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The Growth of *Tagetes patula* and Its Ability to Reduce Cr(VI) with the Addition of *Microbacterium* sp. SpR3

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Abstract. Cr(VI) is a heavy metal that has the potential to become a soil pollutant and has an impact on organisms. The contamination caused by Cr(VI) could be alternatively treated with bioremediation techniques. The current study aimed to determine the most potential combination of *Tagetes patula* Linn. and *Microbacterium* sp. strain SpR3 for remediation of soil Cr(VI) contamination based on growth of *T. patula*. The application of SpR3 applied at the 1st day (T0) and 20th day (T20) with 10 g (M10), 30 g (M30), 50 g (M50) of bacterial inoculum to *T. patula* grown under Cr(VI; 100 mg/L). The results showed that T0M50 treatment resulted in the highest values of growth traits of *T. patula* grown under Cr(VI) metal stress. The highest BC value (0.36) was obtained from plants treated with T2M10 and T2M50, while the highest TF value (0.08) obtained from plants treated with T0M50. BC value <1 means that the combination of *T. patula* and SpR3 bacteria for heavy metal Cr(VI) can be classified as an excluder and the TF value <1 means that the combination can act as a phytostabilization in handling Cr(VI) contamination. In conclusion, the application of SpR3 using T0M50 can enhance the growth of *Tagetes patula* Linn. grown under Cr(VI) stress condition. The outcome of the study are expected to advancement in the application of rhizobacterial and plant combined system in the bioremediation of soil Cr(VI) contaminated.

Keyword: Bioremediation; Cr(VI); *Microbacterium* sp. strain SpR3; *Tagetes patula*

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INTRODUCTION

Tagetes is a genus belonging to the Asteraceae family which has members of the species including *Tagetes erecta, Tagetes patula, Tagetes minuta, Tagetes signata* and others (Priyanka *et al*., 2013). *Tagetes* is a plant that has the potential as a phytoremediator for several heavy metals such as Zn, Cr, Ni, Pb, and Cd (Awan *et al*., 2020; Biswal *et al*., 2021; Miao & Yan., 2013; Sathya *et al*., 2019; Thongchai *et al*., 2018). *T. patula* is one species with potential in heavy metal phytoremediation. According to a study by Sun *et al.* (2011), *T. patula* showed that it has strong tolerance to single B[a]P and HM-B[a]P, combination heavy metal was Cu, Pb, and Cd. *T. patula* has highest accumulation to Cd-B[a]P treatment, the accumulation Cd in shoots was higher than the roots, and the BF and TF values were more than 1. *T. patula* indicates as hyperaccumulator for Cd and Ni with TF values 3,51 and 14,9. *T. patula* is effective in removing Cd and Ni from soil, it can translocate heavy metals from root to shoots (Biswal *et al*., 2021). In other studies, it showed that in the metal accumulation pattern from *T. patula*, the maximum to minimum accumulation values of each heavy metal was observed to be Fe>Cr>Zn>Cu>Pb>Ni>Cd (Chaturverdi *et al*., 2013).

Heavy metals naturally occur in the environment, they are durable and nonbiodegradable. They can be toxic in large amounts. Accumulation in living organisms may cause biological and physiological complications while in the environment changing to pollutant (Briffa *et al*., 2020). One of the heavy metals which the existence is abundant naturally in the earth's crust, scattered in rocks, animals, plants and soil is chromium (Gomes *et al*., 2017).

