74	0.97	0.07	0.29

Values labeled with the same letter are not significantly differing at 0.05 probability level. (C) Control, (AT) atrazin, (N–Se10 and N–Se20) N–Se concentrations and (AT+10 and AT+20) are combination between Atrazine and N-Se.

when combined with Atrazine, the Atrazine treatment recorded the lowest value for root length.

In relation to shoot length, the data did not record significant effects and the N–Se 10 ppm showed the same value for control. From previous results, it can be concluded that N–Se (10) had the best record in both germination as well as shoot and root length. These results may be in agreement with Zhang et al. (2001) who reported that N–Se have a high biological activity, an excellent bioavailability and low toxicity.

Changes in H_2O_2 and MDA values

Atrazine treatment showed a significant increase in both of H_2O_2 (Figure 2A) and MDA (Figure 2B) contents in plant seedlings compared with control treatment. MDA is the decay product of poly unsaturated fatty acids of bio-membranes. Increasing of H_2O_2 and subsequently MDA contents clearly indicate the oxidative status of the cell (Omar et al., 2013). Thus, oxidative stress may be one of the potential mechanisms by which harmful effect of herbicide is occurred (Bongiovanni et al., 2012; Hassan and Alla, 2005). Increasing oxidative stress with Atrazine treatment was reported before in bean and maize (Hassan and Alla, 2005). Treatment with Ne–Se in its two concentrations caused a significant decrease in values of H_2O_2 and MDA contents than control values. Also, the combination addition of Atrazine with N-Se showed a significant reduction in H_2O_2 and MDA contents compared with treatment of Atrazine alone (Figure 2). These results showed the role of N–Se in reducing the oxidative stress of Atrazine. Decreasing oxidative stress could be a result of scavenger role of N–Se on different free radicals *in vitro* (Huang et al., 2003). Also, it has been reported that N–Se has a high efficiency in up-regulating seleno-enzymes (Wang et al., 2007). These results indicated that N–Se can serve as an antioxidant with reduced risk of Se toxicity (Zhang et al., 2007). Using N–Se alone or accompanied with Atrazine, causes an improvement in growth condition for plants which reflected on the growth rate in root and shoot lengths (Table 2), so it can help to reduce the severe effects of Atrazine.

Cytological effects on mitosis of *V. faba* root tips

Table 3 illustrated the effect of Atrazine and N–Se on mitotic index (%) and chromosomal aberrations in *V. faba* seedlings. Cytological analysis showed that the highest value of mitotic index (%) was scored in Atrazine+N–Se20; it showed significant (P< 5%) differences compared with negative and positive control. Application of N-Se alone did not exhibit any differences about negative and positive control but the combination between Atrazine and N–Se exhibited significant differences especially in high concentration of N–Se (Atrazine + N–Se20). In relation to chromosomal aberrations, Atrazine had genotoxic effects. The highest ratio for chromosomal aberrations compared to control and other treatments was in Atrazine, a result that agrees with Srivastava and Mishra (2009) who indicated that Atrazine may produce genotoxic effects in plants. Micronucleus, double nuclei, C–metaphase, lagging chromosome, break and disturbance were prevalent mitotic aberrations. Also, oxidative stress and DNA damage occurred on *V. faba* treated by Atrazine. Song et al. (2009) found significant differences after treatment with different doses of Atrazine compared to the controls in the Olive tail moments of single–cell gel electro-phoresis of root cells which are enhanced by Atrazine.

The combination between N-Se and Atrazine reduced the undesirable side effects of Atrazine; the results also indicated that the low concentrations of nanoparticles are better than the high concentrations alone or in combination with Atrazine. These results agree with the studies which indicated the protective effect of selenium nanoparticles against many materials induced cytotoxicity and genotoxicity effects, where N–Se caused significant reduction in chromosomal abnormality in bone marrow, and DNA harm in lymphocytes as well as bone marrow in mice treated with Cyclophosphamide-induced hepatotoxicity and genotoxicity (Bhattacharjee et al., 2014).

