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Assessment on Drought Stress Resistance, Salinity Endurance, and Indole Acetic Acid Production Potential of Dryland-Isolated Bacteria

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Abstract

Dryland lacks water, unfulfilled nutrient supply, lack of abiotic and biotic elements, and very little rainfall. The area of dry land in Indonesia reaches 144.47×10^6 ha. About 99.65×10^6 ha are dry land with potential for agriculture, and around 44.82×10^6 ha are dry land with no possibility for agriculture. Many arid lands in Indonesia cause significant problems, especially in the agricultural sector. The problem often occurs due to the constraints of plant growth factors, including planting media, water, light, wind, and nutrients. This study aims to determine the resistance of bacteria to drought stress and high salinity as well as their ability to produce IAA (Indole Acetic Acid). The method used is *in vitro*. The results obtained are various kinds of bacteria, the nature of bacteria in binding gram, and osmoprotectant bacteria. Bacteria tested from the dry land of annual plants could effectively survive on PEG 6000 media NaCl even at high concentrations. The best bacteria that can survive in the saline conditions were B7, B8, B9, B10, while the potential as plant growth hormone bacteria are B6, B7, B8, B9, B10.

Keywords: Arid land, Biofertilizer, Dry land microbe, Environmentally friendly, Improve soil quality, *In vitro* screening, Plant growth promoting rhizobacteria, Soil biological fertility

1. Introduction

Indonesia has a vast dry land potential reaching 148×10^6 ha. The dry land area with potential for agriculture is estimated at 76.22×10^6 ha (52 %), and most are in the lowlands (70.71×10^6 or 93 %) (Budiono, *et al*. 2019). There are several obstacles faced in developing dryland agriculture (Adinurani *et al*., 2018; Bafdal *et al*., 2015; Noerwijati, *et al*. 2021). Water availability is highly dependent on rainfall. In general, dry land in Indonesia is degraded due to erosion. Land degradation causes dryland organic matter to be at the lowest level (Goenadi *et al*., 2021; Prasetyo *et al*, 2022a; Purbajanti *et al*., 2016; Vincevica-Gaile *et al*., 2021). Rhizosphere bacteria are soil microorganisms that can establish a relationship with the physical and chemical conditions of the soil (Ekawati, 2019; Sukmawati *et al*. 2021; Xiong *et al*. 2021). These bacteria help plants absorb nutrients and minerals and increase tolerance to environmental stresses (Ahmed and El-Sayed, 2021; Dimkpa *et al*., 2009; Ekawati, 2019; Muhammad *et al*. 2021). Bacteria in the root rhizosphere are bacteria cultured in soil with 10^{-6} to 10^{-9} cm^{-3} living cells (Hafeburg and Kothe, 2007).

However, further research on dryland bacteria is needed to determine how bacteria survive and adapt to dry land. Under drought conditions, the development of microbial communities will be hampered. Thus, the application of plant growth-promoting microorganisms has been suggested (Anjori *et al*., 2021; Marulanda *et al*., 2006). In addition, the ability of certain bacteria to reduce the detrimental effects of stress on plants has been previously reported (Kasim *et al*., 2013; Marulanda *et al*., 2009). Therefore, it is necessary to apply inoculation technology to strengthen the potential of microbes in dry land to improve the soil's physical, chemical, and biological conditions (Barea *et al*., 2002; Ekawati, 2019; Goenadi *et al*., 2021; Prasetyo *et al*., 2022b; Sukmawati *et al*., 2021).

The role of bacteria in growth, nutrition, and drought tolerance under nutrient-limited conditions is based on various physiological and cellular mechanisms (Medina and Azcon, 2012; Shinwari *et al*., 2019). In this case, microorganisms can also reduce water stress by reducing cellular oxidative damage produced by plants under drought conditions (Ahmed and El-Sayed, 2021; Potters *et al*., 2010). Inocula formation in dry soil activates antioxidant metabolic pathways (Benabdellah *et al*., 2011; Nguyen *et al*., 2020). The dry environment determines the ability of microorganisms to reproduce in that habitat. Remarkable similarities exist between plants and bacteria in their cellular responses to osmotic pressure (Csonka, 1989; Purbajanti *et al*., 2019).

