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Evaluating Growth-Promotion and Drought Tolerance Properties of Endophytic Methylobacterium spp. from Semi-Arid Kenya Soil

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Abstract: This study presents the first report of isolating and characterizing root-associated endophytic methylotrophic bacterial strains from maize (*Zea mays L.*) adapted to the semi-arid region of Mbooni Kasikue in Makueni County, Kenya. The findings hold significant promise. A total of 50 unique, colonial, and distinctive endophytic methylotrophic bacterial strains were isolated and subsequently purified. The isolates were subjected to polyethylene glycol (PEG) 6000-induced stress to assess their sensitivity to drought. The isolates were then classified based on their level of drought sensitivity. The highly drought-tolerant isolates were screened for various plant-growth-promoting and drought-tolerance properties, including their ability to produce siderophores, synthesize indole-3-acetic acid, solubilize essential macro-elements, synthesize proline, salicylic acid, and exhibition of catalase activity. Two elite strains, (EMK13 and EMK27) based on their drought tolerance and growth-promoting capabilities were identified as *Methylobacterium variabile* and *Methylobacterium komagatae*, respectively, through 16S rRNA gene sequencing and BLAST analysis. The biotization effect of these elite strains was tested on maize seeds. The co-inoculation of strains EMK13 and EMK27 showed synergistic potential in improving growth metrics such as germination rate (86.67%), vigor index (650.03), and seedling length (7.2 cm) under drought stress conditions compared to the control.

Keywords: Endophytes, Drought, PGPR, Antioxidant Activity, *Methylobacterium spp.*

1.0 Introduction

Globally, agriculture faces significant challenges due to limited water availability, poor soil fertility, and climate change impacts, which are worsened by drought conditions (Ahmad et al., 2023). In Kenya, where agriculture is a critical component of the economy and food security, developing sustainable practices to enhance crop productivity and resilience is essential (Wafula et al., 2020). Traditional methods of increasing crop yields, such as extensive irrigation and chemical fertilizers, are not without practicality and environmental sustainability limitations. Therefore, exploring biological alternatives, such as plant growth-promoting bacteria (PGPB), offers a promising avenue for enhancing plant growth and drought tolerance (Ehinmitan et al., 2024).

Endophytic bacteria, which live symbiotically inside plant tissues without causing harm, have gained attention for their potential to promote plant growth and improve drought stress tolerance. The genus Methylobacterium is fascinating due to its unique metabolic capabilities and ecological roles. Methylobacterium spp. are methylotrophic bacteria capable of utilizing methanol and other single-carbon compounds as energy sources (Rahim et al., 2021). They are commonly found in the phyllospheres, rhizoplanes, rhizospheres and as endophytes within plant tissues. These bacteria can enhance plant growth by various mechanisms (direct and indirect), and their ability to produce osmoprotectants and other stress-related compounds suggests they contribute to plant drought tolerance (Gamit & Amaresan, 2023; Juma et al., 2022). Methylobacterium spp. colonize a wide range of plant species, including important crops such as maize, wheat, and rice. This unique group of PGPB can influence plant physiology by producing phytohormones like auxins, cytokinins, and gibberellic acids, which regulate plant growth and development. They can also enhance nutrient uptake by solubilizing phosphates and producing siderophores, compounds that chelate iron and make it more available to plants (Knief et al., 2010). Moreover, Methylobacterium spp. have been reported to improve plant water use efficiency and photosynthetic rates under drought conditions, although the underlying mechanisms remain to be fully elucidated (Jorge et al., 2019).

The interaction between Methylobacterium spp. and plants has been studied in various contexts, but there are still gaps in knowledge regarding their role in enhancing crop drought tolerance. Semi-arid regions are characterized by low and erratic rainfall, high temperatures, and nutrient-poor soils, making them particularly vulnerable to climate variability (Magnan et al., 2021). Methylobacterium spp. that survive, thrive, and interact with plants under these conditions possess enormous potential as biostimulants for sustainable agricultural practices to enhance economic crops' resilience to abiotic stress.

These bacteria may offer a natural and sustainable means to improve crop yields and stability in the face of abiotic stresses. However, the diversity, abundance, and specific plant growth-promoting (PGP) traits of Methylobacterium spp. in these soils must be

better understood. This knowledge is crucial for developing strategies to utilize these bacteria effectively in agricultural systems. Therefore, this study aims to identify and evaluate the growth-promotion and drought-tolerance properties of endophytic Methylobacterium spp. isolated from semi-arid Kenyan soils.