Chromium can also be produced from human activities in industrial activities, including the metal industry for electroplating, the steel-making industry, leather tanning, and textile coloring (Sharma *et al*., 2020). Cr(VI) are primary states and more stable than other valence states. It is more toxic than other valences because it is very reactive with other elements (Shahid *et al*., 2017). Cr(VI) exists as $H2CrO_4^-$, CrO_4^{2-}, and $Cr_2O_7^{2-}$. In plants, Cr(VI) transportation is an active mechanism by SO_4^{2-} transporter due to similarity anions (Choppala *et al*., 2013). Chromium can be toxic to living things and the ecosystem if the concentration exceeds 10-100 mg/kg soil (Srivastava *et al*., 2021). One of the alternative treatments for chromium metal contamination in the soil is phytoremediation using *Tagetes.*

The phytoremediation process carried out by plants was influenced by the presence of rhizosphere bacteria that colonized around plant roots (Kumar *et al*., 2016). Some of the roles of microorganisms in the remediation process are influencing the mobility of heavy metals in soil by releasing chelating agents, changing the redox potential, dissolving phosphate, stimulating growth hormone, and influencing the absorption of organic matter (Aheemad 2015). Rhizobacteria will affect the ability of plants against heavy metals, it can increase metal uptake in plant tissues (phytoextraction) or decrease metal uptake into plant tissue (phytostabilization) (Pramono *et al*., 2012). One of the bacteria that is reported to be able to tolerate and be resistant to several heavy metals such as Cr, Ni, Cd, and As is *Microbacterium* sp. (Ouertani *et al*., 2020; Meitiniarti *et al*., 2014). Several types of bacteria *Microbacterium* sp. reported specifically to have the ability to tolerate Cr(VI), and enzymatically reduce and change the form of Cr(VI) to Cr(III). In several studies that have been carried out the *Microbacterium* spp. genus has a tolerance regulation of Cr(VI) (Elahi *et al*., 2019; Focardi *et al*., 2013; Learman *et al*., 2019). In addition to the ability to reduce and change the redox potential of Cr(VI), *Microbacterium* sp. is known to be capable of becoming growth-promoting bacteria by initiating the production of plant growth hormones under normal conditions as well as with certain biotic or abiotic stresses (Ouertani *et al*., 2020). This study used the bacterial isolate of *Microbacterium* sp. strain SpR3 which had been tested for the reduction of Cr(VI) in LB media with a Cr(VI) content of 100 mg/L (Meitiniarti *et al*., 2014) and tested for the reduction of soil medium containing Cr(VI) with vermicompost carrier (Innation *et al*., 2021).

The potential *T. patula* treated with the *Microbacterium* sp. strain SpR3 has not been well studied for remediation of Cr(VI) in polluted soils. Therefore, the current study aimed to investigate the effects of different levels of *Microbacterium* sp. strain SpR3 on growth of *T. patula* grown under Cr(VI) stress conditions. The application of *Microbacterium* sp.strain SpR3 in these study is expected to enhance the growth of T. patula cultivated in soil contaminated with Cr(VI). The outcome of the study are expected to advancement in the application of rhizobacterial and plant combined system in the bioremediation of soil Cr(VI) contaminated.

Material and Methods

The study was conducted to investigate the effects of different SpR3 inoculum levels (i.e. 0, 10, 30, and 50 g) on *T. patula* grown under the stress of Cr(VI; 100 mg/L) at a greenhouse using a completely randomized design with five replications for each treatment. The SpR3 treatments were applied at two different dates as follows: 1st day (T0) and 20th (T20).

Plant Preparation and Material

T. patula was taken from nursery shops in Kopeng, Semarang Regency (-7.40342, 110.41854). It was selected at 3 weeks of age, with a height of approximately 4.5-5 cm, and then it was being grown in the medium with the addition of 100 mg/L Cr(VI). A treatment was given by adding *Microbacterium* sp. strain SpR3 inoculum from the Microbiology Laboratory of UKSW, Salatiga using vermicompost carrier material 10 g, 30 g, and 50 g and the inoculation of bacteria on the media at different times, at the first day of planting and the 20th day. The bacterial culture used Luria Bertani medium: Tryptone, NaCl, Yeast extract, and Bacteriological Agar. The Cr(VI) solution used $K_2Cr_2O_7$. Determination of Cr(VI) content using $C_{13}H_{14}N_4O$ plant tissue reagent using dry extraction with HCl and HNO_3, soil extraction using KH_2PO_4 and K_2HPO_4.