Expression patterns of selected genes

sqRT–PCR analysis showed a differential expression

Table 3. Mitotic index, percentage of mitotic phases, types and percentage of abnormalities of *V. faba* root tip cells under different treatments: control(C), atrazin (AT), N–Se (N–Se10 and N–Se20) and combination between Atrazine and N–Se (AT+10 and AT+20).

Treatment	No. of examined cells	No. of dividing cells	No. of abnormal cells	Mitotic phase (%)				Mitotic aberration (%)						Mitotic index (%)	Abnormalities (%)
				Prophase	Metaphase	Anaphase	Telophase	Micronucleus	C-metaphase	Laggard	break	Disturbance	Double nuclei		
C	3512	155	13	42.58	20.00	8.39	29.03		46.15		7.69	7.69	38.46	4.42±0.59^{c}	8.03±5.30^{b}
AT	3124	159	45	48.43	15.10	11.32	25.20	2.22	20.00	13.33	8.88	22.22	33.33	5.14±2.24^{bc}	26.68±10.14^{a}
N–Se10	3163	123	12	66.66	19.51	2.43	11.38		41.66				58.33	3.54±1.91^{c}	11.96±6.71^{b}
N–Se20	3173	135	22	77.77	8.15	2.96	11.11		27.27	4.54		9.10	59.10	4.23±0.88^{c}	17.18±6.22^{ab}
AT+10	3367	266	30	57.52	11.65	9.02	21.80	3.33	33.33	6.66	16.66		40.00	7.85±1.75^{ab}	11.73±3.01^{b}
AT+20	3183	337	50	71.81	7.12	6.82	14.24	4.00	34.00		8.00	30.00	24.00	10.56±1.54^{a}	14.42±3.59^{b}
														0.001	0.046

Values within columns followed by the same letter(s) are not significantly differing at 0.05 probability level.

pattern for all selected gene (Figures 3 and 4). In general, treatment with Atrazine caused suppression in the expression of all studied genes compared with control treatment. Analysis of IDV values (Figure 4) showed that expression level of all studied genes was clearly decreased compared with control treatment. Inhibition effect of Atrazine on antioxidant enzymes were recorded in broad bean and maize (Hassan and Alla, 2005). Thus, effect on antioxidant genes (*CAT, cu/zn–SOD and fe–SOD*) could explain the associated increase in H_2O_2 and subsequently MDA contents in Atrazine treated seedling (Figure 1). Reduction in PSII expression level could be as a result of oxidative stress induced by Atrazine. PSII expression reflects the photosynthesis activity of the cell. So, the reduction in PSII expression explains the reduction in growth rate as root and shoot's length. Damaging of photosynthesis apparatus PSI and PSII under oxidation stress has been reported at many plant species (Van Breusegem et al., 1999; Allakhverdiev et al., 2000; El-Shihaby et al., 2002). Treatment with N–Se in its two concentrations induced a great increase in expression level of all studied genes compared with both control and Atrazine treatments (Figures 3 and 4). That could explain the reduction of H_2O_2 contents accompanied with N–Se application. Also, the addition of N–Se to Atrazine treatment reduces the suppression effects of Atrazine on gene expression of all studied genes.

Two studied *HSPs* genes in this study showed different responses to Atrazine, where the one with high molecular weight (*HSP70.1*) was more affected by Atrazine treatment than that with low molecular weight (*HSP 17.9*). Also, (*HSP70.1*) showed less response to N–Se treatment than (*HSP17.9*). Metallothioneins (MTs) as low molecular weight metal binding proteins showed an increase in their expression along with increasing N–Se concentrations. Increasing the expression pattern of MTs was reported in sugarcane treated with graded concentration of Se (Jain et al., 2015). This result revealed that N–Se cause an induction of MTs expression. For authors' knowledge, this is the first investigation for ability of Nano particles to induce MTs expression.

