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Assessing the superiority of *Bacillus songklensis* strain kca6 along with lime and cow manure to increase white bean yield in cadmium contaminated soil

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Abstract: A study on the co-ordinate use of cow manure and lime with nitrogen-fixing bacteria was conducted on the white bean variety at Phuoc Hung commune. This study was comprised of four treatments T0 (NPK: 40 kg Urea-60 kg P2O5-60 kg K2O per ha); T1 [NPK +2.0 $tCaCO_3ha$-1+ *Bacillus songlensis* strain KCa6; T2 (NPK+10.0t cow manure ha^{-1}+ *Bacillus songklensis* strain KCa6) and T3 (NPK+10.0t cow manure ha^{-1}+ *Bacillus songklensis* strain KCa6) repeated four times. Fertilization with cow manure, lime, along with inoculation of BS strain KCa6 had the best outcome, positively affecting pH, soil organic matter, total nitrogen, available phosphorus and exchangeable potassium. Similarly, the best plant growth and yield traits of white bean such as height, number of pods per plant, seeds number per pod, 1,000 seed weight and fresh yield were achieved under this treatment. The fresh seed productivity increased remarkably up to 16.6% and reduced the cadmium (Cd) accumulation of white bean stems ~38.0%, compared to the control treatment. In addition, cadmium amount in white bean seeds was undetectable across all treatments. The application of N, P and K fertilizers, lime, cow manure associated with *Bacillus songlensis* strain KCa6 inoculation promoted soil nutrients, plant growth as well as enhanced yield and quality of white bean. Therefore, indigenous *Bacillus songlensis* strain KCa6 species discovered as a promising species in combination to increase crop yield and improve crop quality on the cadmium contamination soil.

Keywords: *Bacillus songklensis* strain KCa6, cadmium, cow manure, lime, white bean.
Abbreviations: Cd_Cadmium; WB_white bean; ENFB_endophytic nitrogen-fixing bacterium; CNF_chemical nitrogen fertilizer.

Introduction

White beans (*Phaseolus vulgaris* L.) are commonly known as common beans (Ahmad et al., 2023; Swegarden et al., 2016). Santosa et al., (2017), reported that white bean (WB) contains great compounds of macronutrients, micronutrients, and other beneficial compounds. It contains macronutrients, protein, lipid, carbohydrate and micronutrients such as calcium, phosphorus, iron and other beneficial compounds such as fiber, complex carbohydrates, which are a very good source of nutrients for the body. The previous researches of Chuong and Bush, (2021); and Camara et al., (2013) found that beans contain highly diverse chemical compounds. In addition, beans contain some composition that can protect negative conditions, such as heat stress, plant disease, diabetes, metabolic syndrome, and many other diseases. Therefore, legumes are a great functional food. In the recent years, arable soils have considerably affected by cadmium (Cd) contamination and low nutrients, which are main causes for the continuous reduction of the WB growth and yield (Nguyen and Tran, 2021). Agricultural production has fierily faced by environmental changes that leaded to increasingly evident soil degradation (Chuong, 2024; Barrow, 2012). Farmlands can lose their nutrients due to a highly sandy content in soil structure and become weak in soil organic matter content due to easy leaching (Yunilasari et al., 2020). Farmers have been using many chemical fertilizers and pesticides. This is due to environmental pollution leading to yearly decline in crop yield. Amendment of organic manures combined with nitrogen-fixing bacterium inoculation may improve soil fertility and reduce chemical fertilizer usage (Nguyen, 2024).

The Cd accumulation in agricultural products and its transfer to humans through the food chain is a major environmental problem around the world (Ete Aydemir et al., 2023). Cadmium that is known a pollutant and toxicant element to organisms in the ecosystem, has an average content of 0.1 mg per kg in the atmosphere and 0.53 mg per kg in many agricultural soils. When the Cd content exceeds 3.0 mg kg^{-1} in soil, it becomes toxic to plants. The Cd concentrations in farmland have increased worldwide, mainly due to sources of fertilizers and pesticides in agricultural production and activities of phosphate fertilizer production and use (Li et al., 2024).