This study reports information about the relevance of cellular metabolic processes carried out on the production of microbes that survive unfavorable conditions (stress and

salinity) and the potential of Indole Acetic Acid (IAA) in growing media added with PEG (Polyethylene Glycol) to create osmotic pressure (Gordon and Paleg, 1957; Purbajanti *et al*, 2019). Bacterial IAA production will be used as a plant repair effect and under stress conditions to protect cells against the adverse effects of ROS and stabilize proteins. This compound increases resistance against water shortages, which is considered a good stress indicator. In addition, previous studies reported the mechanisms generally involved in plant growth-promoting bacteria, such as the production of phytohormones, especially IAA, which plays the most crucial role in promoting plant growth (Adinurani *et al.* 2021; Glick, 1995). Thus, it was selected as a representative index of bacterial efficiency. Plants and microorganisms that live in dry soil adapt to stressful conditions.

The microorganisms are applied to form vegetation cover to restore soil conditions (Tanveer *et al*., 2022) . However, adaptable/tolerant bacteria can increase plant growth and nutrition in drought conditions. Besides, physiological mechanisms can increase plant resistance to water stress. Increasing cellular osmolyte accumulation can increase drought tolerance by reducing stomatal conductance and evapotranspiration (Bates *et al.* 1973; Purbajanti *et al*., 2019).

Microbe-producing Plant Growth Promoting Rhizobacteria (PGPR) use different mechanisms to promote plant growth (Ekawati, 2019; Goenadi *et al*. 2021; Parmar and Dadarwal, 2003). Tryptophan – gained from root exudates or decaying cells – replaces IAA microbial biosynthesis in the soil. Endophytic and rhizosphere bacteria have other properties that allow them to reach and establish themselves more effectively in plants' rhizosphere and inner tissues (Martinez-Viveros *et al*, 2010). Therefore, *in vitro* screening of rhizosphere and endophytic bacteria for IAA potency can provide a reliable basis for selecting effective PGPR bacteria.

Based on this research method, the tested bacteria will be selected to stimulate plant growth. After choosing the best isolate, the ultimate goal is to introduce the isolate as a biological fertilizer (Ekawati 2019; Li *et al.*, 2008, Muhammad *et al*. 2021). In addition, several studies have reported that the endophytic microbial community originating from the soil and rhizosphere were the best isolates promoted as biofertilizer or biodegradation (Afzal *et al.* 2017; Elvira-Rescuenco and van Vuurde, 2000; Hallman *et al.*, 1997; Sturz *et al.*, 2000).

2. Materials and Methods

2.1. Soil sampling

Soil samples were taken from the annual plant rhizosphere of Shrub (*Chamaedaphane* Moench), rhizosphere of Sonokeling/Java palisander (*Dalbergia latifolia* Roxb.), and rhizosphere of Coconut (*Cocos nucifera* L.) located in the dryland forest area of of South Malang, East Java, Indonesia *i.e.* Pagak districts (112°29’66″ to 112°33’12″ BT and 8°11’46″ to 8°18’27″ LS) and Bantur districts (8°18′59.14″S to 112°34′40.91″E). The average annual rainfall (2013 to 2021) in Pagak is 152 mm, while in Bantur, it is 194 mm. According to Oldeman's criteria, climate types are categorized as D and C (Harahap *et al*., 2021). The average annual rainfall in South Malang is slightly lower than in North Malang (climatological station at Abdulrahman Saleh Airport), which is 225 mm (climate type C). The soil samples (Pagak — mediteran type and Bantur — alfisol type) were collected from the surface 10 cm below the surface and processed immediately.

2.2. Preparation of bacterial isolation media

Alternative growth media was needed for growing microorganisms containing nutrient-rich carbohydrates and proteins (Medina and Azcon, 2012). The media used was PDA (Potato Dextrose Agar — Merck P2182). Isolation of antagonistic bacteria was according to the method used by Chilcott and Wigley (1993) and Limbu *et al*. (2020). The 1 g of the soil sample was suspended in 9 mL sterile distilled water and shaken vigorously for 2 min. Then, the liquid was serially diluted in sterile distilled water, and a 0.1 mL sample of the dilutions 10^{-4} to 10^{-7} was added to 20 mL of melted 92008 Tryptone Bile Agar (TBA — Merck 92008). After solidification of the medium, the plates were incubated at 30 ℃ for 24 h to 48 h. Finally, the bacterial colonies that showed antagonism to the adjacent colonies were picked up and subcultured to make pure cultures, as explained by Dubey and Maheshwari (2002) and Kumar *et al*. (2021).

