تاثیر باکتری‌های حل کننده سیلیکات‌ها و همزیستی مایکوریزایی بر نیاز کودی گیاه توتون

The effect of silicate solubilizing bacteria and mycorrhizal symbiosis on potassium fertilizer requirement of tobacco (*Nicotiana tabacum* L.)

Rahmatollah Ranjbar[1]- Ebrahim Sepehr[2]- Abbas Samadi[3]- MirHassan Rasouli Sadagiani[4]- Mohsen Barin[5], Behnam Dovlati[6]

PhD Student[1], Associate Prof.[2], Prof.[3], Prof.[4] Assist. Prof.[5] and Assist. Prof.[6], Soil Science Department, College of Agriculture, Urmia University

Abstract

K plays a vital role in increasing the tobacco yield and controlling quality parameters such as leaf combustibility. Soil has rich reserves of K, among which only 1–2% can be directly absorbed by plants. It may be more economically viable to transform the fixed slow-release K into available K. The ability of some microorganisms to dissolve soil K-bearing minerals is used in tobacco farming. The present study was conducted to screen the KSB isolates from tobacco-cultivated soils and evaluate the effects of potassium-solubilizing bacteria (KSB) isolates and arbuscular mycorrhiza fungi (AMF) on the K fertilizer requirement of tobacco. A study was conducted in factorial completely randomized design (CRD) with three factor with three replications. Potassium fertilizer factor included four recommended dose of fertilizer (RDF) for potassium (0, 50%, 75%, and 100% RDF), KSB factor included without KSB inoculants and KSB inoculants, and AMF factor included without AMF inoculant and AMF inoculant. The nine KSB isolates were isolated, purified and evaluated. Some of studied bacteria isolates included KSB20, KSB22, KSB30, KSB40, KSB42, KSB90, KSB92 and KSB10 isolates were more effective in releasing potassium from soil potassium-bearing minerals. In case of low amount of potassium fertilizer application (0 and 50% RDF), the AMF and KSB inoculants significantly increased the shoot and leaf dry weight of tobacco in comparison with control**.** The KSB inoculant led to a 6% increase in the tobacco shoot dry weight in comparison with control**.** KSB inoculation significantly increased leaf K concentration and AMF inoculation led to a 10% reduction in leaf K concentration in comparison with control. KSB can be used to increase the potassium concentration to about 3% in leaf dry matter and improving tobacco leaf, and reduced applied potassium fertilizer application by about 25%.

Key words: Tobacco, Potassium (K), mycorrhizal fungi, Potassium solubilizing bacteria (KSB), K fertilizer requirement

Introduction

Potassium (K) is one of the major essential macronutrients for plant growth. Soil has rich reserves of K, among which only 1–2% can be directly absorbed by plants (Malinovskaya et al., 1990). It may be more economically viable to transform the fixed slow-release K into available K that can be absorbed by plants. The ability of some microorganisms to dissolve soil K-bearing minerals, such as micas is an important feature for increasing the yield of high-K-demand crops such as tobacco (Friedrich et al., 2004 ; Liu et al., 2006). Also, these microorganisms have both economic and environmental advantage (Nihala, 2017). A large number of saprophytic bacteria such as *Bacillus mucilaginosus* and fungal strains such as *Aspergillus spp*. are known for their potential in releasing insoluble native K-source in soil into a plant available nutrient pool (Liu et al., 2012; Nihala, 2017). Tobacco (*Nicotiana* spp.) is one of the most important industrial crops (Subhashini et al., 2016). K plays a vital role in increasing the tobacco yield and controlling quality parameters such as leaf combustibility that is one of the key criteria taken into account by the tobacco industry for assessing quality. Thus, high ranges of K fertilizers applied in tobacco fields based on plant K requirement and build up soil K in tobacco producing countries (Subhashini, 2013 and Vann et al., 2012). Increasing cost of the fertilizers and that's environmental risks necessitates alternate means to fertilizers such as application of microorganisms. The use of chemical K fertilizers can be reduced by exploiting the potential of bio-inoculants which are inexpensive and eco-friendly. Information related to K-solubilizing microorganisms in tobacco rhizospher and their suitability in increasing the available K in tobacco-cultivated soil are not well-documented. Hence, the present study was conducted to screen the KSB isolates from tobacco-cultivated soils and evaluate their potential in dissolving K bearing silicate minerals and increasing soil available potassium.