The recent researches proved that the lime amendment on acidic and Cd-contaminated soils can significantly increase wheat yield, compared to the non-lime treatment. The reduction of grain Cd accumulation and Cd toxicity could be

prevented or alleviated by lime application. Cropping on Cd-contaminated soils should prioritize addition of lime due to its significant effect on reducing Cd concentration in wheat grain (Ete Aydemir et al., 2023).

The amendment of soil with animal manures and lime has successfully reduced Cd accumulation in spinach and increased spinach leave chlorophyll concentration during the development period. This research concluded that the co-application of lime and organic manure is an effective way to remediate Cd-contaminated soil (Pandit et al., 2012). A field experiment was carried out at two consecutive years and showed the effect of chicken manure and endophytic nitrogen-fixing bacterium (ENFB) inoculation on the bean agronomic, yield traits, and yield (Kazim et al., 2023). There is a consensus in all studies that the second crop is higher than the first crop. Chicken manure application had a positive effect on key nutrient compositions and WB outputs. In general, the ENFB inoculation and/or combination had a positive effect on yield and yield components of crops. The highest yield was obtained from the combination of *Rhizobial* and *Bonteral* species (Chuong, 2024). Therefore; inoculation with ENFB + chicken manure, had highly beneficial effects in all bean compositions (Chuong, 2023).

White beans, like many other legumes, have a root symbiosis system combined with ENFB inoculation such as *Rhizobium* sp. and other ENFB. These species have the ability to fix nitrogen and are responsible for fixing free nitrogen in the air for plants (Krishnan et al., 2018; Chuong, 2023). The N fixing amount from ENFB that is normally around 94 kg N/ha/crop, but could take up to 168 kg N/ha/crop in a well environmental condition (Krishnan et al., 2018). Inoculation of indigenous N-fixing bacterium species have sufficiently shown a 50% reduction in chemical nitrogen fertilizer (CNF), which still yielded up to 39% higher than non-inoculated and insignificant difference in yield compared to 100% CNF fertilization. Furthermore, researches on peanut seeds under ENFB inoculation showed lower arsenic (As) content, compared to a control (non ENFB inoculation) (Nguyen 2024). Many recent studies have significantly proven that the positive effects of well-decomposed organic manures had a significantly positive impact on the N-fixing ability of ENFB and enriched the microbial community in rice paddy soil (Gu and Yang 2022.). However, well-decomposed organic manures alone or/and in combination with CNF at a low rate (below 262 kg N ha^{-1} per year), promoted the N fixation process of ENFB. The opposite effect was observed when high rates of inorganic fertilizers, up to 420 kg N ha−1 per year, were applied (Chen et al., 2021; Ma et al., 2022). Due to poor soil nutrients, both the yield and quality of white bean (WB) have decreased with each crop grown on An Phu soil. Therefore, further research on the addition of lime, cow manure, and ENFB inoculation is needed.

Results

Effects of Lime, cow manure and BS strain KCa6 on chemical traits of the soil

The soil chemical properties were significantly affected by the type of cow manure, lime, and BS strain KCa6. Lime, cow manure and BS strain KCa6 increased the soil pH at T1, T2 and T3 to 5.45, 5.90 and 6.17, respctively, compared to intial soil pH (control), while 40 kg urea:60kg P_2O_5:60 kg K_2O per ha lessened it to 4.92 and significant differences at 5 and 1% level. Amendment with cow manure and/or lime, combined with BS strain KCa6 inoculation, resulted in higher outcomes compared to no amendment and BS strain KCa6 inoculation alone. The best chemical values of soil, pH (6.17), SOM (2.87%), total N (0.258%), available P (20.8 mg 100. gr^{-1}) and exchangeable K (16.7 mg. 100gr^{-1}), was acheived in T3 (40 kg urea:60kg P_2O_5:60 kg K_2O ha^{-1} + 10 tcow manure ha^{-1} + 2.0t $CaCO_3$ ha^{-1} combined with BS strain KCa6 inoculation), while the soil chemical traits was pH (H_2O:4.92), SOM (2.31%), total N (0.180%), available P (11.3 mg 100. gr^{-1}) and exchangeable K (12.2 mg. 100gr^{-1}), lowest in 40 kg urea:60kg P_2O_5:60 kg K_2O (in control: T0). The results showed that there are fluctuations between NPK fertilizer combined with cow manure, lime and BS strain KCa6 inoculation, having the best results, while only applying chemical fertilizer had the lowest values.

