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Letter to the Editor

A Modified Plate Assay for Rapid Screening of Potassium-Solubilizing Bacteria

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ABSTRACT

The utility of microorganisms for solubilizing the unavailable forms of potassium (K) from soil has led to renewed interest in fabrication of rapid and sensitive plate assays for their isolation and screening. The present study developed a modified plate assay and compared it with previously reported methods for the isolation and screening of K-solubilizing bacteria. The newly developed plate assay is based on improved visualization of halo zone formation around the colonies on agar plates, through inclusion of an acid-base indicator dye, bromothymol blue (BTB), to modify the previously reported Aleksandrov medium. The halo zone exhibited a significant correlation ($R = 0.939$) with K released in liquid medium. The visualization of potential K solubilizers was improved using this method, which would help in detection of weak/non-acid producers based on secretion of organic acids in the medium. Organic acids in plate diffuse radially and form halo zones in response to reaction with the acid-base indicator dye BTB. Furthermore, K solubilization on plates with this method can be observed within 48–72 h, against the incubation time of 4–5 d needed in the earlier method. Therefore, the newly developed protocol for the plate assay was time saving, more sensitive, and beneficial in comparison to the previously reported Aleksandrov plate assay.

Key Words: bromothymol blue (BTB), Aleksandrov medium, halo zone formation, indicator dye, K solubilizers, microorganisms

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INTRODUCTION

Potassium (K), a major macronutrient for plant growth and development, is essential for many functions in the plant system. The significant role of K on growth, development, yield, and disease resistance has been illustrated in a wide variety of plant species (Ma, 2004; Ahmad and Maathuis, 2014). It remotes many activities such as the metabolism of carbohydrates, organic acids, fats, and nitrogenous compounds, besides involvement in protein synthesis, photosynthesis, resistance to drought, and water use efficiency. The concentration of soluble K in soil is very less, ranging from 4.0 to 30.0 g kg^{-1} (Sparks and Huang, 1985); hence, there is a growing interest in the role of microorganisms in the dissolution of K-bearing minerals. There are four pools of K in soil, *i.e.*, mineral K, available K or exchangeable K, non-exchangeable K, and soluble K (Goldstein, 1994; Zarjani *et al.*, 2013). Most of the K deposits in soil (90%–98%) are in the form of soil minerals, which can not be taken up directly by plants (Huang and Kiang, 1972; Goldstein, 1994). Several investigations showed that microorganisms increase the available K in culture medium. *Bacillus mucilaginosus* enhances the decomposition rate of aluminosilicates and dissolves K and SiO_2 from insoluble minerals by producing organic acids (Welch and Vandevivere, 1994; Ehrlich *et al.*, 2010). Several microorganisms are able to solubilize insoluble forms of K-bearing minerals such as feldspar, mica, illite, and orthoclase, by secreting organic acids that either directly decompose K or chelate the silicon ion to release K in solution (Toba *et al.*, 1991; Bennett *et al.*, 1998). Capsular polysaccharides and carboxylic acids such as citric acid, tartaric acid, and oxalic acids are known to be involved in the solubilization of feldspar by *Bacillus mucilaginosus* and *Bacillus edaphicus* (Richards and Bates, 1989; Lin *et al.*, 2002).

At present, no rapid assay system is available to identify K-solubilizing microorganisms directly on the plates, similar to those available for the identification of phosphorus (P) solubilizers on Pikovskaya agar pla-

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tes which contain insoluble sources of P. Potassium-solubilizing bacteria are generally screened by a plate assay using Aleksandrov agar medium (Aleksandrov *et al.*, 1967), based on exopolysaccharide production; however, the reliability of this exopolysaccharide-based screening for K solubilizers is questionable. Several bacterial isolates solubilize various types of insoluble K minerals in medium, but do not produce any exopolysaccharide on agar plates. Therefore, an assay for screening, based on better visualization techniques, needs to be developed as a prerequisite for identification of promising K solubilizers, as biofertilizer options for K-deficient soils. The present investigation was focused towards the development of rapid plate-based assay system for screening K solubilizers from soil samples.

