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Effective combination of *Lysinibacillus sphaericus* and Phytoremediation in Soil Contaminated with Chromium

Hung Nguyen Thanh[1] | Ha Nguyen Thi Ngoc[1] | Tra Mai Huong[2] ✉ *

1. Ho Chi Minh City Open University
2. Faculty of Food Science and Engineering, Lac Hong University, Vietnam

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ABSTRACT

In the framework of a project aiming to phytoremediate contaminated soils with heavy metal Cr in Long Khanh city, Dongnai province, Southeast of Vietnam, a series of greenhouse experiments followed by field trials were performed in order to evaluate the effect of *L. sphaericus* on the Cr phytoextraction by S. nigrum. The results showed that *L. sphaericus* improved the Cr uptake efficiency of S. nigrum through changing growth parameters such as root length, height, biomass and the ability to accumulate Cr in plants. At an application rate corresponding to the T3 treatment in this experiment, *L. sphaericus* can stimulate the dry biomass of S. nigrum by 143%, increase the Cr concentrations in the aerial part by 70%, the content of Cr extracted in a single plant up to 293% compared to treatment without *L. sphaericus*.

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INTRODUCTION

Studies have shown that Cr can cause severe biotoxicity in plants such as growth inhibition, biomass reduction, enzyme function changes (Qing et al, 2015), photosynthesis injury and nutritional interference (Handa et al, 2018), damage to membrane cell structures and chloroplasts (Zeng et al, 2011a), overproduction of reactive oxygen species, disturbances in antioxidant defense mechanisms (Singh et al, 2013). Cr increases soil ecological safety risks, including soil enzyme activity and microbial community in soil (Shahid et al, 2017; Yang L & cs. 2013; Huang Z & cs. 2014). All Cr (VI) compounds have been classified as human carcinogens by the International Agency for Research on Cancer (IARC). Therefore, Cr needs to be removed from the soil contaminated (Cadel L. M., 1988; Agency for Toxic Substances and Disease Registry, 1993).

Long Khanh city, Dong Nai province, in Vietnam is a perennial agricultural land, where Vietnamese famous fruit trees are grown (Long Khanh, 2022). However, the soil has been contaminated with Cr and exceeded the limits of Vietnamese agricultural land standards from 1.3 to 2 times (Hung Nguyen Thanh et al, 2021). Therefore, finding solutions to reduce Cr in soil to ensure safety for agricultural products is necessary and urgent (Hung Nguyen Thanh, 2021).

*Corresponding Author Email: *hungphuocan@gmail.com*

Currently, there are many methods of treating heavy metals in soil with different advantages and disadvantages such as: soil washing, chemical or physical immobilization of pollutants, heat treatment, ion exchange, oxidation or reduction of pollutants, transfering contaminated soil to suitable landfill sites. Most of these methods are expensive in terms of cost, limited technique and area (Glass, 1999; Vo Chau Tuan, 2012). Recently, using plants in combination with some chemical modifiers to enhance the heavy metal absorption and shorten time of treating heavy metals has been particularly interested by scientists because of low investment cost, safety and environmental friendliness through understanding the mechanism of absorption, metabolism, tolerance and heavy metal removal by some plants (Chaney, 1997; Lombi E, 2001). However, the use of chemical amendments could impact adverse on the soil microflora (Lasat, 2002). Romkens et al. found that chemical agents (EDTA) adversely affected enzyme activity in soil, EDTA decreased the number of nematodes (Romkens et al, 2002). To overcome these disadvantages, we screened and isolated plant growth promoting rhizobacteria (PGPR) capable of producing plant growth regulator IAA, siderophore and ACC deaminase enzyme which is important in plant growth and biomass, opening prospects of remediating contaminated soil by plants. Despite such potential, this method has not become commercially available technologies yet.

To evaluate the effectiveness of metal phytoremediation technology in soil, according to Doty, firstly the separation of metals from contaminated soil to aerial parts of plants was required (Doty, 2008). Therefore, BF (Nadan Kumar, 1995) and TF (Wei, 2006) values were very important to assess the heavy metal removal efficiency of the method using *L. sphaericus* combined with *S. nigrum* (Cruse, 1972).