Bacterial Culture Preparation

The cultivation of *Microbacterium* sp. strain SpR3 to new media by adding 10% inoculum from the bacterial liquid culture to the new culture medium (Meitiniarti *et al*., 2012). The culture was incubated for 48 hours with a shaker speed of 125 rpm until OD (λ 600 nm) = ±1 was obtained (Pramono *et al*., 2012). The cell concentration with OD 1 was then applied to 1000 g of

vermicompost (5 ml of suspension/10 grams of vermicompost), then it is incubated in an incubator for 14 days.

Measured Parameters

T. patula was grown on a medium containing 100 mg/L Cr(VI) for 49 days. Cr(VI) was watered twice during the planting period. The measurement parameters included plant height, and the number of leaves were measured every week, while root length, root dry weight, and shoot dry weight were measured at the beginning and end of the study.

Determination of Cr(VI) levels

The shoots and roots were oven-dried at 80ºC for 2 days. Then the sample was mashed with a blender and then it weighed as much as 0.1 g followed by burning the sample with furnish at 600°C for 9 hours, then dissolved in 2 M HCl and 1M HNO_3 1:1 with a volume of 10 ml followed by vortex and filtering. 2 ml of the extracted sample was taken, then it is added with 0.1 ml of diphenylcarbazide (0.25% (m/v)) and 2 drops of H_2SO_4, following the step, it is allowed to stand for 15 minutes. The mixture was then measured for its absorbance at a wavelength of 540 nm by spectrophotometry (Shimadzu UV mini1240).

As much as 5 g of soil. The soil was put into 50 ml of phosphate buffer (0.005 M KH_2PO_4 and 0.05 M K_2HPO_4 1:1) and incubated for 24 hours with a shaker speed of 100 rpm. The supernatant and pellet were separated by centrifugation at 9000 rpm for 5 minutes, then filtered and the filtrate obtained was transferred to a new Erlenmeyer flask. The volume was determined as the original volume of 50 ml with phosphate buffer. 2 ml of soil sample was taken then it is added with 0.1 ml of diphenylcarbazide (0.25% (m/v)) and 2 drops of H_2SO_4, then incubated for 15 minutes. The absorbance was measured at a wavelength of 540 nm (Gheju *et al*., 2009).

Translocation Factor and Bioaccumulation Coefficient

The determination value of the translocation factor and bioaccumulation coefficient was calculated as:

$$\text{BC} : \frac{Cp}{Cs}$$

Bioaccumulation coefficient to determine the number of heavy metals absorbed from soil in plant tissue.

$$\text{TF} : \frac{Cx}{Cr}$$

Translocation factor to determine the ability of the plant to translocate heavy metal from roots to shoots.

Note: Cp (Concentration Cr(VI) in plant), Cs (Concentration Cr(VI) in soil), Cx (Concentration Cr(VI) in shoot), Cr (Concentration Cr(VI) in radix) (Miao & Yan., 2012).

Statistical Analysis of Data

Data analysis with a completely randomized design was carried out using the SAS ver. 9.3.1. Normality and homogeneity tests were used, followed by the two-way analysis of variance (ANOVA) test with the Duncan Multiple Range Test (DMRT) at a level of 5%.

RESULT AND DISCUSSION

Based on the results obtained after 49 days of treatment, it showed differences in growth in each treatment. Table 1 shows that the root length at the beginning of the study was quite diverse, while the shoot height was quite the same. In terms of root length, it is known that the T2M30 treatment has a significant value compared to the other treatments, namely 69.70 cm. For the height of the treated shoot, T0M30 and T0M50 were not significantly different respectively 30.72 cm and 29.60 cm.