Materials and Methods:

Rhizosphere soil sampling and preparation: Soil samples were randomly collected from the rhizosphere of tobacco (at flowering stage) from 25 different locations in northwest of Iran. Soil samples kept in the refrigerator at four degrees Celsius until the bacteria were isolated**.**

Preparation of mica minerals: Pure mica and feldspar minerals were prepared from the Soil Science Engineering Department of Urmia University. The elemental composition of the studied minerals by XRF was shown in Table (1). The mineral powders were passed through a 60 μm (230 mesh) sieve. The amount of absorbable potassium in the mineral powder was extracted using one molar of calcium chloride and 0.01 M hydrochloric acid (Ashrafi Saeidloo and Rasouli Sadaghiani, 2017). To ensure complete removal of usable potassium, potassium was extracted and measured with 1 M ammonium acetate, and acid washing was continued to ensure the removal of usable potassium. The minerals were then thoroughly washed with water and dried at 50 ° C.

Table 1- The percentage of chemical element oxides in studied clay minerals by XRF

Mineral name	SiO_2	Al_2O_3	K_2O	Na_2O	Fe_2O_3	MgO	CaO	MnO	P_2O_5	TiO_2	LOI*	Total
Feldspar	64.50	17.44	13.67	2.76	0.35	0.01	0.11	0.01	-	0.01	0.42	99.28
Ilite	47.32	34.24	10.74	0.62	1.82	0.08	0.09	0.11	0.03	0.06	4.44	99.26

*: Loss on ignition

Isolation of potassium-solubilizing bacteria from rhizosphere: The serial dilutions of the soil samples were made up to 10^{-4}. After the deposition of fine soil particles in the suspensions, 5 µl of diluted soil suspension was added on solid Aleksandrov medium plates (on the agar-based culture medium). Dissolve potassium minerals in the culture medium. Aleksandrov medium was containing (5.0 g Glucose, 0.5 g $MgSO_4.7H_2O$, 0.1g $CaCO_3$, 0.006 g $FeCl_3$, 2.0 g Ca_3PO_4, 2.0 g insoluble mica and feldspar powder as potassium source and 20.0 g agar) in 1 liter of deionized water. The plates were incubated at 28±2°C in incubator for 10 days. The clearance zone around the bacterial colony on the Aleksandrov solid medium indicates the ability of the bacterium to dissolve the potassium minerals present in the Aleksandrov medium. Clearance zone of colonies was isolated and cultured several times linearly on agar nutrition on purification and after obtaining pure isolate, the bacterial isolates were re-cultured pointwise and linearly on solid Aleksandrov medium. Through purification, nine isolates of bacteria soluble in potassium-bearing minerals were obtained and cultured in Slant. After incubation, these colonies were refrigerated at 4 ° C to evaluate some characteristics and evaluate the quality and quantity of the isolates (Ashrafi Saeidloo and Rasouli Sadaghiani, 2017).

Qualitative and quantitative evaluation bacterial isolates: For qualitative and quantitative evaluation of the isolates 25 ml of the nutrient broth culture medium was poured into each of the nine erlenmeyer 250 ml (equal to the number of purified isolates) and sterilized. The culture medium inside the erlenmeyers was inoculated with each of the purified bacterial isolates and shaken overnight at 28 ° C at 150 rpm. Then, Solid and liquid Aleksandrov media applied for qualitative (solubility index) and quantitative (K concentration in liquid Aleksandrov medium) evaluation, respectively, by using the completely randomized design (CRD) with three replications (Hu et al., 2006.). Bacterial isolates that created high solubility index (equation 1) on solid medium and released more K from K-bearing minerals into liquid medium, were selected as effective isolates. In qualitative evaluation, solubility index on solid Aleksandrov medium was measured by the use of equation 1 (Ebrahimi Karim-Abad et al., 2016).