The dilution method was used to isolate bacteria. This isolation was carried out by taking 10 L of homogenized soil samples with different dilution levels. The samples were then flattened before poured into the Petri dish using a multilevel triangle. Finally, planting was made in three cups per dilution to maximize bacterial growth *in vitro*.

2.3. Purification

Purification was carried out by taking a suspension of bacteria that had grown to the same size and volume and streaking them on new solid PDA media. Purification was carried out by taking a growing bacterial suspension of the same size and volume. After streaking on new solid PDA media then storing in a sterile room for 3 d to 4 d, the suspension was ready for further testing.

2.4. Gram stain and bacterial morphological identification

To observe microscopic characterization, gram staining method was used (Thairu *et al.* 2014). It was carried out to determine bacteria morphology and their gram properties. It used four different colors: crystal violet, Lugol's iodine, 96 % ethanol, and safranin. Bacteria with gram-positive properties will be blue-purple, while bacteria with gram-negative properties will be red or pink.

2.5. PEG 6000 — drought test

Drought stress test using PEG 6000 + NB media (PEG —Merck, Sigma-Aldrich) (Nutrient Both — Chemical Athaya/Tokopedia), according to Susilowati *et al.* (2018). The concentrations tested in the drought stress test were different: 1 %, 2 %, 3 %, 4 %, 5 %, 6 %, 7 %, 8 %, 9 %, and 10 %. An amount of 10 mL of the media was put into the test tube according to the concentration, and two inoculating loops of bacteria inoculums were added. This drought stress test was carried out to determine the ability of bacteria to survive in drought conditions with relatively little water conditions and organic matter that did not match the needs of bacteria and very minimal nutrients. Observations were made by reading the Optical Density

(OD) value on a spectrophotometer (Genesys 10 S UV, USA) and recording each phase of bacterial growth marked on the number of OD values produced. Observation variables start from the lag phase, the exponential/logarithmic phase, the stationary phase, and the death phase (Myers *et al*., 2013).

2.6. NaCl salinity test

The salinity test was carried out to produce bacteria that can survive in osmotic stress or low water content due to the salt's high nature. The salinity test used NaCl + NB media (NaCl — Merck, CAS Number:7647-14-5) (Nutrient Both — Chemical Athaya/Toko Pedia) with 4 %, 5 %, 6 %, 7 %, 8 %, 9 %, 10 %. Observation variables include the value of OD (Optical Density), with an observation time interval of 3 h for 24 h (Khanghahi *et al.*, 2021). The OD value obtained will be recorded and presented on a bacterial growth. Suppose the resulting OD value increases at certain time intervals for 24 h. In that case, the bacteria can survive unfavorable conditions and if the suspension becomes cloudy, the bacteria can adapt to the media.

2.7. IAA potency test

Testing the potency of IAA using NB + L-Tryptophan media (Merck, CAS Number: 73-22-3) and added with Salkowski reagent (CV Nitra Kimia/Tokopedia) as a precursor to induce IAA in bacterial samples, according to Gang *et al*. 2019. Take the media at the concentration of 10 mL, add two inoculating loops of bacteria inoculum, homogenize it, and add 2 mL to 3 mL of Salkowski's reagent. Observations were conducted qualitatively after being in OD for 24 h with a 24 h observation interval. The bacteria can produce IAA hormone if the sample is pink after adding Salkowski's reagent.

2.8. Data analysis technique

Data analysis is based on in vitro experiments with qualitative descriptive study. The data obtained will be analyzed by bacterial growth during the initial 0 h observation and the final 24 h. The data will be presented by displaying macroscopic and microscopic observations of the morphology of bacterial growth, the shape of the bacteria, and the nature of the bacteria in binding color (Adinurani, 2022).

