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Indole-3-acetic acid production by rhizobacteria *Bacillus* spp. to various abiotic stress factors

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ABSTRACT

Indole-3-acetic acid (IAA) phytohormone plays an essential role in forming and initiating main, lateral, and adventitious roots in vegetative propagation. Plants are receiving IAA naturally from a diverse group of soil-plant associated rhizobacteria. However, IAA synthesis by rhizobacteria is influenced by abiotic growth condition. Three indigenous *Bacillus* isolates were subject to *in vitro* assay for the effects of abiotic factors (temperature, salinity and pH) on growth and IAA production. All isolates grew well between 25 - 40°C, and only *B. megaterium* UPMLH3 was capable of synthesising IAA (21.18 µg/ml) at 40°C. All three bacterial growth under saline stress were slightly dropped over control (0% NaCl), but still producing IAA up to 1% NaCl condition. *B. cereus* UPMLH24 revealed high resistance to salinity up to 5% NaCl. Optimum growth of all three *Bacillus* spp. was at pH 7. *B. cereus* UPMLH1 and UPMLH24 discovered higher IAA production in slightly alkaline condition (pH 8). Each rhizobacterium shows different physiology trait against each abiotic factor. However, the multiple tolerance ability of PGPR against abiotic factors is an indication that its ability to survive under harsh soil and plant environments while delivering benefits to the plant. Thus, *B. cereus* UPMLH1, *B. megaterium* UPMLH3 and *B. cereus* UPMLH24 might serve as potential biofertiliser, enhancing the growth performance of test plants at various environmental conditions.

KEYWORDS: Acidity, *Bacillus cereus*, *Bacillus megaterium*, Rhizobacteria, Salinity, Temperature

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Author note:
This paper is dedicated to the memory of the late Dr. Wong Sing King.

INTRODUCTION

Plant growth-promoting rhizobacteria (PGPR) enhancing plant growth and yield by direct and indirect mechanisms through increasing nutrients availability, producing phytohormone, and controlling plant diseases (Gouda *et al.*, 2018). Somehow, 80% of PGPR can synthesise and export phytohormones such as auxins, gibberellins, cytokinins, ethylene and abscisic acid (Kumar *et al.*, 2019). Indole-3-acetic acid (IAA) is a type of auxin involved in the rapid initiation and formation of central, lateral and adventitious roots of plants (Raheem *et al.*, 2018). Several studies clearly showed the effects of various PGPR on the growth of different crops in different climates, soils and temperatures (Fan *et al.*, 2018; Mathes *et al.*, 2020). Among PGPR, genus *Bacillus* is imperative potential bioinoculants in conventional organic agriculture, as bioenhancer (Probanza *et al.*, 2001; Zakry *et al.*, 2012a), bioregulator (Zakry *et al.*,

2010; Zakry *et al.*, 2012b) and biocontrol (Domenech *et al.*, 2006; Herman *et al.*, 2008). Other than that, Bacilli are novel rhizobacteria that proficient to adapted and stimulated various type of economic plants; including pine, *Pinus* sp. (Probanza *et al.*, 2001), oil palm, *Elaeis guineensis* (Zakry *et al.*, 2012a), black pepper, *Piper nigrum* (Aziz *et al.*, 2015), tomato, *Lycopersicon esculentum* (Domenech *et al.*, 2006), rice, *Oryza sativa* (Thakuria *et al.*, 2004), pigeon pea, *Cajanus cajan* and wheat, *Triticum aestivum* (Tilak and Reddy, 2006). Bacilli can survive for prolonged periods in the soil in the form of hardy spores (Paul *et al.*, 2019).

Environmental stresses, including temperature, soil pH, salinity and drought, are among the most limiting factors to plant productivity. Among these, salinity is one of the most unfavourable factors that could limit agriculture's future in most productivity areas of the world (Ashraf, 1994). Environmental

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abiotic factors affect the diversity and biological activity of microorganisms in the soil (Khan *et al.*, 2020). Delivering multiple abiotic stress-resistant PGPR as biofertiliser helps improve the total resistance system of those plants against stress environment while enhancing plant biomass and yield (Banerjee *et al.*, 2010).