Table 1. The length of root and height of the shoot of *T. patula*

Treatments	Length of Root (cm)		Height of Shoot (cm)	
	Before	After	Before	After
T0M0	8.12^{B}±0.94	55.06^{C}±1	4.3^{C}±0.16	26.60^{CD}±1.4
T0M10	8.94^{B}±2.46	47.96^{D}±1.6	4.5^{AB}±0.10	26.48^{CD}±1.2
T0M30	7.14^{C}±0.96	49.34^{D}±1.9	4.4^{ABC}±0.16	30.72^{A}±1.3
T0M50	7.10^{B}±2.87	60.40^{B}±1.6	4.6^{A}±0.10	29.60^{AB}±0.5
T2M10	6.02^{D}±1.15	56.62^{C}±1.9	4.4^{BC}±0.14	26.60^{CD}±1.8
T2M30	9.98^{A}±0.53	69.70^{A}±2.2	4.4^{ABC}±0.10	28.04^{BC}±1.2
T2M50	6^{D}±1.90	43.38^{E}±1.5	4.5^{ABC}±0.10	25.88^{D}±0.6

Different letters within the treatments indicate significant differences at the 5% level according to the DMRT test.

Cr(VI) in high concentrations exerts various effects on plants such as inhibiting physiological processes, and affecting the development of morphology and metabolic activity of cells in plants (Srivastava *et al*., 2021). The presence of Cr(VI) in the medium affects the uptake and ion exchange of cell membranes in roots. Absorption of heavy metals in plants does not have a specific pathway but could be through a passive transport with water pressure through the roots or active transport through the plasma membrane in root epidermal cells (Yoon *et al*., 2006). The Cr(VI) absorption in root cells from the medium can be via the sulfate or phosphate pathway due to the similarity in structure and the number of anions (Joutey *et al*., 2015; Shahid *et al*., 2017). In general, sulfate transport occurs through an active transporter called a sulfate transporter. This transporter can take up sulfate ions that are in apoplastic pathway (Takahashi, 2019). The changes in ion exchange adjust the transport pathways in cells, and may cause ROS in root cells as a result of electron reduction and defense mechanisms, as well as suppress antioxidant enzymatic systems. ROS can inhibit the initiation of growth hormone and the process of cell division in roots causing slow root growth. In terms of morphological appearance, the treatment with the addition of Cr(VI) changed structure and color as a form of oxidative stress. In control, roots have a finer structure and a lighter color. However, in the T0M30, T0M50, and T2M30 treatments, although the roots changed structure and color, the roots grew thick and long. According to the study by Singh & Singh (2019) the presence of *Microbacterium* sp. in lindane stress conditions had a positive impact on increasing the production of the hormone auxin (IAA), ammonia, and ACC deaminase activity. Rhizobacteria stimulates IAA production thereby initiating cell division and the formation of lateral and adventitious meristems in roots.

The shoot growth in the T0M30 and T0M50 treatments had a significant effect. In this treatment, it is known to be able to support crown growth at the optimal point, normally the crown growth of *T. patula* is ±15-40 cm in each individual (Priyanka *et al*., 2013). Compared to the control treatment (without giving bacterial inoculum), it is known that *Microbacterium* sp. strain SpR3 can act as plant growth-promoting bacteria. The growth-promoting bacteria have an effect on increasing the absorption of nutrients from soil contaminated with heavy metals, inducing and producing growth regulatory hormones and spurring resistance mechanisms in plants so that they affect the formation of new tissues in the shoot (Aheemad, 2015).