In this study, small-scale field and pot experiments were performed using *Solanum nigrum* L. (*S. nigrum*), the native plant accumulating Cr in above-ground parts more than 1% mg/kg of dry biomass (Hung Nguyen Thanh et al, 2021), in combination with *Lysinibacillus sphaericus* (*L. sphaericus*) isolated in the study area, to evaluate the efficiency of heavy metal uptake and removal of Cr from contaminated soil, which is a promising and sustainable path.

MATERIALS AND METHODS

The soil was used in the experiment was collected from Long Khanh city, Dong Nai province, in Vietnam ($10^0$58'32.3"N, $107^0$18'19.5"E). Soils were collected from the experimental site from 0 to 20 cm depth at different points and a composite sample was made. The composite sample was air- dried, gently ground to pass through a 2-mm sieve, homogenised and used to fill the pots (10 kg soil per pot).

An aqueous cell suspension (10^8 cells ml^{-1}) of *L. sphaericus* isolated from Cr contaminated

Table 1. Properties of the experimental soil

Parameter	Units	Value[a:]
Soil type		Sepia clay
pH		5.02±1.07
N	%	0.11±0.01
P_2O_5	%	0.43±0.18
K_2O	%	0.10±0.02
OC	%	1.56±0.11
Easily digestible potassium	mg/100g	9.74±4.93
Easily digestible phosphorus	mg/100g	23.5±17.7
Cr	mg/ kg	263.8±7.98

a: Mean± Standard Error (each analysis was performed three times).

Table 2. Soil contaminated with Cr supplemented *L. sphaericus* % (v/w) in pot experiment

	Soil contaminated with Cr (263.8 mg/kg soil)	***L. sphaericus* % (v/w)**	**Formula in pot experiment (10kg soil/ pot)**		
1	Polluted soil	0	CK	CK	CK
2	Polluted soil	5	T1	T1	T1
3	Polluted soil	10	T2	T2	T2
4	Polluted soil	20	T3	T3	T3

Table 3. Concentration of *L. sphaericus* in small-scale field experiment

TT	**Concentration of *L. sphaericus* % (v/v)**	**Plot with size 5m x 5m**
1	0	CK2
2	5	T4
3	10	T5
4	20	T6

soil in Long Khanh city, Dong Nai province, was used as amendments by thorough mixing with the soil.

One month before the experiment, *S. nigrum* seedlings were cultivated as follows: locally obtained seeds were uniformly sown in a seedling plate and propagated in a greenhouse, maintained under humid conditions: natural sunlight, temperature 25°C, relative humidity 40–60%. Seedling propagation was continued for about a month until 4–6 mature leaves.

Twelve experimental pots with a diameter of 45 cm and a depth of 35 cm were each contained 10 kg of contaminated soil. Five seedlings of *S. nigrum* L. with 4-6 mature leaves were planted in each pot, watered once a day to ensure 80% moisture in the soil. The experiment was repeated 3 times as shown in Table 2.

The experimental pots were grown in greenhouse from Febuary to May 2021 covered with a shading net, not exposed to natural light with an average annual temperature of 24 - 29°C, an average number of sunny hours of 4-9.5 hours/day, and an average annual humidity of 80-82%. After 90 days of planting, samples were collected and analized.

At the same time as pot experiment, more than 300m^2 of soil at the experimental site (10.98774, 107.23434) was plowed (25cm), then it was divided into independent experimental plots (5m x 5m). Plots were treated with *L. sphaericus* (aqueous cell suspension diluted in water as shown in Table 3 then sprayed on the soil). On February 10th, 2021, *S. nigrum* seedlings were planted in experimental plots with a distance of 10cm and harvested after 120 days from sowing. The experiments were performed in triplicate.

The bulk soil was removed and the roots were carefully washed with irrigation water to maintain the root system integrity, and the tap-root length and the plant height (the aerial part) was measured using a flexible tape. Plants were separated into roots, stems and leaves. Each component was rinsed with tap water to remove remaining surface dirt, and carefully washed with deionized water. The fresh samples were weighed after the surface deionized water had completely evaporated, and then oven-dried at 120^{0}C for 30 min, then at 70^{0}C until constant weight was obtained and then ground to powder, sent for Cr analysis (Le Van Khoa, 1996; Lu, R. K, 2000).