MATERIALS AND METHODS

Isolation of bacteria and screening of K solubilizers

Soil samples were collected from the rhizosphere of vegetable crops and isolation of bacteria was done on a nutrient agar medium (pH 7.0 ± 0.2) containing 5 g L^{-1} peptone, 3 g L^{-1} beef extract, 5 g L^{-1} sodium chloride, and 15 g L^{-1} agar, using standard spread plate technique. A total of 85 bacterial isolates exhibiting different morphological characteristics were purified and spot inoculated on Aleksandrov agar medium plates (Hu *et al.*, 2006). The plates were incubated for 5 d at 30 °C and observed for the formation of halo zones around the colonies. Cultures positive for K solubilization based on plate assay were grown in Aleksandrov broth individually and solubilized K in culture supernatant was quantified with a flame photometer. All chemicals used were of analytical grade.

Qualitative analyses of K solubilization

Qualitative analysis of K solubilization was carried out using the Aleksandrov medium (pH 7.2 ± 0.2) containing 5.0 g L^{-1} glucose, 0.5 g L^{-1} magnesium sulphate, 0.005 g L^{-1} ferric chloride, 0.1 g L^{-1} calcium carbonate, 2 g L^{-1} calcium phosphate, and 2 g L^{-1} K-bearing minerals (Hu *et al.*, 2006). Potassium aluminosilicates were purchased from HiMedia Labs, Mumbai (India). All chemicals used were of analytical grade.

Optimization of assay using dyes

A modified Aleksandrov medium was prepared by amending the Aleksandrov medium with different concentrations of 3 different acid-base indicator dyes, bromocresol purple, phenol red, and bromothymol blue (BTB), from stock solutions (5 g L^{-1}) prepared in 70% (weight/volume) ethanol. Different amounts of stock dye solution ranging from 0.25 to 2.5 mL were mixed in 100 mL of Aleksandrov agar medium to achieve final concentrations of 12.5, 25.0, 37.5, 50.0, 75.0, 100.0 and 125.0 mg L^{-1}. After adding the measured amounts of dye solution, the medium was autoclaved and poured into Petri plates. Plates containing Aleksandrov medium without dye solution served as a control. The halo zone size and colony diameter were measured after 72 h. The halo zone size was calculated by subtracting the diameter of colony from the total diameter (Fig. 1).

Quantitative analysis of K solubilization

Quantitative assay of K solubilization was carried out in the same Aleksandrov medium as the qualitative assay of K solubilization. The cultures were inoculated in 150 mL conical flasks containing 40 mL of Aleksandrov broth and incubated for 5 d at 30 °C on a rotary shaker at 100 r min^{-1}. Autoclaved broth served as a control. The pH of the medium was checked at the end of incubation. After incubation, medium was centrifuged at 10 000 r min^{-1} for 10 min, and the superna tant was used for estimation of soluble K with a flame photometer. Different concentrations (20, 30, and 40

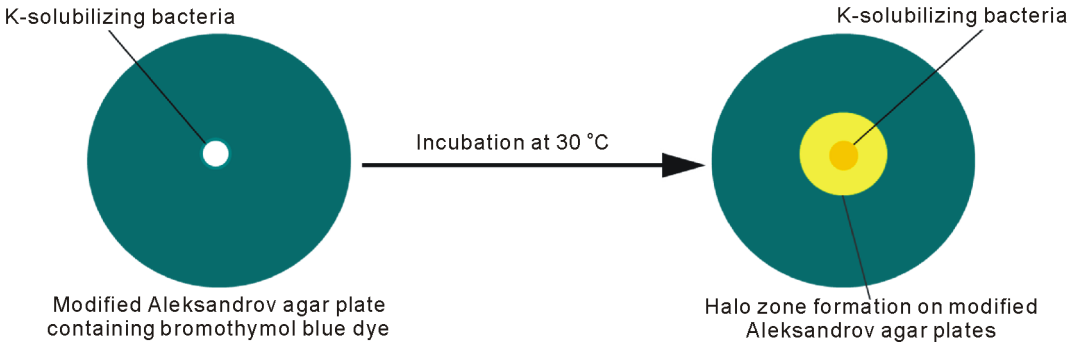

Fig. 1 Schematic diagram of plate assay for rapid screening of K-solubilizing bacteria.