Our results showed that treatment with N–Se either alone or with Atrazine cause a noticeable increase in the expression level of antioxidant genes and some protected genes such as *HSP17.9* gene, thus cause a reduction in oxidative stress and improve growth condition. For authors' knowledge, this is one of rare studies that investigate the biological effects of N–Se *in vivo* as growth stimulator, and could be the first record for N–Se anti-mutagenesis effect of Atrazine as an herbicide widely applied on many plants.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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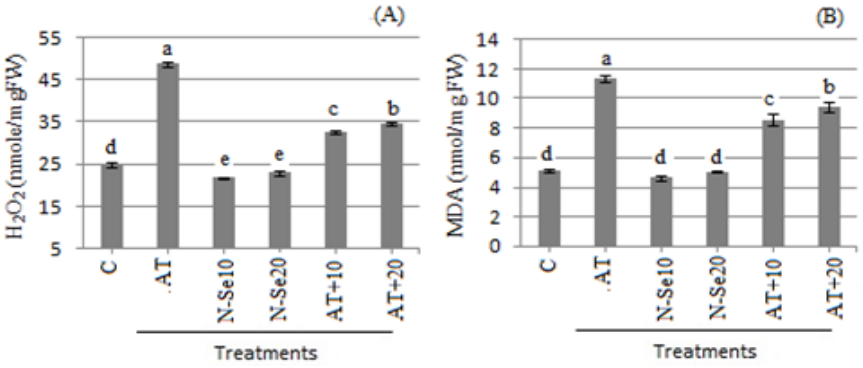

Figure 1. Changes in MDA content (A) and rate of electrolyte leakage (B) of *V. faba* seedlings under different treatments: control(C), atrazin (AT), N–Se (N-Se10 and N–Se20) and combination between Atrazine and N-Se (AT+10 and AT+20). Values are the main of three replicates ±SE. Values labeled with the same letter are not significantly differ at 0.05 probability level.