2.0 Materials and Methods

2.1 Sampling site

The Zea mays L. root material was collected from Makueni county of Kenya (-1° 48' 14.72" S and 37° 37' 13.22" E). The plant was chosen based on its known adaptation to semi-arid climatic conditions. Five healthy adult plants were sampled aseptically and transported at 4°C to the laboratory, Next, tap water was employed to remove loose and adhering soil.

2.2 Isolation of endophytic methylotrophic bacteria

The surface sterilization process followed the five-step method outlined by (Sahu et al., 2022). Initially, the samples were submerged in a 5% hypochlorite solution for 10 minutes. This was followed by immersion in a 2.5% sodium thiosulfate solution for another 10 minutes. The third step involved a 5-minute wash with 75% ethanol. Afterwards, a 10-minute rinse with 10% sodium bicarbonate was conducted. Root tissue was pulverized, diluted, and cultured on ammonia mineral salts (AMS) agar, using methanol as the only carbon and energy source. Cycloheximide and nalidixic acid were incorporated to suppress fungal proliferation. Distinct colonies were isolated and preserved in glycerol for further use (Udochukwu et al., 2016). The sterilization efficacy was confirmed by culturing the final washing water. The purified isolates were assessed for drought tolerance by incorporating -1.5 MPa of polyethylene glycol 6000 (PEG 6000) into AMS broth. Growth was assessed by measuring the optical density at 600 nm (OD600) using a spectrophotometer. The isolates were classified into highly sensitive, sensitive, tolerant, and highly tolerant following the criteria outlined by (Ashry et al., 2022).

2.3 Characterization of plant growth-promoting properties of the endophytic methylotrophic isolates

2.3.1 Siderophore production

Chrome Azul-S culture medium was used for the isolates' siderophore production assay. A positive result is indicated by the formation of an orange halo surrounding the growing colonies. The diameter of the halo was measured for quantification (Kumar et al., 2024).

2.3.2 Evaluation of indole-3-acetic acid (IAA) synthesis

Bacterial isolates were cultured in 100 mL of AMS supplemented with 1.0 mM of L-tryptophan. The cultures were incubated at room temperature in darkness for five days. After incubation, 1.5 mL of the bacterial culture was centrifuged at 8,000 rpm for 10

minutes. The resulting supernatant was mixed with 4 mL of Salkowski reagent (comprised of 2% 0.5 M $FeCl_3$ in 35% $HClO_4$). The mixture was then left in a dark environment at room temperature for 24 hours. The absorbance of the resulting IAA was measured at a wavelength of 520 nm using a spectrophotometer. IAA concentration was calculated by comparing the absorbance with a standard IAA curve (Egamberdieva, 2009).

2.3.3 Assessing gibberellic acid (GA) synthesis

Gibberellic acid production by bacterial isolates was measured using a spectrophotometric technique (El-Meihy et al., 2019). Bacterial cultures were grown in AMS medium at ambient temperature for a period of five days. Following incubation, a cell-free extract was prepared by centrifuging the culture at 8,000 rpm for 10 minutes. This extract was then transferred to a 15 mL reaction tube. Zinc acetate (2 mL) and potassium ferrocyanide (2 mL) solutions were added to the extract, and the mixture was centrifuged again at 8,000 rpm for another 10 minutes. Afterwards, 5 mL of the resulting supernatant was mixed with 5 mL of 30% hydrochloric acid and incubated at 28°C for 75 minutes. The absorbance of the solution was recorded at a wavelength of 254 nm, and the GA concentration was calculated by comparing the results to a standard curve of gibberellic acid.

2.3.4 Salicylic acid production

The production of salicylic acid (SA) by the bacterial isolates was quantified using the method outlined by (Abou-Aly et al., 2019). To begin, the pH of a 4.0 mL bacterial supernatant sample was adjusted to pH of 2 by adding 1 N HCl. Salicylic acid was then extracted by mixing the supernatant with an equal volume of chloroform ($CHCl_3$). Subsequently, 4.0 mL of distilled water and 5.0 mL of 2M ferric chloride ($FeCl_3$) solution were introduced into the mixture. The absorbance was read at 527 nm with a spectrophotometer, using chloroform as the blank control.