$$\text{Solubility Index} = \frac{\text{Diameter of clearancezone} + \text{Diameter of colony}}{\text{Diameter of colony}} \qquad \text{(Equation 1)}$$

The unit of diameter in clearance zone around the bacterial colony is in millimeters. Eight bacterial isolate that had high solubility index were selected for quantitative evaluation in liquid Aleksandrov medium. In quantitative evaluation of bacteria, 1 ml of nutrient broth medium containing bacterial isolate into 50 ml of Aleksandrov liquid medium containing potassium minerals (2 g / l mixture of mica and feldspar clay minerals) was cultured in three replications separately at the same time. The control was also considered and one milliliter of sterile nutrient broth medium was used. Samples were shaken at 150 rpm for 10 days at 28 °C. Then, 10 ml of the liquid medium was centrifuged and filtered. The amount of potassium released in the liquid medium was measured using a flowmeter (Sugumaran P., and Janarthanam B. 2007). Bacteria isolates with high Solubility index and high ability to release potassium in the liquid medium were selected as efficient isolates

In order to evaluate the efficiency of the potent bacterial isolates for increasing soil available K, an experiment was conducted by using the completely randomized design (CRD) with three replications and eight potent bacterial isolates along with a control (non-inoculated soil). Sterilized soil samples were inoculated with bacterial isolates separately and incubated at 25°C, with 75% field capacity moisture levels for 90 days. After incubation, available K in soil samples were extracted with Ammonium Acetate 1M.

Biochemical evaluation of bacterial isolates

Starch hydrolysis test: For this purpose, nutrient agar medium containing 0.2% water-soluble starch was prepared and sterilized. Bacterial isolates were cultured on media in plates. After 72 hours of incubation, logol solution was added to expose the starch in the culture medium inside the plates. The presence of a yellow clearance zone in the blue background indicates the activity of the bacterium alpha-amylase (Schaad et al., 2001; Khoshrou et al., 2013).

Test of sugars (glucose and sucrose): Two separate culture media were prepared from peptone fluid, one containing 1% glucose and the other containing 1% sucrose. Peptone medium into test tubes was sterilized and inoculated with a specific bacterial isolate. After three days of incubation, if the desired sugar is consumed by the bacterium, the culture medium becomes acidic and as a result appears yellow. (Schaad et al., 2001; Khoshrou et al., 2013).

Fluorescent test: Sterilized King B culture medium into plates was inoculated with bacterial isolates. After 72 hours, the fluorescent properties of bacteria were determined under ultraviolet light by observing blue-green light (Schaad et al., 2001; Khoshrou et al., 2013).

Gram test: A good spread of bacteria was created and fixed on the slide. The bacterial slide was first covered with crystal violet solution and then with iodine solution for one minute. The bacterial mass on the slide was rinsed with alcohol, then covered with safranin for 45 seconds, and then washed with water after each step. After drying, the slides were prepared for microscopic examination (Khoshrou et al., 2013.

Data analysis: Variance of solubility index, K concentration in to liquid Aleksandrov medium and soil available K were analyzed by using SPSS (Statistical Package for the Social Sciences). Student-Newman-Keuls (SNK) test comparisons were made to compare available soil K using SPSS 16.0.

Results and Discussion

Isolation and quantitative and qualitative evaluation of bacterial isolates: Nine KSBs isolates, including KSB20, KSB30, KSB40, KSB22, KSB42, KSB90, KSB92, KSB70 and KSB10, were isolated and purified as effective isolates for dissolving of mica and feldspar minerals. According to the results of analysis of variance of the data obtained from the qualitative evaluation of 9 bacterial isolates, bacterial isolates had a significant difference ($P \leq 0.01$) in solubilizing potassium minerals and creating a clearance zone on Aleksandrov solid culture medium (solubility index). The highest solubility index (2.8, 2.7 and 2.5) obtained from the activity of KSB22, KSB42 and KSB10 isolates in solid Aleksandrov medium, respectively (Figure 1). The solubility index of KSB30, KSB40 and KSB92 was less than two (1.8, 1.8 and 1.9, respectively). The solubility index of KSB70 was the least (1.3), thus, this bacterial isolate was not selected for quantitative and biochemical investigation.