3. Results and Discussion

3.1. Purification, bacterial morphology, and bacterial physiology test

The results of bacterial purification that were successfully isolated and purified with different dilution levels from the Rhizosphere of Shrub, Rhizosphere of Sonokeling/*Java palisander*, and Rhizosphere of Coconut were shown in Table 1.

Table 1. Bacterial Isolation Results Based on The Bacterial Purification Index

No.	Inoculum sources	inoculums	Inoculum code
1.	Shrubs Location 1	B1 (five colonies)	B1
2.	Shrubs Location 2	B2 (three colonies)	B2
3.	Shrubs Location 3	B3 (two colonies)	B3
4.	Coconut Location 1	B1 (four colonies)	B4
5.	Coconut Location 2	B2 (three colonies)	B5
6.	Coconut Location 3	B3 (three colonies)	B6
7.	Coconut Location 4	B1 (two colonies)	B7
8.	Sonokeling Location 1	B2 (four colonies)	B8
9.	Sonokeling Location 2	B3 (two colonies)	B9
10.	Sonokeling Location 3	B4 (four colonies)	B10

Table 1 shows the purification results of bacteria from different plants with different dilution levels. In regards of the isolated sample selected based on the isolate purification index above, 32 inoculants were produced but only ten colonies were the difference. The morphological characteristics of bacterial colonies were presented in Table 2 to Table 4.

Table 2. Morphological characteristics of bacterial colonies of shrubs rhizosphere

Observation	Bacteria 1	Bacteria 2	Bacteria 3
Form	Circular	Circular	Circular
Elevation	Raised	Raised	Raised
Surface	Shiny	Shiny	Shiny
Edge	Entire	Entire	Entire
Color	Milky white	Milky white	Milky white
Cell shape	Coccus	Basil	Basil
Gram stain	Positive(+)	Negative (-)	Negative (-)

Table 3. Morphological characteristics of bacterial colonies of coconut rhizosphere

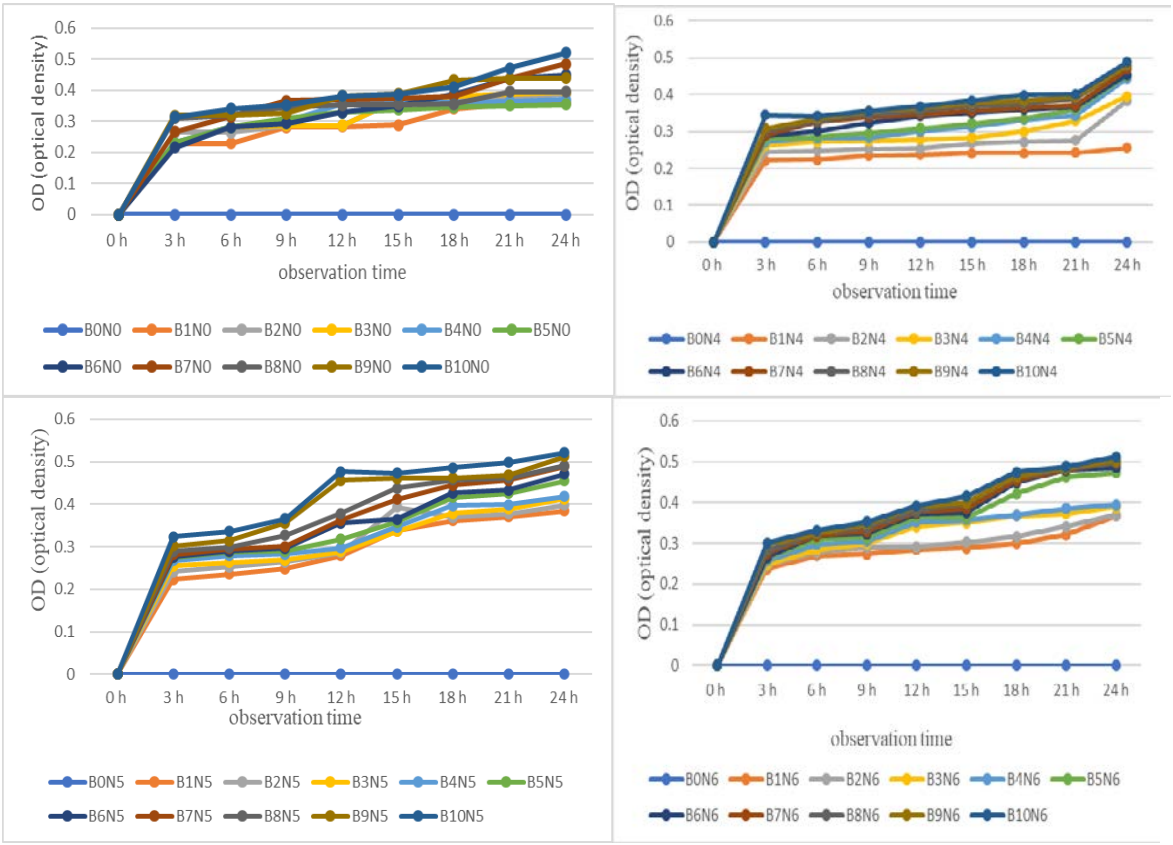
Observation	Bacteria 1	Bacteria 2	Bacteria 3	Bacteria 4
Form	Circular	Circular	Circular	Circular
Elevation	Raised	Raised	Raised	Raised
Surface	Shiny	Shiny	Shiny	Shiny
Edge	Undulate	Undulate	Undulate	Undulate
Color	Milky white	Milky white	Milky white	Milky white
Cell shape	Coccus	Basil	Coccus	Coccus
Gram stain	Positive (+)	Positive (+)	Positive (-)	Positive (+)