2.3.5 Mineral solubilization

The dissolution of inorganic minerals like phosphate, potassium, and zinc by bacterial strains was examined using various media: Pikovskaya, Aleksandrov, and Bunt & Rovira (Wang et al., 2022). To quantify the extent of dissolution, the solubilization index (SI) was determined with the formula below:

$$\text{Mineral Dissolution Index (SI)} = \frac{(\text{Colony Diameter} + \text{Halo Zone Diameter})}{\text{Colony Diameter}}$$

2.4 Characterization of drought tolerance properties of the endophytic methylotrophic isolates

2.4.1 Proline synthesis

Proline content was measured following a modified approach by (Chandra et al., 2018).. In this procedure, 0.5 mL of the bacterial supernatant was mixed with an equal volume of glacial acetic acid and 0.5 mL of an acid ninhydrin solution. The ninhydrin reagent was made by dissolving 2.5 g of ninhydrin in 80 mL of 6 M phosphoric acid and 60 mL of glacial acetic acid, and then heating the mixture until fully dissolved. The resulting solution was transferred into a clean glass tube, where it was vigorously shaken with 1.0 mL of toluene for 15-20 seconds. The absorbance of the toluene layer was subsequently read at 520 nm to quantify proline levels.

2.4.2 Catalase activity

Catalase activity (CAT) was assessed by observing the reduction in optical density at 240 nm, which is a reliable marker for the decomposition of hydrogen peroxide (H_2O_2). The procedure was conducted in accordance with the methods outlined by (Ahmed et al., 2021) The experiment involved using 250 µL of a 75 mM H_2O_2 solution, 750 µL of a potassium phosphate buffer at 100 mM concentration and pH 7.0, 100 µL of the enzyme extract, and 400 µL of distilled water.

2.5 Molecular characterization of the endophytic Methylobacterium spp

2.5.1 DNA extraction and PCR amplification

The bacteria's genomic DNA was isolated using the ZymoBIOMICS DNA Miniprep kit, and the Universal 16S rRNA gene was amplified using primers 27F and 1492R. PCR was performed using a ProFlex thermal cycler, with a reaction mixture including primers, dNTPs, buffer, $MgCl_2$, Taq DNA polymerase and nuclease free-sterile water (De Oliveira et al., 1999).

2.5.2 Sequencing analysis

Following PCR, DNA amplicons were purified using the QIAquick purification kit (USA), according to the manufacturer's instructions. Sequencing was then conducted, and the resulting 16S rRNA sequences were compared with those in the NCBI nucleotide database using the BLAST algorithm to identify related bacterial species (Woese & Fox, 1977)

2.6 Evaluation of seed biotization effectiveness

The study evaluated the effectiveness of Methylobacterium spp. in improving drought tolerance in maize seeds under stress and non-stress conditions. Drought-sensitive maize seeds were sterilized by soaking them in 70% ethanol and a 2% sodium hypochlorite solution. After treatment, the seeds were rinsed with distilled water. Petri dishes were filled with bacterial suspension prepared with -1.5 MPa PEG 6000, and 15 sterilized seeds

were placed in each dish. The seeds were incubated at 25°C for ten days, with germination counts recorded and seedlings length measured. Germination percentage and vigor index were calculated using the method described by (Ashry et al., 2022)

2.6 **Statistical analysis**

All experiments were performed in triplicate, and statistical evaluation was carried out using analysis of variance (ANOVA). The mean outcomes were subjected to Tukey's post-hoc test through SPSS version 14.0. Statistical significance was determined at a p-value below 0.05.

3.0 Results

A total of 50 endophytic methylotrophic bacterial strains were isolated from the root tissue of maize (Zea mays l.) cultivated in Makueni County, Kenya, a semi-arid region. The isolates were subjected to an osmotic pressure of -1.5 MPa, induced with polyethylene glycol (PEG) 6000, and then classified based on their drought sensitivity levels. Of the isolates, 52% were highly sensitive, 32% were sensitive, 10% were tolerant, and only 6% were highly tolerant. The three highly tolerant isolates (EMK13, EMK27, and EMK38) were selected for plant growth promotion and drought tolerance assays.