Influence of Lime, cow manure and BS strain KCa6 on WB height and branch number

Lime, cow manure and BS strain KCa6 significantly affected the growth performance of plant (Table 4). Treatment T3 had the highest plant height and branch number cultivated in the sandy silt soils of Phuoc Hung community. Plant height (62.1cm) and branch number per plant (9.98 branches) were the highest in T3 after 60 DAS. The lowest plant height (54.4cm) and branch number per plant (8.68 branches) observed at control treatment T0 after 60 DAS, all significantly different at 5 and 1% level (Expect branch number in 20 DAS).

Influence of lime, cow manure and BS strain KCa6 on yield parameters and seed yield

The WB yield traits and fresh seed productivity remarkably changed (*P-value* = 0.000**) along allg four treatments. The treatment T3, which was co-application of NPK, lime and cow manure with BS strain KCa6 inoculation, acheived the highest values of pod and seed number, 1,000 weight and fresh seed yield with 39.5 pod $plant^{-1}$, 14.3 seeds pod^{-1}, 200 gr and 4.53 t ha^{-1}, respectively, followed by T2, T1 and lowest result in T0 (control) with 27.8 pod $plant^{-1}$, 9.50 seeds pod^{-1}, 188 gr and 3.78 t ha^{-1} (Table 5). Sufficiently associated fertilization of all experimental materials showed statistically significant differences (*P-value* = 0.000**) in all WB yield parameters and seed yields. The treatment T3 produced the highest fresh seed yield with 4.53 tha^{-1}, followed by the treatment T2 (NPK+ 10 cow manures+ BS strain KCa6 inoculation) with 4.1 tha^{-1}, treatment T2 (NPK+ 2.0 $CaCO_3$ tha^{-1} + BS strain KCa6 inoculation) with 4.0 tha^{-1} and lowest with 3.78 tha^{-1} in treatment T0 (NPK only).

Influence of lime, cow manure and BS strain KCa6 on Cd accumulation of farmland, WB stems and seeds

Concentration of Cadmium in soil (initial and end experiment), stems and seeds (*P-value* = 0.00**) varied significantly among treatments (Table 5). The highest soil Cd concentration observed at treatment T2 (113 mg kg^{-1}) in the first experiment, and lowest with T3 (89.1 mg kg^{-1}), followed by treatment T0 and T2 (96.7 mg kg^{-1}). Furthermore, the highest soil Cd concentration showed at treatment T1 and T2 (108 mg kg^{-1}) in the end experiment, and lowest with T0 (84.4 mg kg^{-1}), followed by treatment T3 (102 mg kg^{-1}). Table 5 shows that no Cd content was detected in WB seed samples at harvest season. However, the Cd content of WB stem valued from 57.0 to 92 µg kg^{-1}, which was significantly different at the 1% level. The highest Cd concentration in stem was observed in T0 with 92 µg kg^{-1}, and lowest in treatment T3 with 57 µg kg^{-1}, followed by treatment T2 (61.0 µg kg^{-1}) and T1 (83.0 µg kg^{-1}). Table 5 results reveals that the combined fertilization of cow manure, lime, and BS strain KCa6 inoculation remarkably reduced Cd accumulation in WB stems compared to control treatment, which applied chemical fertilizer. Further, WB seeds did not

Table 1. Different dosages of experimental meterials in all treatments.