In previous studies (Zakry *et al.*, 2010; Zakry *et al.*, 2012b), *B. cereus* UPMLH1, *B. megaterium* UPMLH3 and *B. cereus* UPMLH24 showed different responses to the early development of shallot (*Allium ascalonicum*) and mustard (*Brassica juncea*) plants. They have different shallot and mustard development responses with additional IAA production capability. PGPR signals and reactions to plants in the rhizosphere are different between species, variety, and plant vegetative stage. Other than plant-bacteria association, abiotic environmental factors are also involved in manipulating a complex communication system between PGPR and plants in the rhizosphere. Nevertheless, association of IAA bacteria to the IAA pool in the rhizosphere depends on several factors, including population size of IAA synthesising bacteria and amount of IAA produced by an individual cell in rhizosphere. Thus, this study was conducted to assay the tolerance capability of indigenous PGPR *Bacillus* spp. isolates on IAA production against *in-vitro* temperature, saline and pH stress conditions.

MATERIALS AND METHODS

Strain *Bacillus cereus* UPMLH1, *Bacillus megaterium* UPMLH3 and *Bacillus cereus* UPMLH24 with GenBank/EMBL/DDBJ accession number HQ876003, JN012241, and HQ876004, respectively; used in this study were previously isolated from soil and root samples of pepper vine (*Piper nigrum* L.) of Kuching and Semengok Emas varieties grown under stress and low soil fertility condition and characterised as indole-3-acetic acid synthesiser (Zakry *et al.*, 2010).

Overnight grown bacterial culture (1000 µL) was transfer into 50 mL of sterile nutrient broth and incubated at different temperatures (25, 28, 35 and 40°C) for 96 h and shaken at 140 rpm. Fully grown bacterial cultures were harvested and centrifuged at 4000 rpm for 20 min. The supernatant (2 mL) was mixed with 4 mL of Salkowski reagent (50 mL, 35% of sulphuric acid, 1 mL 0.5M FeCl₃ solution) and kept in the dark for 25 min as described by Gordon and Weber (1951). The development of the pink colour in the mixture indicates IAA production. Optical density was taken at 530 nm using a UV-Vis spectrophotometer. Development of pink colour measured at 540 nm, and the IAA production by rhizobacteria estimated based on a standard graph of IAA in the range of 0-100 µg/mL. Bacteria were grown in different NaCl concentrations [0.1, 0.5, 1.0 and 5.0 % (w/v)] and various pH (4.0 to 10.0 with 1.0 interval) for 96 h at 28±1°C and IAA production was quantified as described earlier. All data (except optical density of bacteria cell) subjected to Analysis of Variance (ANOVA). The mean separation was done using the least significant difference (LSD) at $p < 0.05$.

RESULTS

In general, the growth of all three *Bacilli* spp. decreased as the incubation temperature increased (Figure 1). Even though

B. cereus UPMLH1 had better growth at OD_{530nm} of 1.77-1.83 under 25-40°C incubations than *B. megaterium* UPMLH3 and *B. cereus* UPMLH24, but the highest growth was achieved at 25°C (OD_{530nm} of 1.83). *B. cereus* UPMLH24 recorded highest growth at 28°C (OD_{530nm} of 1.79) and followed by 25°C (OD_{530nm} of 1.78). Strain *B. cereus* UPMLH1 and UPMLH24 synthesised higher IAA at 25°C (with 23.97 µg/mL and 28.56 µg/mL), and no IAA detected under incubation of 40°C. IAA production by *B. megaterium* increased as the incubation temperature increased from 25 to 35°C (with the range of 15.46-27.34 µg/mL) and sudden fall at 40°C. However, *B. megaterium* was the greatest IAA producer among all three strains after 28°C of incubation.

In general, all three *Bacillus* spp. strains have good growth under 0-5% saline condition, and *B. cereus* UPMLH1 revealed better overall growth (Figure 2). Strain *B. megaterium* has the same OD (1.78 respectively) with 0 and 1% saline conditions. Strain *B. cereus* UPMLH24 has higher growth rate with 0.5% NaCl (with OD 1.80) and it followed by 0% NaCl (with OD 1.79). On the whole, *B. cereus* UPMLH24 has higher IAA production than *B. cereus* UPMLH1 and *B. megaterium* UPMLH3 under 0-5% saline conditions. IAA production by *B. cereus* UPMLH24 dropped (from 28.56 µg/mL to 5.14 µg/mL) as the salinity level increased. However, strain *B. megaterium* UPMLH3 synthesised higher amount of IAA under 0.5% saline (with 18.21 µg/mL) than 0% NaCl, and no IAA was detected at 5% saline stress.