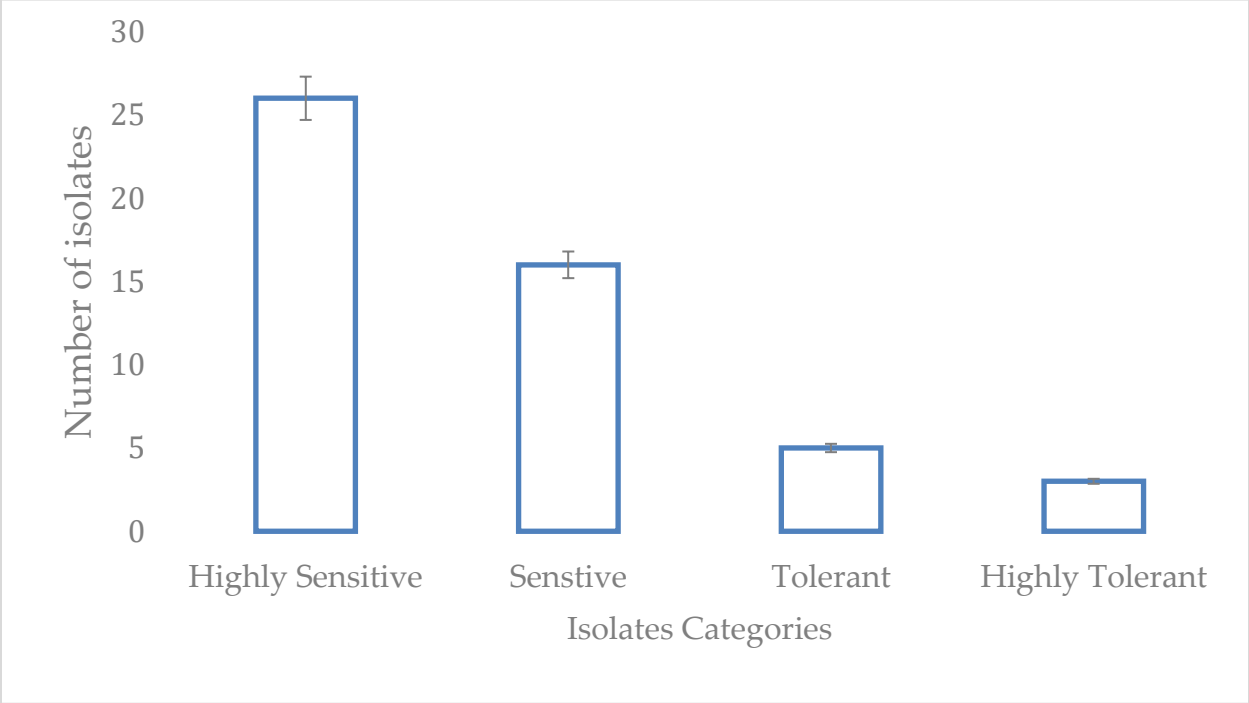

Figure 1. Tolerance percentages of bacterial isolates grown on Methanol+AMS medium supplemented with PEG 6000 (-1.5 MPa)

3.1 Qualitative evaluation of plant growth-promoting properties of the isolates

Siderophore production in the tested isolates was observed EMK13 and EMK27 tested positive, whereas EMK38 tested negative (Table 1). However, the isolates showed varying efficiency in the solubilization of potassium, phosphate, and zinc. EMK27 and EMK38 were able to solubilize inorganic phosphate by visible clear zone on growth media, but EMK13 tested negative. Regarding potassium and Zinc solubilization, isolates EMK13 and EMK 27 tested positive, whereas isolate EMK38 tested negative.

Table 1 Quantitative Plant Growth Promoting Properties of the Isolates

PGP properties	EMK13	EMK27	EMK38
Inorganic phosphate utilization	-	+	+
Zinc solubilization	+	+	-
Potassium solubilization	+	+	-
Siderophore production	+	+	-

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3.2 Quantitative evaluation of plant growth-promoting properties of the isolates

All the tested isolates synthesized indole acetic acid with varying efficiency. EMK13 was the best producer at 27.56 µg/mL, followed by EMK27 with 22.04 µg/mL and EMK38, with 18.43µg/mL production, is the least efficient producer (Figure 2a). In terms of Gibberellic acid production, EMK27 is the most potent producer at 33.42 µg/mL, closely followed by EMK13 with 30.77 µg/mL and EMK38 with 16.53 µg/mL respectively (Figure 2b). Salicylic acid production was assayed in the three highly drought-tolerant isolates. EMK27 produced 31.32 mg/mL, EMK13 with 28.07 mg/mL and EMK38 with 18.56 mg/mL (Figure 2c).