Treatment	BS strain KCa6 (10^8CFU/g)	Lime (t $CaCO_3$ ha^{-1})	Cow manure (t ha^{-1})	Urea-P_2O_5-K_2O (kg ha^{-1})
T0	No	0.00	0.00	40-60-60
T1	Yes	2.00	0.00	
T2	Yes	0.00	10.0	
T3	Yes	2.00	10.0	

Table 2. Soil chemical properties of experimental samples at harvest season.

Properties	Treatments				
	T0	T1	T2	T3	*P-value*
pH	4.92d	5.45c	5.90b	6.17a	0.000**
SOM(%)	2.31d	2.51c	2.61b	2.87a	0.001**
Total N (%)	0.180 c	0.177d	0.210b	0.258a	0.000**
Available P (mg 100. gr^{-1})	11.3c	20.0 a	15.5b	20.8a	0.003**
Exchangeable K (mg. 100gr^{-1})	12.2c	15.0ab	13.2b	16.7a	0.045*

*: *P-value* ≤ 0.05; **: *P-value* ≤ 0.01; (±): presents the mean standard deviation of four repeats; The same letter denotes insignificantly different means in a row for each property.

Table 3. The WB height and branch number impacted by chemical fertilizers, cow manure, lime combined with BS strain KCa6 inoculation

Treatments	Plant height (cm)			Branch number per plant (branches)		
	Days after sowing (DAS)					
	20	45	60	20	40	60
T0	14.3c	47.7c	54.4d	1.0a	5.10b	8.68d
T1	15.1b	49.1b	56.3c	1.0a	5.30ab	8.78c
T2	15.5b	49.0b	57.1b	1.0a	5.30ab	9.28b
T3	18.6a	53.4a	62.1a	1.1a	5.45a	9.98a
P-value	0.000**	0.000**	0.000**	0.970ns	0.049*	0.000**

ns: no significant differences (*P-value* <0.05); *: *P-value* ≤ 0.05; **: *P-value* ≤ 0.01; (±): presents the mean standard deviation of four repeats; The same letter denotes insignificantly different means in a column for 20, 45 and 60 DAS.

Table 4. yield parameter and seed yield of WB at harvest season.

Treatments	Parameters			
	Pod number (pods $plant^{-1}$)	Seed number (seeds pod^{-1})	Weight of 1,000 seeds (gr)	Fresh seed yield (t ha^{-1})
T0	27.8c	9.50c	188b	3.78d
T1	36.3b	10.5b	197ab	4.00c
T2	36.5b	11.0b	195ab	4.10b
T3	39.5a	14.3a	200a	4.53a
P-value	0.000**	0.000**	0.000**	0.000**

*: *P-value* ≤ 0.05; **: *P-value* ≤ 0.01; (±): presents the mean standard deviation of four repeats; The same letter denotes insignificantly different means in a column for each parameter.

show Cd accumulation with a low soil Cd contents (< 100 mg kg^{-1})

Discussion

In the face of increasingly severe climate change, sustainable and resilient agricultural practices are essential to ensure global food security, quality, and environmental protection (Beddington et al., 2012). Overuse of chemical fertilizers to increase crop output in traditional farming methods is the main cause leading to soil fertility and crop productivity decline. On the contrary, applying N-fixing microorganisms is one of the potential strategies to improve the quality of agricultural soils and crops (Pylak et al., 2019). Endophytic N-fixing bacteria can fix nitrogen from the atmosphere (Miao et al., 2020), and they can produce organic acids, enzymes, and hormones; thereby, performing beneficial activities for agricultural soil such as disease resistance, and nitrogen fixation (Olanrewaju et al., 2017), promoting the development of beneficial microbial communities for agricultural soil (Bhardwaj et al., 2014; Rahman and Zhang 2018). These ENFB strains that have relation and interaction with the rhizosphere soil and plant root system, increase the natural N contribution and nutrient uptake ability of plants (Chuong, 2021; Armanhi et al., 2017). Many ENFB strains, which had an ability to fix atmospheric nitrogen, need high amount of soil calcium and carbon for their living and action. Therefore, lime and cow manure application not only reduce the plant Cd uptake but also provides nutrients for crops and soil bacteria (Pitiwittayakul et al., 2021; Bisht et al., 2020; Bononi et al., 2020). The results of this research found out that the inoculation with BS strain KCa6 to the farmlands, coordinated with chemical fertilizers, lime and cow can improve all the traits such as pH, SOM, total N, P and K concentration in the soil as well as the yield traits, yield and Cd uptake of WB (Table 3-6). Wang et al. (2021) and Lui et al. (2015) found out the superior release of SOM, which was similar to the results of this research. Agricultural soils and crops, especially food crops, have seriously polluted Cd