Table 2. The effects of different treatments on leaves growth of *T. patula* grown under Cr(VI)

Treatments	Number of Leave (sheet)		Length of Leave (cm)	Width of Leave (cm)
	Before	After		
T0M0	2^{A}±0	20.20^{F}±1.3	9.5^{B}±0.92	6.2^{BC}±1.27
T0M10	2^{A}±0	19.60^{F}±1.5	9.8^{B}±1.01	5.6^{C}±0.86
T0M30	2^{A}±0	27.80^{D}±1.6	11.1^{AB}±1.56	6.6^{BC}±1.33
T0M50	2^{A}±0	38.20^{A}±1.3	10.9^{AB}±1.90	6.7^{BC}±0.70
T2M10	2^{A}±0	25.20^{E}±1.3	9.7^{B}±1.37	7.2^{AB}±0.40
T2M30	2^{A}±0	35.80^{B}±1.6	12.1^{A}±0.43	8.2^{A}±0.91
T2M50	2^{A}±0	33.40^{C}±1.7	9.9^{B}±1.27	6.8^{BC}±0.88

Different letters within the treatments indicate significant differences at the 5% level according to the DMRT test.

The highest number of leaves was found in the T0M50 treatment with 38.2 strands while the lowest leaf growth was shown in the T0M10 and T0M0 treatments with an average number of leaves of 19.6 and 20.2 leaves. From this observation, it is proven that in general the treatment with the addition of *Microbacterium* sp. strain SpR3 inoculum affected the formation and growth of the leaf blade. The length and width of the leaves of *T. patula* which received 30 g of inoculum on day 20 showed the highest growth with a leaf length of 12.1 and a leaf width of 8.2 cm. These values were significantly different from the control. According to Suryani et al. (2017), the application of *Ceratophyllum demersum* in liquid chrome leather tanning was found to disrupt the photosynthesis process. This disruption was characterized by increased chlorosis on the leaves and shedding of leaf buds.

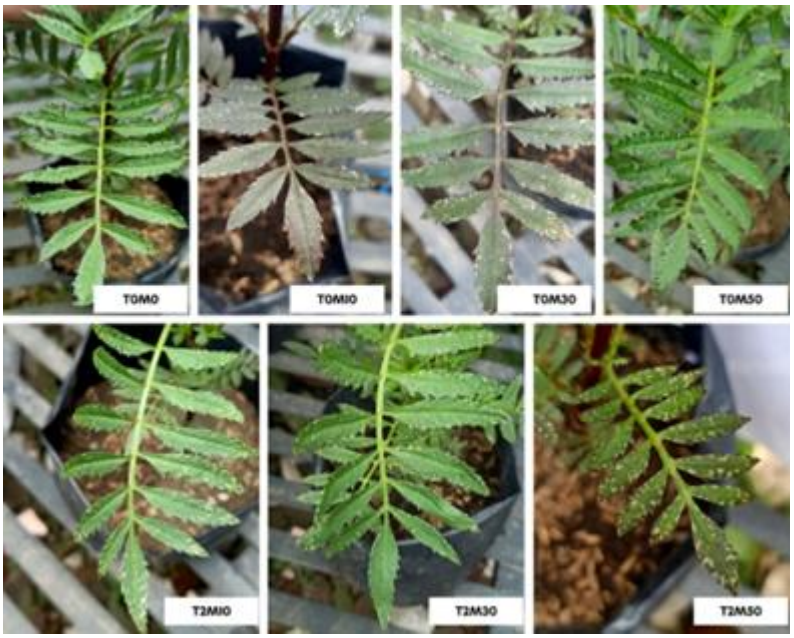

Figure 1. Leaf morphology of *T. patula* under Cr(VI) stress with treatment of the number and time of *Microbacterium* sp. strain SpR3. (T0M0= control, T0= inoculum given day, T2= inoculum given day 20, M10= 10 g inoculum, M30= 30 g inoculum, M50= 50 g inoculum).