Soil samples, after being collected, was dried in a sunny, well-ventilated place, ground and 2mm-sieve, continuously ground and sieved through a 0.25mm-sieve, then sent for analysis of heavy metals (Le Van Khoa, 1996; Lu, R. K, 2000).

Determine the content of Cr: weighing 0.2000 - 0.2500 g of the sample into a 25 ml PTFE

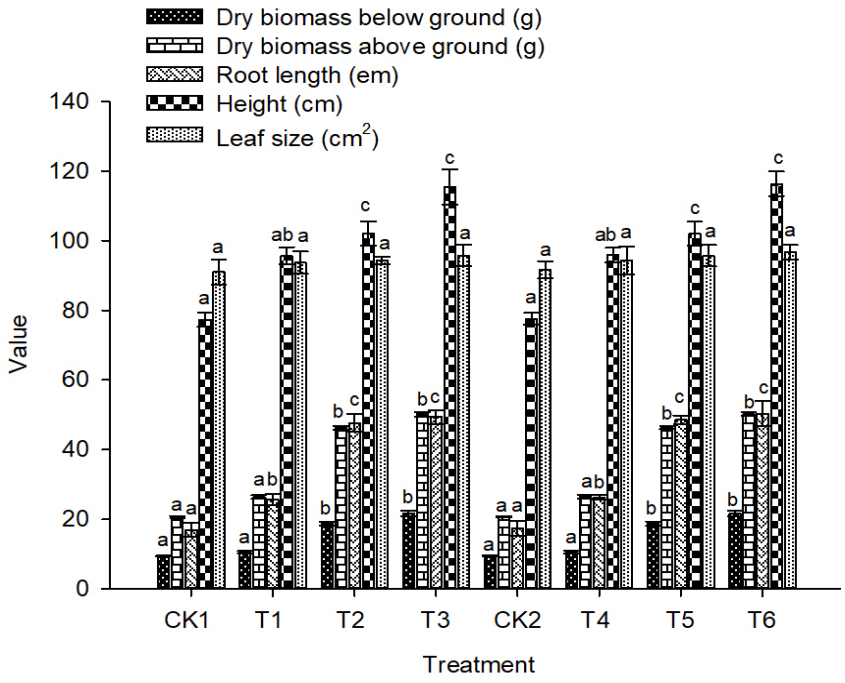

Fig. 1. Physiological effects of *L. sphaericus* on *S. nigrum*

digestion tube, adding 9 ml of HNO_3 and 3 ml of $HClO_4$, and putting it in the oven at 130°C to decompose. After boiling for 1 hour, the solution was added 5 ml of HF to continue decomposition until yellow or colorless solution appeared in the tube. Using distilled water Mili-Q to transfer all solution in PTFE tube to a 10 ml plastic tube, shaking well. The Cr content was determined using a Perkin Elmer model AA-400 spectrophotometer (Perkin Elmer Corporation, USA) (Le Van Khoa, 1996; Lu, R. K, 2000).

The Bioconcentration Factor (BF) of each metal in plants was calculated by dividing the total content in shoots by the total content in soil (Brooks, 1998). Further, the Translocation Factor (TF) was calculated by dividing the total metal content in shoots by the total metal content in roots (Brooks, 1998). Both factors were calculated on a dry mass basis.

Data were expressed as Means ± SD, and the statistical significance of the differences between groups was evaluated by analysis of variance (ANOVA) at $p < 0.05$. The Pearson correlation coefficients were calculated to examine the relationships with 95% confidence intervals.

RESULTS AND DISCUSSION

The effect of *L sphaericus* on the growth of *S. nigrum* was shown in Figure 1. In Cr-contaminated soil, the height and biomass of *S. nigrum* plants grown in green house pots compared to small-scale field were the same, which was no statistically significant difference. In treatments supplemented with *L. sphaericus* (T1, T2, T3, T4, T5, T6), we found that adding *L. sphaericus* increased significantly the growth parameters such as height, biomass, root length of *S. nigrum* grown in pots and small- scale field, except leaf size. Specifically, the root length and the height in treatments T2≈ T5 and T3≈ T6 increased by 180% and 190%, 32% and 50%, respectively, leading to an increase in biomass considerably. The biomass of plants increased by 143% (T3 ≈ T6), ie doubled compared with the treatment without adding *L. sphaericus* whereas it increased by 24% and 128% (T1 ≈ T4, T2 ≈ T5 treatment) respectively.