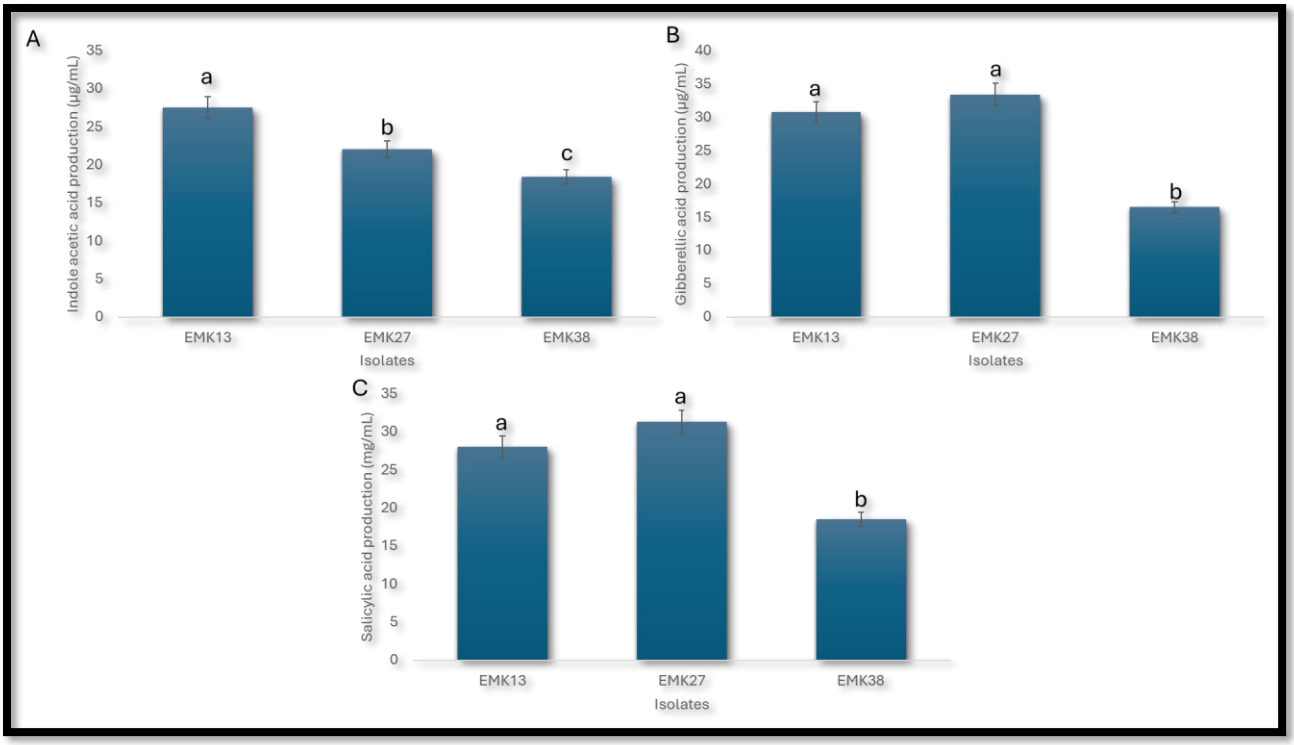

Figure 2. Production of (A) IAA, (B) GA and (C) SA by elite drought-tolerant isolates. The data shown are the average results from three repeated experiments, with error bars representing the measurement variation. Means that share the same letters are not significantly different at the 0.05 significance level, as determined by the Tukey test.

3.3 Quantitative evaluation of drought tolerance properties of the isolates

The production of the antioxidant enzyme catalase and the osmoprotectants proline by the three elite strains with high drought tolerance was evaluated (Figure 3). The maximum proline production was observed in EMK27 with 2.65 µg/mL followed by EMK13 with 1.69 µg/mL and EMK38 with 0.72 µg/mL (Figure 3a). Catalase enzyme activity for the tested isolates showed varying efficiencies. EMK13 showed a maximum CAT activity of 1.43 mg/mL, followed by EMK27 with 1.22 mg/mL and the least activity was observed at 0.73 mg/mL in EMK38 (Figure 3b). Based on preliminary testing, isolates EMK13 and EMK27 were selected as two elite isolates for further testing.