mg L^{-1}) of KCl solution were used as standards for the determination of available K (Jackson, 1967). All the tests were conducted in triplicates. All chemicals used were of analytical grade.

Statistical analysis

Correlation analyses on solubilized K obtained with flame photometric method and the halo zone sizes generated using the newly developed plate assay were undertaken using the SPSS 16.0 statistical software package (SPSS Inc., Chicago, USA).

RESULTS

Screening of rhizosphere soil isolates for K solubilization

A set of 85 bacterial isolates were screened for K solubilization using qualitative assay involving spot inoculation on Aleksandrov agar medium plates. Based upon the halo zones observed in the plate assay, the positive isolates were grown in Aleksandrov broth. Quantification of solubilized K led to the selection of 11 promising isolates, AKSB 1, AKSB 7, AKSB 12, AKSB 15, AKSB 16, AKSB 22, AKSB 24, AKSB 25, AKSB 37, AKSB 40, and AKSB 41, which were used for further study.

Optimization of dye concentrations

Among the 3 different acid-base indicator dyes used, BTB was found to be superior in producing clear and distinct halo zones around the colonies for all 11 promising strains; therefore, BTB was selected for further analyses (Fig. 2). The tests with different concentrations of BTB showed that the visibility and clarity of yellow-coloured halo zones improved with increases in BTB concentration. A concentration of 100 mg L^{-1} of BTB was found to be optimum for the detection of K-solubilizing bacteria (Fig. 3).

Qualitative analysis of K solubilization

Among the 11 bacterial isolates analysed qualitatively for K solubilization, only four (AKSB 7, AKSB 24, AKSB 40, and AKSB 41) showed clear halo zones on Aleksandrov agar plates. However, on the modified medium developed using BTB, all the 11 isolates showed distinct halo zone formation (Fig. 4).

Quantitative analysis of K solubilization

All the tested bacterial cultures were able to solubilize potassium aluminosilicates in liquid medium and release K^+ in available form. The pH of the medium was found to be lowered. Solubilization in broth was done to compare the results with modified plate assay.

The mean values of halo zone size and K quantification at the 0.01 level of significance are given in Table I. Among all the bacterial cultures, a direct correlation was observed between the size of halo zone with modified plate assay and the solubilized K in broth. The scatter plot between the size of halo zone with modified plate assay and the solubilized available K in broth is shown in Fig. 5. The correlation coefficient was found to be 0.939 at the 0.01 level of significance.

DISCUSSION

In optimum crop production a continuous supply of nutrients, through replenishment by release of insoluble or mobilization of unavailable forms, is achieved through chemical or biological processes. The imbalanced use of chemical fertilizers is slowly leading to K deficiency in soils and poor growth and development of crops. It becomes imperative, therefore, to look for biological means to conserve our existing biodiversity and natural resources for sustaining crop production. Microorganisms are known to play a significant role in improving K availability in soil through the production of protons, siderophores, organic acids, organic ligands,