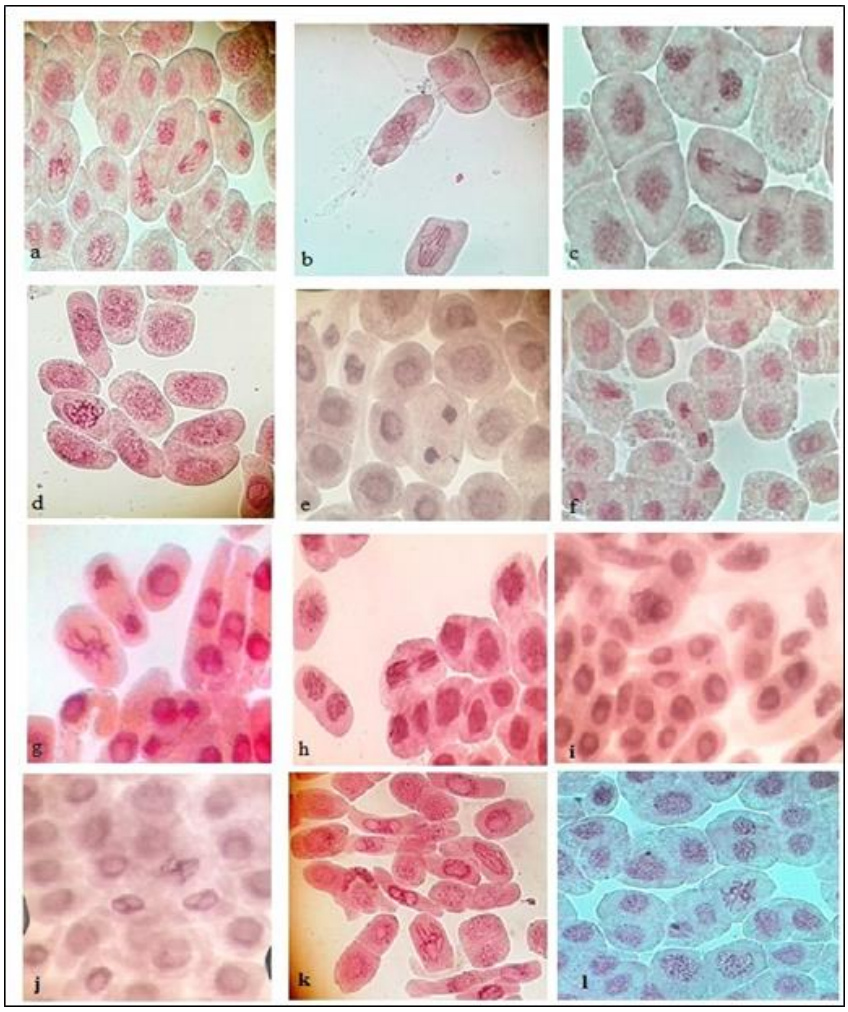

Figure 2. Types of abnormalities observed in *V. faba* root tips cells under different treatments: Control(C), atrazin (AT), N–Se (N–Se10 and N–Se20) and combination between Atrazine and N–Se (AT+10 and AT+20). (a) C–metaphase with fragment, (b) Disturbed metaphase, (c) Metaphase with laggard, (d) Sticky prophase, (e) Multiple bridges, (f) Telophase with single bridge and laggard, (g) Anaphase with chromosome breakage, (h) Anaphase with laggard (i) Micronucleus.