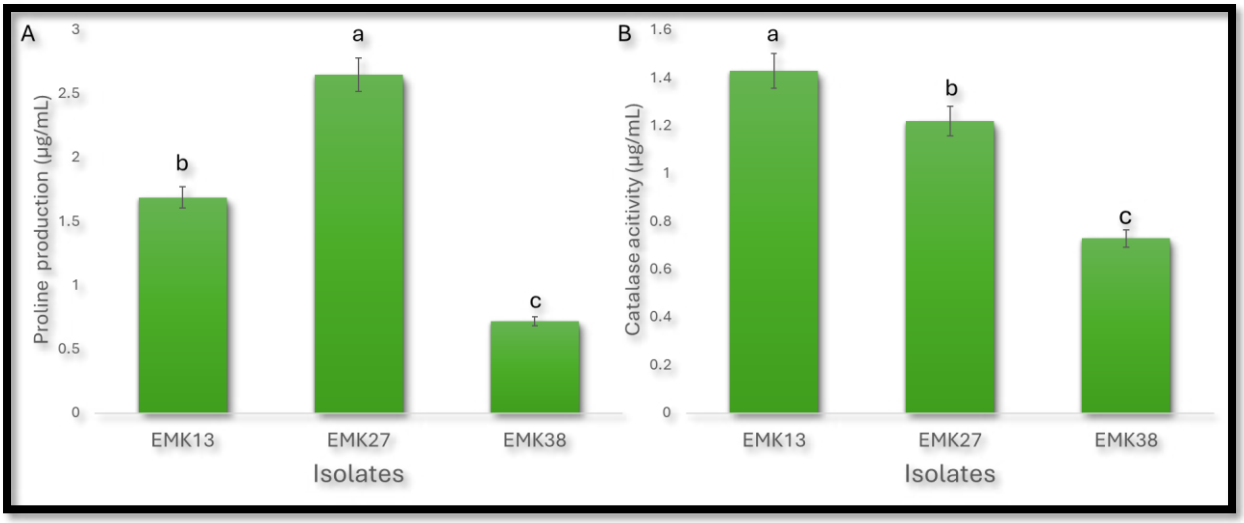

Figure 3. Production of (A) proline and (B) catalase by elite drought-tolerant strains. The data shown are the average results from three repeated experiments, with error bars representing the measurement variation. Means that share the same letters are not significantly different at the 0.05 significance level, as determined by the Tukey test.

3.4 Molecular identification of endophytic methylotrophic strains

The amplification and sequencing of approximately 1.5 kb fragments of the 16S rRNA gene were conducted for two elite drought-tolerant bacterial isolates using polymerase chain reaction (PCR) techniques. This approach was employed to accurately determine the identity of the isolates through analysis of the 16S rRNA gene segments. The isolates were identified as Methylobacterium variabile (EMK13) and Methylobacterium komagatae (EMK27).

3.5 Microbial seed biotization efficiency test

The effects of the EMK13 and EMK27 strains on maize seed growth promotion metric were evaluated under both normal and drought-stressed conditions, with an osmotic pressure of -1.5 MPa, as outlined in Table 2. Under non-stress conditions, seeds treated with either EMK13 or EMK27, individually or in combination (T3, T5, & T7), demonstrated increased germination rates, longer seedling lengths, and a higher vigor index, indicating their potential use as biofertilizers. Additionally, during drought stress, seeds treated with a combination of EMK13 and EMK27 (T8) showed significantly better results, with a vigor index of 650.03, seedling length of 7.2 cm, and a germination percentage of 86.67%, compared to seeds treated with EMK13 or EMK27 alone (T4 and T6).

Table 2. Impact of Microbial Inoculation on Seed Vigor, Seedling Length, and Germination Rate Percentage

Treatments	Description	Vigor index	Total seedling length (cm)	Germination %
T1	Uninoculated seed	233.38	5.60	66.67
T2	Uninoculated seed + (-1.5 MPa)	11.66	0.70	13.33
T3	Seeds + EMK13	660.00	6.60	100.00
T4	Seeds + EMK13 + (-1.5 MPa)	265.00	5.30	50.00
T5	Seed + EK27	610.00	6.10	100.00
T6	Seeds + EMK27 + (-1.5 MPa)	340.02	5.10	66.67
T7	Seeds + EMK13 + EMK27	760.00	7.60	100.00
T8	Seeds + EMK13 + EMK27 + (-1.5 MPa)	650.03	7.20	86.67

4.0 Discussion

The isolation of 50 endophytic methylotrophic strains from maize roots grown in semi-arid Makueni County, Kenya, revealed a significant diversity in drought tolerance, with only a small percentage (6%) being highly tolerant. This highlights the rarity of drought-tolerant endophytes, making identifying EMK13, EMK27, and EMK38 as highly tolerant strains particularly noteworthy. The differential response to osmotic stress suggests these isolates possess adaptive mechanisms that could be explored further for agricultural applications in drought-prone regions.