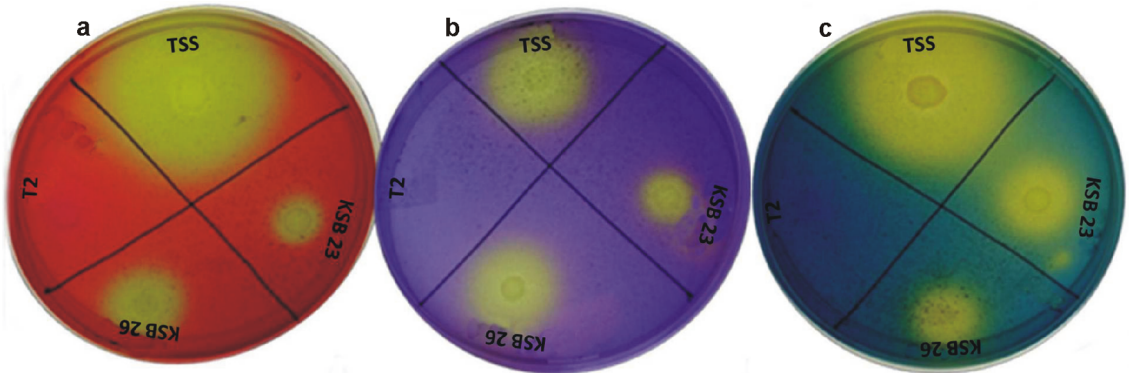

Fig. 2 Screening of different K-solubilizing bacterial isolates on the Aleksandrov agar medium amended with different acid-base indicator dyes (50 mg L^{-1}): phenol red (a), bromocresol purple (b), and bromothymol blue (c).

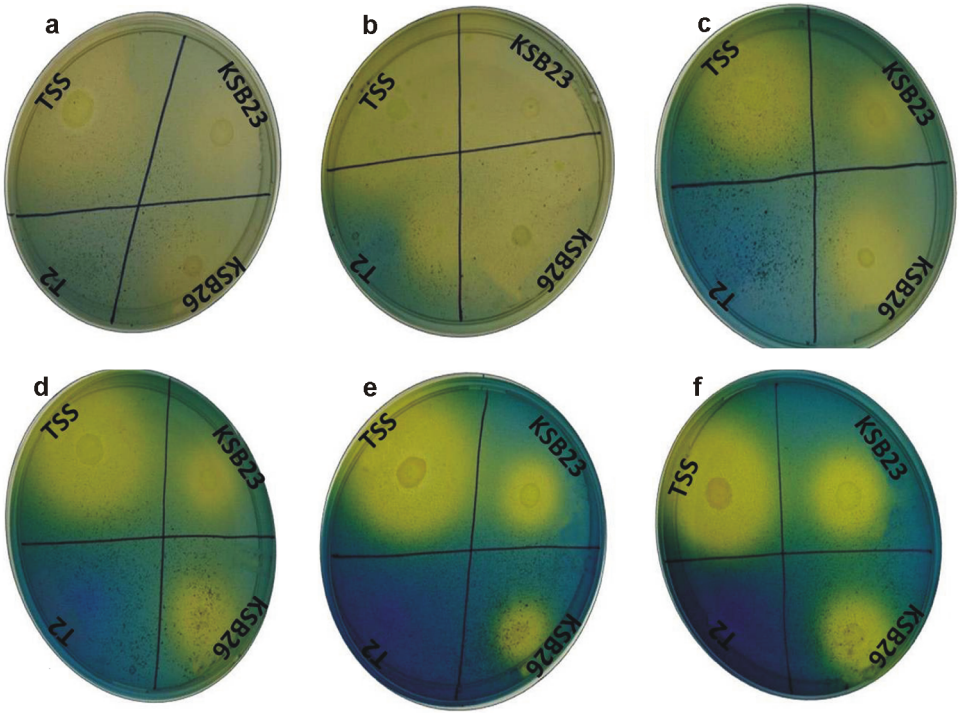

Fig. 3 Optimization of the concentration of bromothymol blue (5 g L^{-1}), an acid-base indicator dye used to amend the Aleksandrov agar medium for screening of K-solubilizing bacterial isolates, using different amounts of stock dye solution (ranging from 0.25 to 2.5 mL) mixed in 100 mL of Aleksandrov agar medium: 0.25 mL (a), 0.75 mL (b), 1 mL (c), 1.5 mL (d), 2 mL (e), and 2.5 mL (f).