All three *Bacillus* sp. strains were grown at pH 5-10, and no growth observed at pH 4 (Figure 3). *B. cereus* UPMLH1, *B. megaterium* UPMLH3 and *B. cereus* UPMLH24 have tremendous growth at pH neutral. Strain *B. cereus* UPMLH1 shown better growth under alkaline conditions (pH 8-10 with 1.3-1.4 OD). In contrast, strain *B. cereus* UPMLH24 has good change under slightly acidic conditions (pH 5-7). However, *B. megaterium* UPMLH3 revealed almost consistent growth in the range of pH 5-10 (with OD 1.59-1.70) than other strains. *B. cereus* UPMLH1 has higher production of IAA under slightly acidic (pH 6) and alkaline (pH 8) conditions at 51.05 µg/mL and 51.94 µg/mL, respectively. *B. cereus* UPMLH 24 produced the highest amount of IAA (56.66 µg/mL) at pH 8.

DISCUSSION

Landa *et al.* (2004) documented a slight temperature change, causing remarkable changes in rhizobacterial population and biological activities. This fact helps explain the findings of the present study. The population size of *Bacillus* spp. strains were inverted to temperature rising. The IAA synthesising activity by *B. cereus* UPMLH1 and UPMLH24 at 28°C was dropped about 72% and 74%, respectively, from 25°C incubation. *B. cereus* UPMLH1 and UPMLH24 produce IAA at an optimal level at 25°C. However, strain *B. megaterium* UPMLH3 has reached a tolerance to high temperature at 40°C on IAA synthesising activity, with optimum productivity was reached under 35°C condition. This phenomenon indicates that *B. cereus* and *B. megaterium* have the differential regulation on the structural development of plant roots by producing a variable level of IAA where the IAA production capacity of bacteria probably influenced by fluctuating soil temperature.

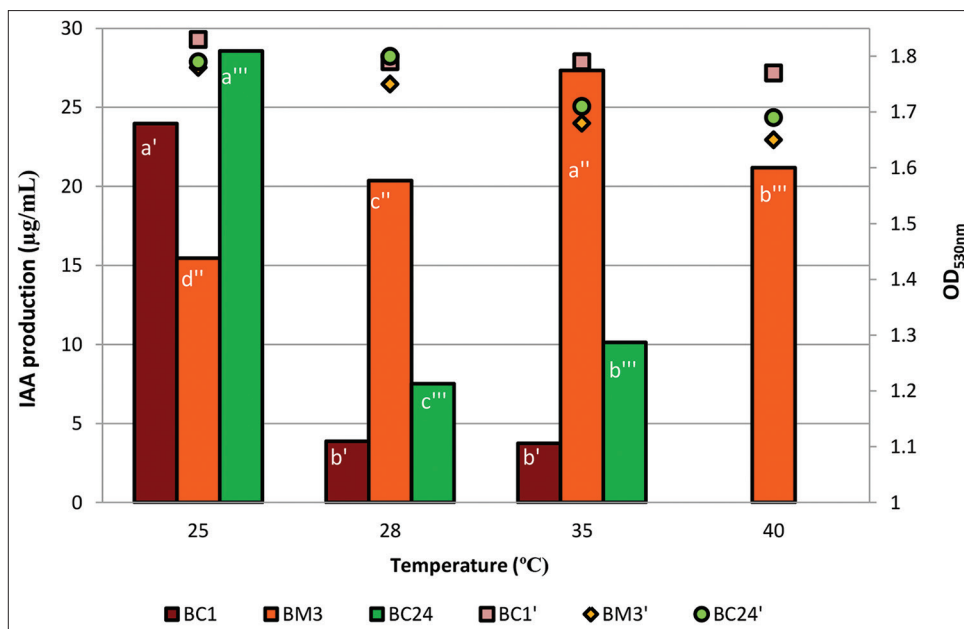


Figure 1: Influence of temperature on growth and IAA production by rhizobacteria
Note: BC1: *B. cereus* UPMLH1; BM3: *B. megaterium* UPMLH3; BC24: *B. cereus* UPMLH24. BC1', BM3' and BC24' represent as OD bacteria cell; Means followed by different letters within single, double and triple primes are significantly different at $p \leq 0.05$