In another of our studies, we found that *L. sphaericus* bacteria are able to produce plant

growth regulators IAA, promote cell division and form branch roots to help plants grow about root length and height (Hung Nguyen Thanh et al, 2021). This finding was consistent with A. M. Aguirre's study "*L. sphaericus* is a thermophilic, Gram-positive bacterium, used to improve soil during replanting thanks to its ability to fix nitrogen, nitrify and dissolve phosphorus, increase soil nutrients, produce indole acetic acid (IAA) to helps plants grow" (AM Aguirre-Monroy et al, 2019). Chaudhry and Egamber said that indole acetic acid (IAA) is capable of increasing root length and shoots for plants living in heavy metal polluted environments (Chaudhry and Rasheed, 2003; Egamber dieva, 2009).

Different letters on the top of the bars indicate they are significantly different from each other at P< 0.05. The CK1, T1, T2, T3, CK2… and T6 stand for the treatment with solution application concentrations: 0, 5, 10 and 20%, respectively.

From the obtained study results, it was confirmed that *L. sphaericus* enhances plant growth, protects *S. nigrum* from Cr toxicity, increase biomass and bioaccumulation capacity in plants.

Statistical analysis indicated that Cr concentration accumulated in the above-ground part (stems, leaves) in treatments T1, T2, T3 and T4, T5, T6 increased significantly (Table 4) and proportionately to the amount of *L. sphaericus* added in soil with the Pearson correlation coefficients were 0.7330 and 0.7261, respectively. Similarly, the amount of Cr extracted by a *S. nigrum* plant was also proportional to the concentration of *L. sphaericus* supplemented in soil with the Pearson factors were up to 0.9322 and 0.9300. This proved that *L. sphaericus* enhanced Cr uptake by *S. nigrum* efficiently.

Data are means ± SD (n=5). One-way ANOVA was performed for each parameter. Different letters following data within the same column mean concentrations of Cr are significantly different at P<0.05. The CK1, T1, T2, T3… and T6 stand for the treatment with solution application concentrations: 0, 5, 10 and 20%, respectively.

It is well-known that plants take up metal ions through membrane transporters, which makes possible the movements of metal ions across the cell membranes (López et al, 2005). Our study suggested that *L. sphaericus* produces auxin which can induce the activation of the ATPases in the plasma membrane, create changes in the transport of metal ions through the membrane (Altabella et al, 1990). The influence of *L. sphaericus* on Cr uptake and accumulation by *S. nigrum* was shown in Table 4. In treatment T3 ≈ T6, Cr concentrations in roots increased by 51%; in leaves by 68% and in stem by 71% as well as the amount of Cr extracted from a single plant increased manifestly by 293% compared with control.

The results of this study showed that no significant difference was detected between the TF values in the formulas (Figure 2), which proved that *L. sphaericus* did not affect TF coefficient of *S. nigrum* plants. However, the BF value increased in all the treatments supplemented with *L. sphaericus*, especially for T3 ≈ T6 increased by 70%.

Table 4. Influence of *L. sphaericus* application on Cr uptake and accumulation of *S. nigrum*

Treat.	Cr concentrations in plants (mg/kg)			Cr extracted by a single plant (µg)
	Stem	Leaf	Root	
CK1	21.63±0.20a	21.82±0.07a	16.36±0.50a	1796.19a
T1	35.63±0.36b	35.63±0.58b	24.26±0.60b	3525.27b
T2	36.29±1.19c	36.29±1.17bc	24.36±0.45bc	6297.55c
T3	36.93±0.40c	36.76±0.67c	24.73±0.06c	7066.14d
CK2	21.64±0.18a	21.83±0.08a	16.37±0.49a	1801.88a
T4	35.79±0.63b	35.78±0.60b	24.27±0.58b	3545.76b
T5	36.49±1.16c	36.54±1.13bc	24.48±0.48bc	6344.43c
T6	36.94±0.41c	36.78±0.71c	24.74±0.05d	7080.59d