Table 5. Cadmium concentration of experimental soil, WB stems and seeds.

Treatments	Cadmium concentration			
	Experimental soil (mg kg^{-1})		While bean (µg kg^{-1})	
	Before	After	Stems	Seeds
T0	96.7b	84.4c	92.0a	undetected
T1	113a	108a	83.0b	undetected
T2	96.7b	108a	61.0c	undetected
T3	89.1c	102b	57.0d	undetected
P-value	0.000**	0.000**	0.000**	0.000**

**: *P-value* ≤ 0.01; (±): presents the mean standard deviation of four repeats; The same letter denotes insignificantly different means in a column for Cd concentration

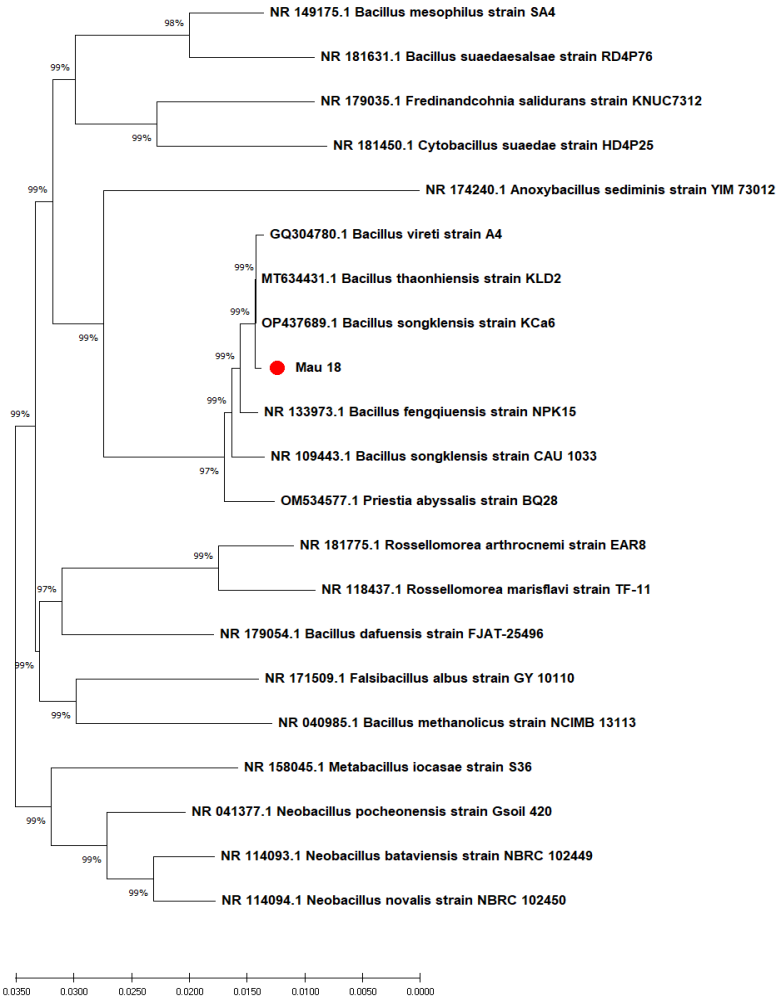

Figure 1. Phylogenetic tree of BS strain KCa6 species relied on 16S rRNA gene sequences.

toxicology and increased foodstuff safe problem around the world. Various methods have been explored to reduce Cd contamination in agricultural products from soil. Soil reclamation, irrigation management, beneficial microbial supplementation and climate change adaptation have been systematically proposed to illustrate developments and achievements in soil and crop management (Rehman et al., 2023; Petry et al., 2015). The use of lime, organic manures and heavy metal-resistant microorganisms have significantly contributed to a safer agricultural production, especially in Cd-contaminated agricultural soils. This review provided an inspiring and promising tool to ensure food safety by reducing Cd uptake of crops (Mubeen et al., 2023).