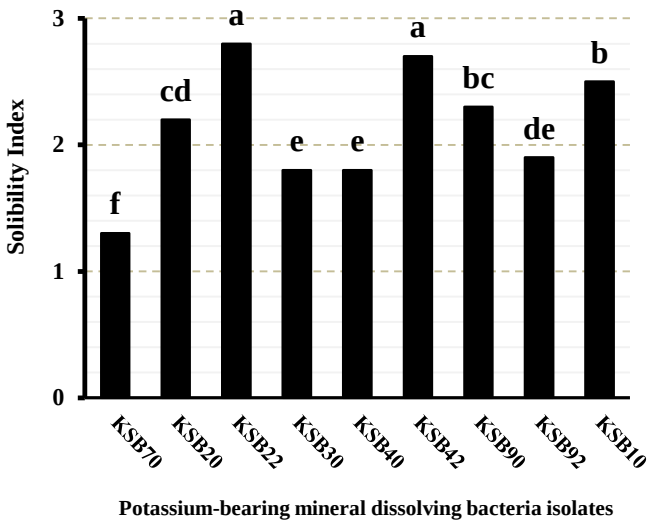

Figure 1- Effect of potassium-bearing minerals dissolving isolates on the mean of solubility index on solid Aleksandrov medium

After qualitative evaluation, bacterial isolates except KSB70 were quantitatively evaluated. The results of variance analysis obtained from quantitative evaluation of 8 bacterial isolates together control showed that the bacterial isolates were significantly different ($P \leq 0.01$) in ability to dissolve potassium minerals and released potassium from mica and feldspar minerals into Aleksandrov liquid medium (Table 2).

Table 2- Variance analysis of solubility index, released potassium in medium and soil available potassium after bacterial inoculation

The source of variation	Freedom degree	The mean of squares	
		Soil available potassium 90days after of inoculation	Released K into liquid medium
Bacteria inoculation	8	620.98**	12.356***
Error	18	13.96	0.139

*** indicate significant differences at 0.1% probability level.

According to means comparison results of potassium concentration in Aleksandrov liquid medium are shown in Figure 2. All 8 bacterial isolates were able to dissolve potassium-containing minerals and caused a significant increase (at least 2.7 times) in potassium concentration in Aleksandrov liquid medium compared to control. The concentration of potassium in control (medium without bacterial inoculation) was 2.96 mg L^{-1}. The highest concentration of potassium into liquid Aleksandrov medium (9.40 and 9.40 mg L^{-1}) related to the KSB42 and KSB10 isolates, respectively. The KSB42 and KSB10 isolates increased medium K concentration approximately three times more than non-inoculated medium (Figure 3). Microorganisms release potassium and other nutrients from minerals by secreting various organic acids depends on the type of potassium-bearing mineral (Sheng and Huang, 2002).

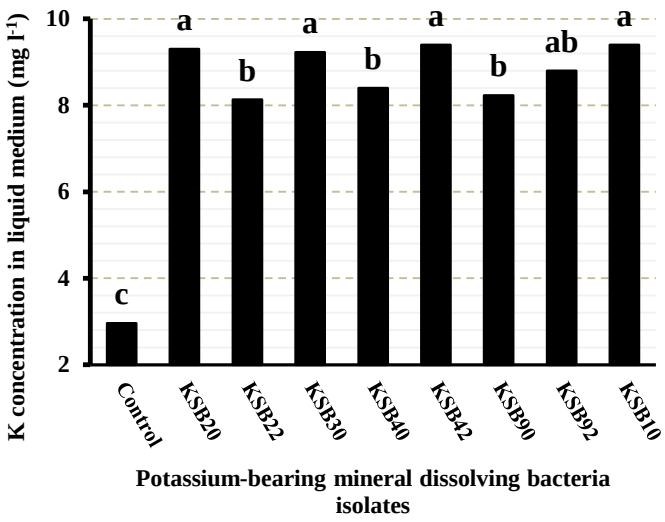

Figure 2- Effect of potassium-bearing minerals dissolving isolates on the mean of potassium concentration in liquid Aleksandrov medium