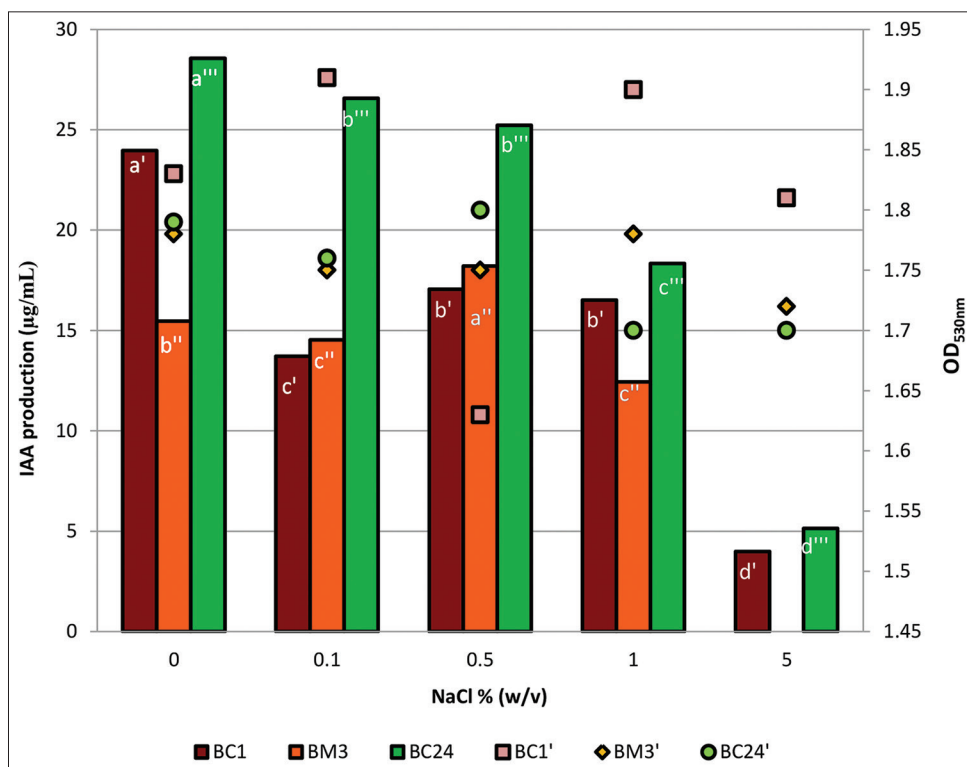


Figure 2: Influence of salinity on growth and IAA production by rhizobacteria
Note: BC1: *B. cereus* UPMLH1; BM3: *B. megaterium* UPMLH3; BC24: *B. cereus* UPMLH24; BC1', BM3' and BC24': OD of bacteria cell; Means followed by different letters within single, double and triple primes are significantly different at $p \leq 0.05$

Even though the population densities of all three strains have fluctuated within a different level of saline conditions, they have high tolerance toward IAA synthesising activity. *B. cereus* had shown more tolerance to salinity than *B. megaterium*. This phenomenon may vary due to different physiological trait with

peptide antibiotics, peptide signal molecules and extracellular enzymes emissions against unfavourable environmental conditions (Gardener, 2004). Saline tolerance PGPR can inhibit and associate with plant roots at various saline environments and enhance plant health (Lugtenberg & Kamilova 2009;