TABLE I

Qualitative and quantitative comparison of K-solubilizing potential of 11 promising bacterial isolates after 72 h incubation between the Aleksandrov plate assay and the Modified plate assay[a)]

Isolate	Aleksandrov plate assay		Modified plate assay		Solubilized available K[b)]	pH of the culture
	Colony parameter	Halo zone size	Colony parameter	Halo zone size[b)]	in liquid medium	filtrate
	mm				µg mL^{-1}	
AKSB 1	7.0	–	6.0	10.17 ± 0.58[c)]	22.50 ± 0.40	6.1
AKSB 7	7.5	0.5	7.0	11.00 ± 0.50	24.83 ± 0.45	6.3
AKSB 12	7.0	–	6.0	10.33 ± 1.04	26.50 ± 0.26	6.5
AKSB 15	7.0	–	6.0	12.13 ± 1.03	21.60 ± 0.36	5.6
AKSB 16	7.0	–	6.0	10.00 ± 0.50	24.70 ± 0.75	6.5
AKSB 22	7.0	–	7.0	13.00 ± 0.50	26.30 ± 0.36	5.7
AKSB 24	7.5	0.5	7.0	8.00 ± 1.32	15.77 ± 1.00	6.6
AKSB 25	7.0	–	6.0	10.00 ± 1.00	17.90 ± 0.70	5.0
AKSB 37	7.5	–	7.0	1.50 ± 0.50	5.50 ± 0.61	6.9
AKSB 40	8.0	1.0	6.0	19.50 ± 1.50	37.30 ± 1.23	5.2
AKSB 41	7.5	2.5	9.0	12.67 ± 0.76	26.50 ± 0.96	5.5

[a)] Insoluble K provided as potassium aluminosilicates.
[b)] The correlation coefficient between the halo zone size with the modified plate assay for K-solubilization and the solubilized available K in liquid medium is 0.939 at the 0.01 level of significance, showing significant correlations among the values obtained from the modified plate assay and K quantification in broth.
[c)] Means ± standard deviations.

or capsular polysaccharides (Sparks and Huang, 1985; Sugumaran and Janarthanam, 2007; Zarjani *et al.*, 2013) and thus in improving crop growth and yields (Zhang and Kong, 2014). In order to identify promising microbes, an easy-to-use method for screening of soil samples to identify K solubilizers is an essential step in this direction.

The plate assay using Aleksandrov medium, which is currently utilized for screening K-solubilizing bacteria, is not rapid and not reproducible. It does not correlate significantly with the quantitative estimation. Screening of phosphate solubilizers using Pikovskaya

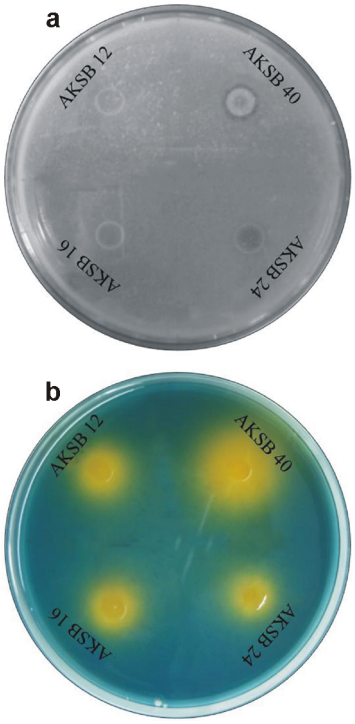

Fig. 4 Comparison of K solubilization on the Aleksandrov agar plates (a) and modified agar medium plates (b) after 72 h of incubation.