Materials and methods

Plant materials

This field study was performed on a silty-sand soil in Phuoc Hung community, An Phu district, An Giang province, Vietnam during the Autumn-Winter crop of 2023, using an irrigation water pump system from deep wells. The soil studied was in the dyke area, and had an average annual rainfall of 400-600 mm. The rainy season between April and October accounts for 50-60% of the total annual rainfall. The temperature ranges from 24-35°C in summer and 22-30°C in winter.

Endophytic nitrogen-fixing bacterium

Twenty root samples of white bean (WB) variety Blue Seeds were collected from Phu Dien company, Vietnam. They were taken from the WB growing area to isolate ENFB, in which was used to arrange field experiments. Root samples (3-5 cm and 3-5 mm in diameter) were collected to isolate ENFB. Step 1: Surface of WB Roots were sterilized with alcohol (70%) for 120 seconds; step 2: sterilized roots were washed many times by sterile distilled water and shaken in 5% (w/v) NaClO solution for 5 minutes and was rewashed 3-4 times by sterile water (Koyama et al., 2012). Sterilized root samples were grown and inoculated on the YMA medium with 0.1 mL grinding solution. After that, the YMA medium having ENFB incubated at 28°C for 3–5 days, continuously isolated and incubated until a single strain remained, followed by gram staining, physical examination, and identification and family tree drawing (Koyama et al., 2012). *Bacillus songklensis* strain KCa6 (BS strain KCa6) was grown and isolated on YMA medium at 28°C for 2 days and were observed by a microscope for the single colony. Genomic DNA of BS strain KCa6 was extracted and amplified using primers 18F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1411R (5′-GGTTACC TTGTTACGACTT-3′) (Weisberg et al., 1991). Based on BLAST search in NCBI, accession number of BS strain KCa6a6 was OP 437689.1 and had 99.93% identity with *Bacillus thaonhiensis* and *Bacillus vireti* strain A4 (Fig. 1). Based on morphological characteristics, 16S rRNA gene sequencing, and identification results, strain Mau 18 was identified as *Bacillus songklensis* strain KCa6, a member of the *Bacillus* genus (Fig. 2).

Experimental design

Field experiment was performed from September to December of 2023 in Phuoc Hung community. White bean cultivar was "BI" obtained from Phu Minh Tam company, Vietnam. *Bacillus songklensis* strain KCa6 was isolated from WB roots in Phuoc Hung, An Phu District, An Giang province. Urea, superphosphate and potassium chloride were used for all experimental treatments. A three-factor experiment was designed by a randomly complete block with four replications. Three factors included: (i) BS strain KCa6 (inoculation and no inoculation); (ii) lime (0 and 2.0 t ha^{-1}); (iii) cow manure (0 and 10.0 t ha^{-1}), and applying the same chemical fertilizer rate of NPK 40:60:60 kg ha^{-1} (NPK rates exchanged from urea (46%N), superphosphate (16% P_2O_5) and potassium chloride (60% K_2O) for all experimental treatments, which had four treatments, which were named T0, T1, T2 and T3, respectively, repeated four times (Table 1).