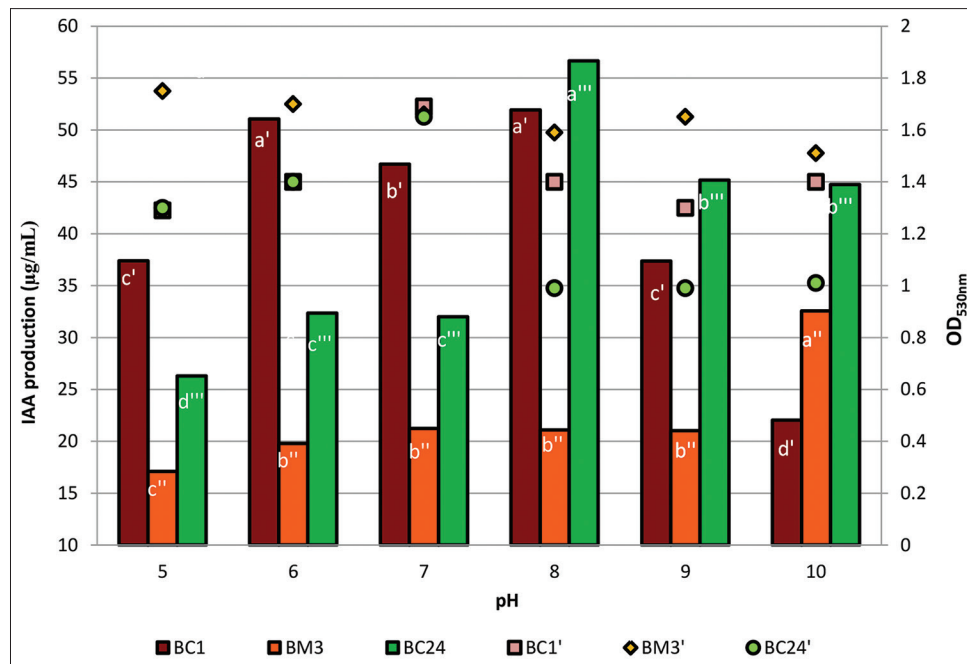


Figure 3: Influence of pH on growth and IAA production by rhizobacteria
Note: BC1: *B. cereus* UPMLH1; BM3: *B. megaterium* UPMLH3; BC24: *B. cereus* UPMLH24; BC1' BM3' and BC24': OD of bacteria cell; Means followed by different letters within single, double and triple primes are significantly different at $p \leq 0.05$

Banerjee et al., 2010). In general, *Bacillus* species fast evolved in adaptation to various saline environments and improve plant tolerances to ecological stress (Marulanda et al., 2010).

Salinity may directly or indirectly inhibit cell division and the elongation growth of plants. On the other hand, plants in the saline agricultural area gave low yield due to osmotic stress and improper water uptake due to transpiration and inadequate nutrition (Singh & Chatrath, 2001). Few researchers are working on the growth enhancement of different plants' types by beneficial saline tolerance PGPR inoculants under saline stress to encounter those problems. Those findings show that saline tolerance PGPR improved biomass growth of plants and improved water status, nutrient content, and yield (Kohler et al., 2009). In count with that, saline tolerant IAA producing *B. cereus* UPMLH1, *B. megaterium* UPMLH3 and *B. cereus* UPMLH24 are proficient to improved morphology and physiology of plants roots and modulate plant responses to saline osmotic stress. However, the actual mechanisms between PGPR and plant at the rhizosphere are still unrevealed puzzles in the scientific world.

A pH factor could put up a barrier to microorganisms population and diversity in soil (Brockwell et al., 1991; Palmer & Young, 2000). Beneduzi et al. (2008b) & Karagoz et al. (2012) discovered that the most prominent bacteria in different soil types are genera *Bacillus* and *Paenibacillus*. All three *Bacillus* strains successfully managed to grow and produce IAA at various pH ranges (pH 5-10). Each strain showed a different characteristic in this study. Both *B. cereus* strains in the current study revealed opposite physiological trait at various pH ranges. Population density of *B. cereus* UPMLH1 more or less retain in both acidic and alkaline conditions, while *B. cereus* UPMLH24

have drastic dropped at alkaline state, but producing a high amount of IAA. Although the optimum pH for Bacilli is 7 (Ash et al., 1993), pH tolerance can vary among species' strains. These diverse responses with pH by *Bacillus* PGPR probably correlated with the complex acidic-alkaline pool composition of root exudates in the plant rhizosphere (Dakora & Phillips, 2002).

Among abiotic factors, soil acidity is the main factor that directly influences soil microorganisms' diversity and crop growth, development, and yield. Acidic soils with a pH less than 5.0 usually have a limited amount of available nitrogen, phosphorus, potassium, calcium, magnesium and other micronutrients (Vijila & Jebaraj, 2008). Improper addition of agrochemical fertiliser to the soil to sustain optimum or high plant yield, however, in the long term it has decreasing the soil fertility by acidifying soil. Several studies clearly showed that the crop inoculated with PGPR enhancing plant growth and yield with the potential to reduce chemical fertiliser application (Naveed et al., 2008; Akbari et al., 2011; Zakry et al., 2012a). IAA producing PGPR with high tolerance to pH can adapt and inhabit various type of acidic and alkaline soil (Beneduzi et al., 2008a; Beneduzi et al., 2008b; Vijila & Jebaraj, 2008). Meanwhile, promoting plant growth, PGPR can also improve soil fertility and health (Haskett et al., 2020).

CONCLUSION

The multiple tolerance ability of PGPR against abiotic factors indicates its ability to survive under harsh soil and plant environments while delivering benefits to the plant. *B. cereus* UPMLH1, *B. megaterium* UPMLH3 and *B. cereus* UPMLH24 have differential responses on IAA-producing capacity to

abiotic parameters. *B. cereus* UPMLH24 appears a more promising biofertiliser under saline conditions. This finding may help determine appropriate strategies for the biofertiliser formulation, storage and application.

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