After quantitative evaluation of bacterial isolates dissolving potassium-bearing minerals in increasing the potassium concentration of the liquid medium, four bacterial isolates KSB20, KSB22, KSB42 and KSB10 were selected as efficient bacterial isolates in dissolving potassium-bearing minerals. Therefore, screening of these effective and native isolates makes it possible to use these bacteria in biological inoculation of tobacco alone or with potassium fertilizer.

The effect of bacterial isolates on soil available potassium

The properties of the studied soil in this part of the experiment are given in Table (3). According to the results of variance analysis, the effect of the studied bacteria on the amount of potassium available in the soil was significant ($P < 0.01$) as shown in table 2. Bacteria Isolates were significantly different in terms of the effect on the amount of potassium available in the soil (90 days after bacterial inoculation of the soil and incubation).

Table 3- The physico-chemical properties of studied soil

pH	Calcium Carbonate Equilibrium, CCE (%)	Cation Exchange Capacity, CEC (Cmol Kg^{-1})	Organic carbon (٪)	Available potassium (mg kg^{-1})	٪	
					Silt	Clay
6.79	0.25	19.2	0.52	158	37.4	39.2

The ability of isolated bacteria to increase the amount of soil available potassium is shown in figure 3. The amount of available potassium in soil for inoculation with KSB42 and KSB10 isolates was maximum (200 and 202 mg kg^{-1}, respectively), which increased to 44 and 46 mg kg^{-1} of soil, respectively, compared to the control (soil without KSB inoculation). The effect of KSB30, KSB40 and KSB20 bacterial isolates on increasing usable soil potassium was less (20 to 25 mg kg^{-1} soil compared to control).

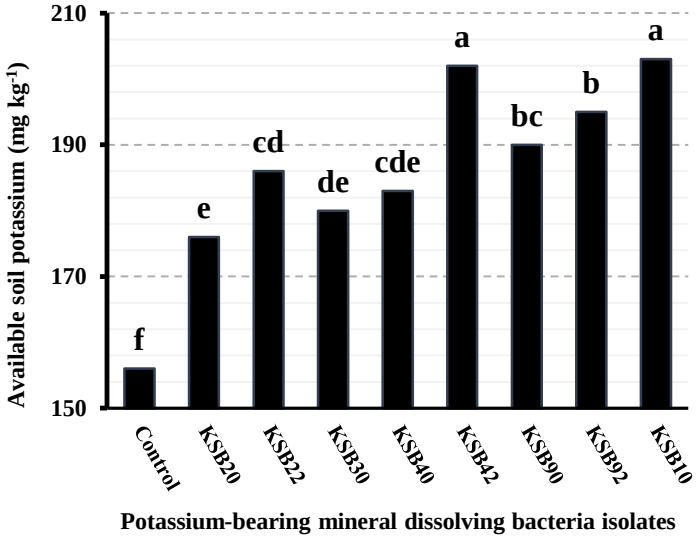

Figure 3- Effect of K-bearing minerals dissolving bacteria isolates on the mean of soil available potassium

The amount of potassium released by the isolated bacteria KSB30, KSB20, KSB42 and KSB10 to the liquid Aleksandrof medium was maximum, but the bacterial isolates KSB20 and KSB30, despite their high ability to dissolve potassium-bearing minerals in the Aleksanderov liquid medium, were less able to increase soil available potassium. KSB42 and KSB10 isolates were more effective in potassium release from soil potassium-bearing minerals, So that the amount of soil available potassium in inoculation with these isolates increased by 29% compared to the control.