The strains demonstrated varied abilities in terms of plant growth-promoting (PGP) properties. EMK13 and EMK27 exhibited strong siderophore production, a trait linked to improved plant iron acquisition, which is crucial under nutrient-deficient conditions often associated with arid soils (Juma et al., 2022; Kumar et al., 2024). The ability of EMK27 and EMK38 to solubilize inorganic phosphate, as well as EMK13 and EMK27's capacity to solubilize potassium and zinc, suggests these strains can enhance nutrient availability, further supporting plant growth under stress (Hawkesford et al., 2023; Lambers, 2022). Interestingly, EMK38 was less consistent, testing negative for siderophore and potassium solubilization, indicating strain-specific capabilities that may influence their utility in different environmental contexts.

The quantitative evaluation of PGP traits revealed that all strains produced indole acetic acid (IAA), gibberellic acid (GA), and salicylic acid (SA), with EMK13 and EMK27 consistently outperforming EMK38. EMK13's superior IAA production suggests its potential to enhance root development, which is crucial for water uptake in drought conditions (Chandra et al., 2018). EMK27's high gibberellic acid and salicylic acid

production positions it as a strain with strong phyto-hormonal regulation, likely to enhance plant growth and stress response (Gutiérrez-Mañero et al., 2001). These hormone-mediated mechanisms align with the enhanced seedling vigor and growth observed in the microbial seed biotization tests.

The role of osmoprotectants and antioxidant enzymes is critical in drought tolerance, as demonstrated by the proline and catalase assays. EMK27's highest proline production underscores its ability to mitigate osmotic stress by stabilizing proteins and membranes, while EMK13's highest catalase activity indicates its capacity to alleviate oxidative stress (Ghaffari et al., 2019). These biochemical traits suggest that both EMK13 and EMK27 are well-equipped to enhance maize resilience under drought stress, making them suitable candidates for further biotechnological applications (Chandra et al., 2018; Li et al., 2022).

Molecular identification confirmed EMK13 and EMK27 as Methylobacterium variabile and Methylobacterium komagatae, respectively. The genus Methylobacterium is known for its plant growth-promoting attributes, and these findings align with previous reports of their ability to modulate plant hormone levels and improve stress tolerance (Gamit & Amaresan, 2023; Vanacore et al., 2022). The identification of these species within the context of drought tolerance and PGP traits adds valuable insights into the functional diversity of methylotrophic bacteria in semi-arid environments (Jorge et al., 2019).

The microbial seed biotization test further validated the growth-promoting effects of these strains. Under non-stress conditions, seeds inoculated with either strain (EMK13 or EMK27) showed improved germination rates and seedling growth, consistent with the observed PGP traits. Under drought conditions, the combination of EMK13 and EMK27 (T8) yielded the most substantial improvements. This synergistic effect could be attributed to the complementary actions of the two strains, where the higher IAA production by EMK13 and the enhanced GA and SA production by EMK27 together promote both root and shoot growth, as well as stress resilience. These findings support their potential application as biostimulants, particularly in semi-arid regions where water availability is a limiting factor. Further field trials are necessary to assess the long-term impact of these strains on crop yields under varying environmental conditions.

5.0 Conclusion

To effectively counteract the negative impacts of water scarcity around crop roots, it is crucial to choose bacterial strains that are resilient to drought and exhibit a range of traits that promote plant development. This study successfully identified methylotrophic strains that exhibit substantial drought tolerance and a range of plant growth-promoting characteristics. These strains were shown to be beneficial in both normal and deought conditions. Two of these isolates, namely EMK13 and EMK27, have been shown to be very efficient plant growth-promoting bacteria (PGPB). Applying EMK13 or EMK27 to maize

seedlings led to a significant enhancement in germination rates, a promotion of seedling development, and a mitigation of the negative impacts of drought.

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