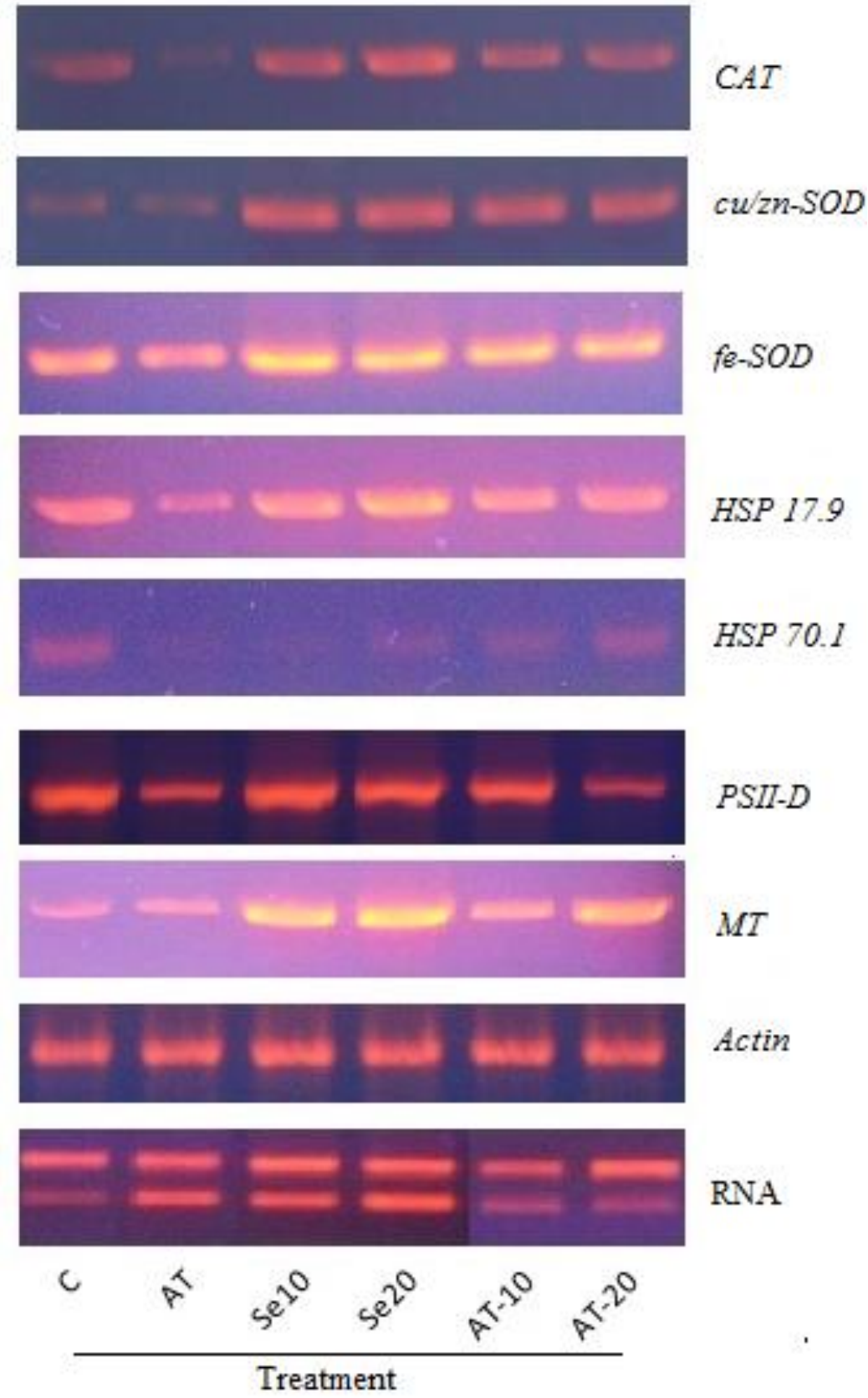

Figure 3. Changes in levels of expression of studied genotypes under different treatments: control(C), atrazin (AT), N–Se (N–Se10 and N–Se20) and combination between Atrazine and N–Se (AT+10 and AT+20).

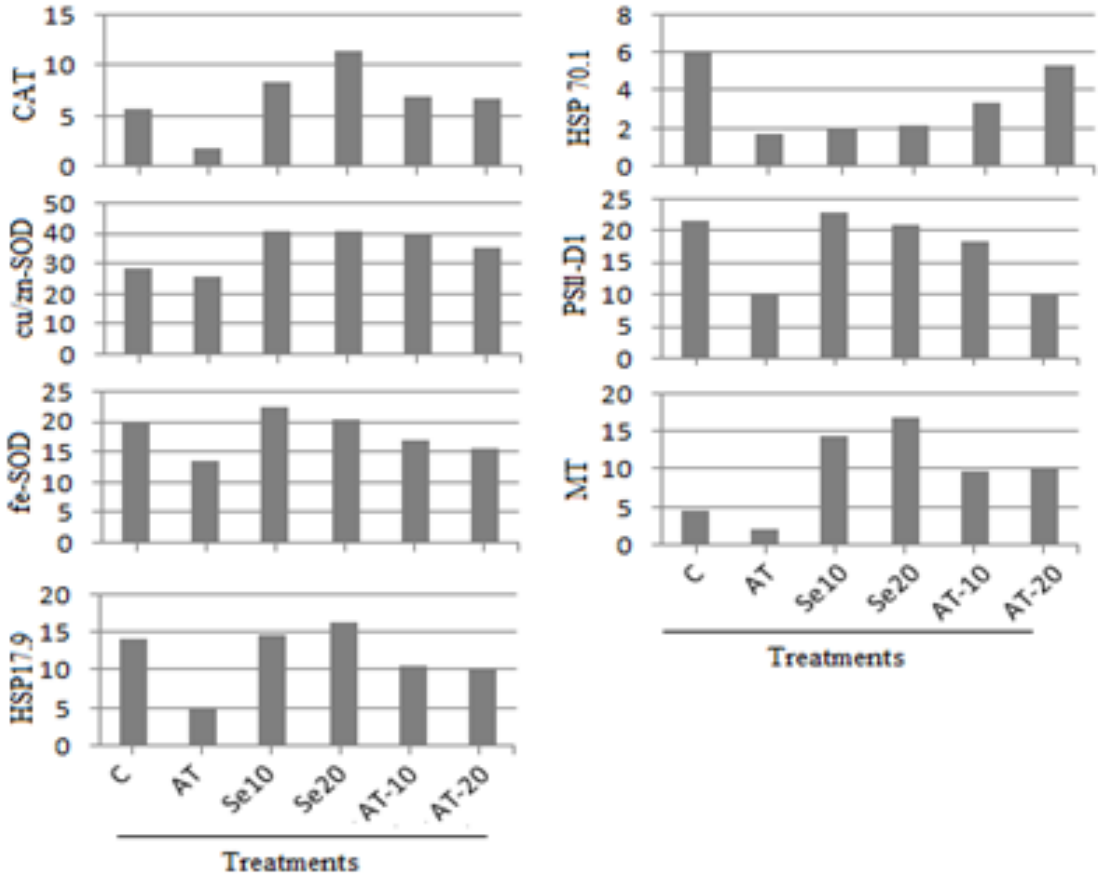

Figure 4. Changes in levels of expression of studied genes expressed as integrative density values (IDV) quantified using Totallab© v13.2 soft wear. IDV thus obtained was divided by 1000 for ease of writing on the y axis.

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