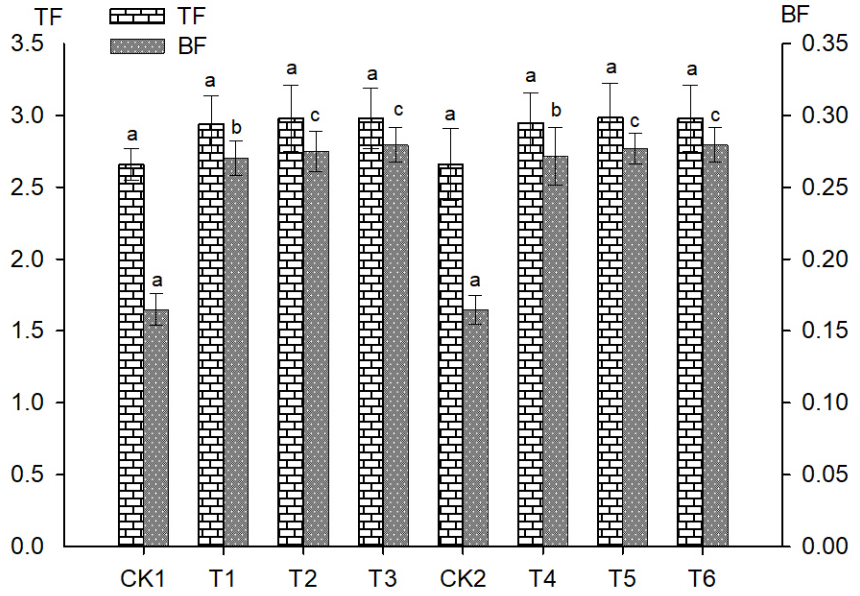

Fig. 2. The bioaccumulation factor (BF), translocation factor (TF) values in *S. nigrum* under different treatments

Different letters on the top of the bars indicate they are significantly different from each other at P< 0.05. The CK1, T1, T2, T3, CK2… and T6 stand for the treatment with solution application concentrations: 0, 5, 10 and 20% (v/ w), respectively.

According to Tu and Ma, if TF is more than 1 plant species, has high capacity for transporting heavy metals (Tu and Ma, 2002). Baker et al. suggested that plants are called Cr-hyperaccumulator when they can store Cr content in the above-ground parts 0.1% higher than dry weight, regardless of the amount of metal present in soil (Baker and Brooks, 1989). TF values were always greater than 1 (TF >1) and the amount of Cr accumulated in above-ground parts of *S. nigrum* always was 0.1% higher than the dry weight of the plant (Fig. 2 and Table 4). These results once again confirmed that the combination of *L. sphaericus* with *S. nigrum* improved the removal efficiency of Cr from the soil, which fully meets the requirements of metal phytoextraction technology.

With a planting density of 10cm x 10cm plants/m2, time to bring the soil back to the safe threshold established by the Vietnamese law will be 4 years instead of 15 years if *L. sphaericus* is not added. Therefore, the use of *L. sphaericus* in combination with *S. nigrum* to enhance the Cr uptake efficiency and shorten the treatment time of contaminated soil is feasible and promising.

CONCLUSIONS

The effect of *L. sphaericus* on the growth and Cr uptake by *S. nigrum* grown in pot experiments and small-scale field was similar, no statistically significant difference.

L. sphaericus could increase the dry biomass of *S. nigrum* by 143%, stimulate the Cr uptake in the above-ground parts to 70%. Furthermore, the Cr content was extracted from the soil by a plant up to 293%. Due to this, phytoremediation by *S. nigrum* can be considered the appropriate choice for soil contaminated with Cr in the studied area.

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CONFLICT OF INTEREST

The author declares that there is not any conflict of interests regarding the publication of this manuscript. In addition, the ethical issues, including plagiarism, informed consent, misconduct, data fabrication and/ or falsification, double publication and/or submission and redundancy have been completely observed by the authors.

LIFE SCIENCE REPORTING

No life science threat was practiced in this research.

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