Colony morphology of bacterial isolates: Eight KSBs isolates, including KSB20, KSB30, KSB40, KSB22, KSB42, KSB90, KSB92 and KSB10, were isolated and purified as effective isolates for dissolving of mica and feldspar minerals. Many bacterial isolates had a white colony (Table 4).

Table 4- The cultural properties of potassium-bearing mineral dissolving bacteria isolates

KSB[1] isolate	Colony morphology	Gram reaction	Colony diameter (mm)	Clearance zone diameter (mm)
KSB20	White, regular margin, slimy surface	+	5.1	11.1
KSB22	White, regular margin, slimy surface	+	5.0	13.3
KSB30	Creamish, regular margin, slimy surface	+	5.3	9.9
KSB40	Creamish white, irregular margin	+	5.4	9.9
KSB42	White, regular margin, slimy surface	+	3.7	10.2
KSB90	White, irregular margin, slimy surface	+	4.3	9.7
KSB92	Creamish, regular margin	+	6.2	12.3
KSB10	Bright, regular margin	-	4.3	10.7

1-KSB: potassium solubilizing bacterium

A clearance zone of bacterial colony appeared within 72 hours of incubation as shown in Figure (4).

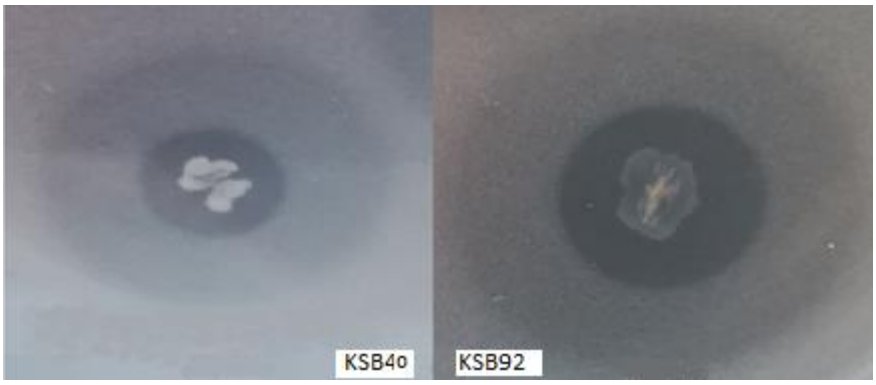

Figure 4- Potassium-bearing mineral dissolving isolates with well-demarcated clearing zones in Aleksanderov agar media

Biochemical properties of bacterial isolates: Most isolates were gram-positive, rod-shaped, and white in appearance. The studied isolates, except KSB22, KSB40 and KSB20, had α-amylase enzyme activity. Bacterial isolates, including KSB20, KSB30, KSB42 and KSB10, were significantly superior in digesting sucrose and glucose. The isolate of KSB10 also had fluorescence properties. (Table 5).

Table 5- Some of biochemical properties of potassium-bearing dissolving bacteria isolates

Bacteria isolate	Gram staining	Fluoresce test	Sucrose test	Glucose test	Starch hydrolysis test
KSB20	Positive	Negative	Positive	Positive	Negative
KSB22	Positive	Negative	Positive	Negative	Negative
KSB30	Positive	Negative	Positive	Positive	Positive
KSB40	Positive	Negative	Negative	Negative	Negative
KSB42	Positive	Negative	Positive	Positive	Positive
KSB90	Positive	Negative	Positive	Negative	Positive
KSB92	Positive	Negative	Negative	Negative	Positive
KSB10	Negative	Positive	Positive	Positive	Positive

Conclusions: Among bacterial isolates that were purified from the tobacco rhizosphere, the isolates of KSB42 and KSB10 were significantly higher in solubilizing potassium minerals and increasing the amount of available potassium in soil compared to other isolates. So that, these bacteria isolate increased potassium concentration into Aleksandrov liquid medium by more than three times and also increased soil available potassium by about 30% compared with the control. As a result, these isolates (KSB42 and KSB10) can be used as a bio-fertilizer to reduce potassium fertilizer application and increase the quality of tobacco after field experiments.

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