Preparation of WB seeds and BS strain KCa6 species

WB seeds were sterilized with 70% alcohol for 2 minutes, and then, washed twice with sterile water, shaken in 1% (w/v) NaClO solution for 5 minutes and rewashed 3-4 times with sterile distilled. White bean seeds were incubated for 36 hours for germination in dark condition. The next step, WB seeds were well sprayed mixed with 10 mL BS strain KCa6 cells (10^8 CFU mL^{-1}) and inoculated seeds were finally dried under natural condition before sowing. White bean seeds in the control pots that similarly prepared the BS strain KCa6 pots, used sterile distilled water instead. Two WB seeds were planted per hole, 20 cm in hole distances, and 30 cm in a row distances. The studied area per replicate was 20 m^2 (1 m in width x 20 m in length). The total area for the two crops was 320 m^2 (20 m^2 x 4 replicates x 4 treatments). *Bacillus songklensis* strain KCa6 preparation: The isolated strains were cultured on N-free Ashby agar plates (Zhang et al., 2022; Sun et al., 2018) at room temperature for 5–7 days before being incubated with WB seeds.

Soil and seed analysis

The soil analysis methods that were used by FAO (2020) and were particularly presented as following: pH_{H2O} were determined by pH meter (1gr soil and 2.5 mL water); the Kjeldahl method was used by analyzing for soil total N; Soil was extracted with 0.1N HCl and 0.03N NH_4F solution to determine phosphorus (P) and potassium (K). The P color solution was emitted by ascorbic acid and colorimetric with a spectrophotometer at 880 nm. The exchangeable K and Cd contents were determined by the flame method on atomic absorption spectrometry (AAS: contrAA 800 platform); Method of Nelson and Sommers (1982) was used by $K_2Cr_2O_7$ to oxidize organic matter and $FeSO_4$ 0.5N solution to determine the residual $K_2Cr_2O_7$ content for determining soil organic matter (SOM) was determined by the. Initial soil chemical properties are presented in Table 1. Soil, plant analysis and BS strain KCa6 isolation was carried out in AGU laboratory and identified by Phu Sa company, Vietnam. Fifteen days after sowing (DAS), lime and cow manure were amended in the soil. Soil samples were collected before and after planting in 20 cm of depth. Physical - chemical characteristics of the Initial soil (before experiment) sowed the following pH (5.02), soil organic matter (0.91%), exchangeable K (12.0 mg.$100g^{-1}$), Total N (0.25 %), soluble P (11.1 mg. $100g^{-1}$) and Cd (92.0 µg kg^{-1}).

Yield traits and seed productivity

Growth traits: plant height and branch number per plant at 20, 45, and 60 days after sowing (DAS) were randomly taken from and 10 plants per replication to estimate the average height and branch number per plant. 10 random plants sample were selected from each plot to determine the plant biomass, number of nodules per plant, dry weight of nodules, fresh and dry weight of filled pods and seeds per plant at harvest time. Plants in the two middle rows in each plot were harvested separately and dried to estimate pod weight per hectare (t ha^{-1}), protein and oil content in seeds.

Statistical analysis

The statistical analysis was carried out according to Steel and Torrie, (1980). All data were collected to variance analysis (ANOVA) for RCBD. The significant differences were tested at P-value ≤0.05, with package IBM SPSS.

Conclusion

The results provide evidences that the associated application of chemical fertilizers, lime, cow manure and BS strain KCa6 inoculation can improved soil fertility and promote white bean growth, increase fresh yield compared to NPK fertilizer application alone. Moreover, Cd accumulation in WB stems was significantly reduced in treatments of the lime, cow manure, and BS strain KCa6 inoculation. Especially, Cd accumulation was undetected in WB seeds. The application of 40 kg urea ha^{-1} + 60 kg P_2O_5 ha^{-1} + 60 kg K_2O ha^{-1} combined with 2.0 $tCaCO_3$ ha^{-1} + 10.0 t cow manure ha^{-1} with BS strain KCa6 inoculation showed a fresh seed yield increase up to 16.6% compared to chemical fertilizers alone. Furthermore, lime application alone and/or combination with cow manure decreased Cd accumulation in WB stems from 9.8 up to 38% compared to the control treatment. These results encourage the co-application of lime, CM, ENFB inoculation, and

chemical fertilizers, which has a WB yield increase and Cd accumulation reduction in Cd polluted soils.

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