The appearance of *T. patula* leaves in several treatments showed changes in the color appearance of the leaves. The leaves turn purplish and paler, have a rough or wavy texture, and flake off the epidermis. In the T2M50 treatment the leaves experienced necrosis marked by death in the leaf cells with a change in leaf color to brown and the presence of white spots on the leaf surface indicating loss of leaf chlorophyll (Srivastava *et al*., 2021). The morphological changes that were shown in the leaf organs indicated a deficiency of essential nutrients needed by plants to sustain growth. Nutrient deficiencies could be caused by various factors, one of which is stress. The discoloration of green leaves to red or purplish specifically indicates symptoms of phosphorus and magnesium deficiency. The presence of the evenly distribution of tissue spots on the leaves at the tips and edges indicates a deficiency of potassium and molybdenum. Deficiency Ca in plants was also found in some treatments, by wilting and death of growing points on young leaves (Veazie *et al*., 2020). The presence of Cr(VI) in toxic concentrations triggers stiffness in the cell wall by disrupting Ca homeostasis in the cell wall. Ca plays a role in the structural function of the cell wall and cell membrane so this may increase damage to cell structures (Murti & Maryani, 2020).

Table 3. Effects of different treatments on fresh and dry weight of root and shoot biomass of *T. patula* grown under Cr(VI) stress

Treatments	Biomass of Root		Biomass of Shoot	
	Fresh Weight (g)	Dry Weight (g)	Fresh Weight (g)	Dry Weight (g)
T0M0	2.3^{EF}±0.08	0.62^{D}±0.04	7.5^{E}±0.52	1.11^{E}±0.18
T0M10	4.1^{D}±0.20	0.96^{C}±0.11	9.4^{D}±1.04	1.32^{D}±0.09
T0M30	7.1^{B}±0.43	1.32^{B}±0.07	16.2^{B}±1.36	2.05^{B}±0.08
T0M50	8.9^{A}±0.45	1.56^{A}±0.09	19.8^{A}±0.40	2.35^{A}±0.06
T2M10	2.8^{E}±0.44	0.56^{D}±0.04	10.4^{D}±0.99	1.15^{E}±0.07
T2M30	5.3^{C}±0.81	1.02^{C}±0.12	17.1^{B}±1.38	1.81^{C}±0.10
T2M50	1.8^{F}±0.35	0.36^{E}±0.03	12.8^{C}±0.96	1.19^{DE}±0.04

Different letters within the treatments indicate significant differences at the 5% level according to the DMRT test.

In terms of biomass parameters, the treatment with the highest biomass was the T0M50 treatment with a wet biomass of 8.9 g of root and 19.8 g of root, 1.56 g of dry root, and 2.35 g of root. The lowest root dry biomass was found in the T2M50 treatment of 0.36 g while the lowest shoot dry biomass was in the T2M10 treatment of 1.15 g. Accumulation of heavy metals in plant tissues affects plant biomass, the higher the absorption and transfer of heavy metals in root tissues and other tissues, the greater the final biomass. Apart from being affected by metal accumulation, according to a study by Ouertani *et al.* (2020), the bacterial isolate *Microbacterium metallidurans* TL13 applied to tomato plants in vivo was able to increase growth and plant biomass, 12.79% on wet weight and 34.62% on the dry weight. On the other hand, in some treatments, the presence of heavy metal Cr(VI) stress triggers oxidative stress which disrupts the photosynthetic process in plants it affects plant biomass. This is supported by research conducted by Aravindhan *et al*. (2019) that high Cr concentrations affect plant biomass. *T. erecta*.