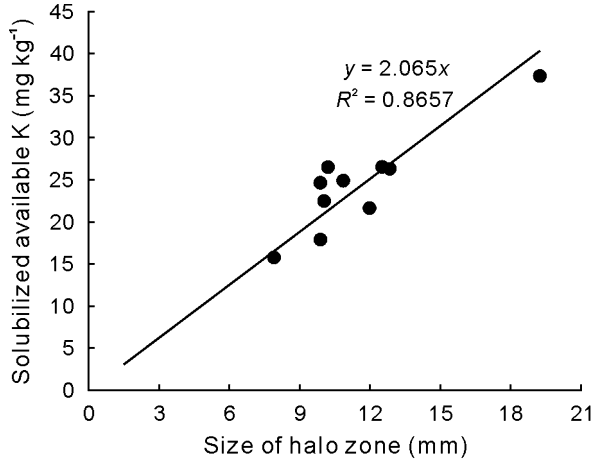

Fig. 5 Scatter plot illustrating the correlation between the size of halo zone with the modified plate assay and the solubilized available K in broth.

medium earlier had encountered similar problem, and the method was modified by amending the medium with indicator dyes such as bromocresol green (BCG) (Gadagi and Sa, 2002) and bromophenol blue (BPB) (Singh *et al.*, 2006). The basic contention is the production of organic acids leading to decrease in pH and the development of yellow color in modified medium. This in turn increases the protonated indicator dye (BPB or BCG) concentration, leading to a change in the colour of the reaction medium from blue to yellow, which can be visually observed. Taking the cue from this modification which helps in better visualization of phosphate solubilizers, we amended the Aleksandrov medium with different concentrations of dyes and compared the observations. Three acid-base indicator dyes, bromocresol purple, phenol red, and BTB, were used to develop an efficient plate assay for screening of K-solubilizing microorganisms. The medium modified with BTB proved more sensitive and most promising with all the 11 isolates showing clear formation of halo zone, whose diameters correlated positively with K quantified using a flame photometer.

In the present study, the BTB indicator, which has a turning range of concentrations that are adequate for monitoring the change in pH due to acid production by microorganisms, was found to be efficient and resulted in distinct halo zones around the K solubilizers. There was a strong positive correlation between halo zone size on modified Aleksandrov medium and quantitative release of K in culture supernatant. During the observation of K solubilization, it was found that many isolates showed the capability to solubilize in liquid broth; however, no polysaccharide production on Aleksandrov agar plates led to their delineation as non-K solubilizers. The use of acid-base indicator dyes, such as BTB, was therefore found to enhance the efficiency of screening P-solubilizing microorganisms.

Earlier reports suggested that the production of polysaccharides and organic acids plays an important role in weathering of K-containing minerals and releasing K (Aleksandrov *et al.*, 1967; Welch and Vandevivere, 1994; Sheng *et al.*, 2008; Meena *et al.*, 2014). In the modified plate assay of this study, halo zone formation based on organic acid production showed a significant correlation with K released in liquid medium. Zarjani *et al.* (2013) observed a similar inverse relationship between the pH value of culture medium and the concentration of solubilized K. Several other studies (*e.g.*, Lian *et al.*, 2007; Lopes-Assad *et al.*, 2010) have reported a positive dependence of pH on K solubilization.

A major advantage of the modified plate assay in this study was that the incubation time for screening K solubilizers was reduced significantly. The minimum incubation time for screening K solubilizers with the modified plate assay was 24 h; with the Aleksandrov medium, it normally exceeds 5 d (Hu *et al.*, 2006). The modified Aleksandra medium was more discriminative for isolating K-solubilizing bacteria from soils, allowing a wide range of bacteria employing different mecha-

nisms for K solubilization to be screened. A direct significant correlation between the size of halo zone and quantitative solubilization of K in liquid medium was observed using this method. It was more sensitive, reliable, and rapid for isolation of K-solubilizing bacteria. Further research needs to focus on in-depth analyses of the promising strains for the various mechanisms employed for K solubilization and development of a consortium for deployments in soils with different types of immobilized K reserves, thereby reducing dependence on K fertilizers in crop production.

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