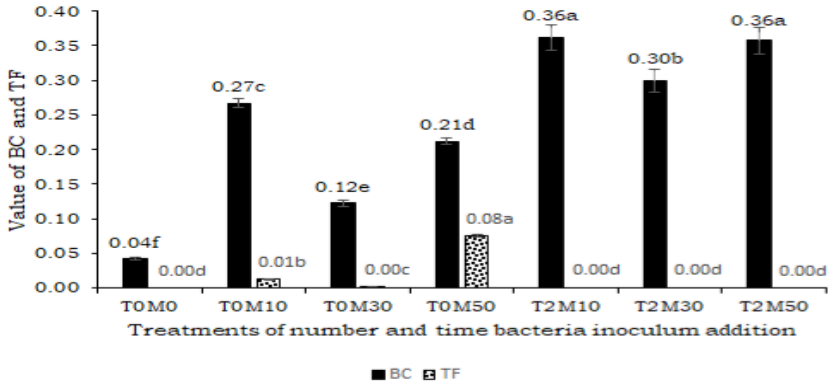

Figure 2. BC and TF values in *T. patula* under Cr(VI) stress with the treatment of the number and time of addition of *Microbacterium* sp. strain SpR3. (T0M10= control, T0= inoculum given on day 1, T2= inoculum given on day 20, M10= 10 g inoculum, M30= 30 g inoculum, M50= 50 g inoculum).

The highest BC values were shown in the T2M10 and T2M50 treatments, the values for which were not significantly different, 0.3616 and 0.3575. The high value of BC indicated a large absorption in the root tissue. High accumulation of heavy metal Cr(VI) in the roots resulted in obstructions to the absorption of nutrients and minerals from the medium and barriers to the transfer of ions to other tissues. Thus, the growth of *T. patula* in the T2M50 and T2M10 treatments was relatively stunted, and had short root lengths. Based on the BC <1 *T. patula* value for heavy metals Cr(VI) it is categorized as an excluder plant. An excluder plant is a plant that is tolerant to heavy metals, able to adapt to the stressed environment as well and absorb heavy metals from the medium. However, excluder plants tend to limit the transfer process from root tissue to aboveground tissue and limit the accumulation of heavy metals into biomass (Yoon *et al*., 2006). Plants are categorized as hyperaccumulators if they have a BC value> 10, as accumulators if BC values are 1-10, and as indicators if BC values = are 1 or close to 1 (Bader *et al*., 2019; Susana & Suswati, 2013).

The T0M50 treatment exhibited the highest translocation factor value at 0.07525, exceeding those of the T0M10 and T0M30 treatments. There were only 3 treatments that showed the transfer of heavy metals to the shoot tissue while the other treatments only accumulated heavy metals in the root tissue. TF value <1 indicates that the combination of *T. patula* and *Microbacterium* sp. strain SpR3 plays a role in the mechanism of phytostabilization of Cr(VI) metal. Based on the category of the translocation factor mechanism, if TF <1 then the plant is categorized as a phytostabilizer. While plants with the value TF> 1 are grouped to the phytoextraction mechanism (Bader *et al*., 2019; Susana & Suswati 2013). Phytostabilizers play a role so that contaminants do not migrate to other places by chelating metals with roots and metabolic products from bacteria. It is evident that *T. patula* demonstrates high adaptability, along with the capacity to accumulate high concentrations of metals in vacuoles and root nuclei, potentially enabling rapid growth (Thongchai et al., 2019)."

The growth of *T. patula* can be enhance by *Microbacterium* sp. strain SpR3 when grown in

contaminated soil by Cr(VI). The combination can be applied to the land that is contaminated by Cr(VI) or planted in solid waste in industry. This is expected to be beneficial in the application of rhizobacterial and plant combined systems in the bioremediation of contaminated soil Cr(VI).

CONCLUSION

Cr(VI) stress treatment in the medium and the addition of inoculums with different amounts and times affected the growth and accumulation of Cr(VI) in *T. patula*. In general, the additional of 50 g of *Microbacterium* sp strain SpR3 inoculum at the 1st day could support the growth of *T. patula* in Cr(VI) polluted environments and increase the translocation of heavy metals from the roots to the shoots. The highest BC value was in the T2M10 and T2M50 treatment with a value of 0.36, and the highest TF value was 0.08 in the T0M50 treatment and the combination of *Microbacterium* sp strain SpR3 can act as a phytostabilization in handling Cr(VI) contamination. Further research needs to be conducted with the bacterial SpR3 gene that plays a role in Cr(VI) reduction, enzyme activity, and protein profiles in the *T. patula* root and to determine the symbiosis process that can occur between the bacteria and *T. patula* root.

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