Table 4. Morphological characteristics of bacterial colonies of sonokeling rhizosphere

Observation	Bacteria 1	Bacteria 2	Bacteria 3
Form	Irregular	Irregular	Irregular
Elevation	Raised	Raised	Raised
Surface	shiny	Shiny	shiny
Edge	Undulate	Undulate	Undulate
Color	Milky white	Milky white	Milky white
Cell shape	Basilus	Basilus	Coccus
Gram stain	Positive (+)	Positive (+)	Positive (+)

Growth of 10 bacteria on media treated with NaCl to test whether the ten bacteria could grow in unfavorable environmental conditions, especially salinity. At 8 % salinity, all bacteria except bacteria B1, B2, and B3 showed an increasing trend, but at 9 % NaCl, only bacteria B7, B8, B9, and B10 still showed a growing trend, while other bacteria experienced a decrease in growth. At 10 % NaCl, only B7 and B10 bacteria survived (Figure 1). Salinity is a severe environmental problem. Salinity causes osmotic stress around the roots and causes a decrease in plant growth and productivity in dry areas that rely on irrigation or in dry areas without irrigation (Cicek and Cakirlar, 2002). Stress caused by salinity will affect plant metabolism. Excess salt in the soil solution can affect plant growth either through osmotic inhibition of water uptake by roots or the effect of specific ions. Salinity will increase Na^+ uptake, which in turn causes a decrease in Ca^{2+} and K^+ uptake (Kusmiyati *et al*., 2009; Yildirim *et al*., 2006). Bacterial B7 and B10 are able to survive at high concentrations of NaCl, and the two bacteria may be halotolerant bacteria. This type of bacteria can grow in the presence of high NaCl concentrations (Tortura, *et al.*, 1998), such as on the surface of the skin, which often has a high NaCl concentration (10 % NaCl) (Tsai *et al.*, 2016). This may suggest that the two bacteria may exhibit a better ability to overcome the osmotic shock due to NaCl. The ability of bacteria to survive in high salinity stress is due to the ability of bacteria to accumulate dissolved organic matter in their cytoplasm. The goal is to prevent the loss of fluid from inside the cell as a result of the high osmotic pressure outside the cell due to the increased concentration of dissolved organic matter NaCl. Halophilic bacteria can produce hydraulic enzymes, one of which is a protease. It can catalyze protein hydrolysis reactions into oligopeptides and their amino acids. That these microorganisms can thrive in hypersaline environments has been correlated with the high content of acidic amino acids in their proteins, which increases the negative surface potential of proteins, since these microorganisms were effectively used.

Bacteria able to survive at high salinity can be used further. It uses in the process of agronomic tests in terms of improving saline soil aggregates.



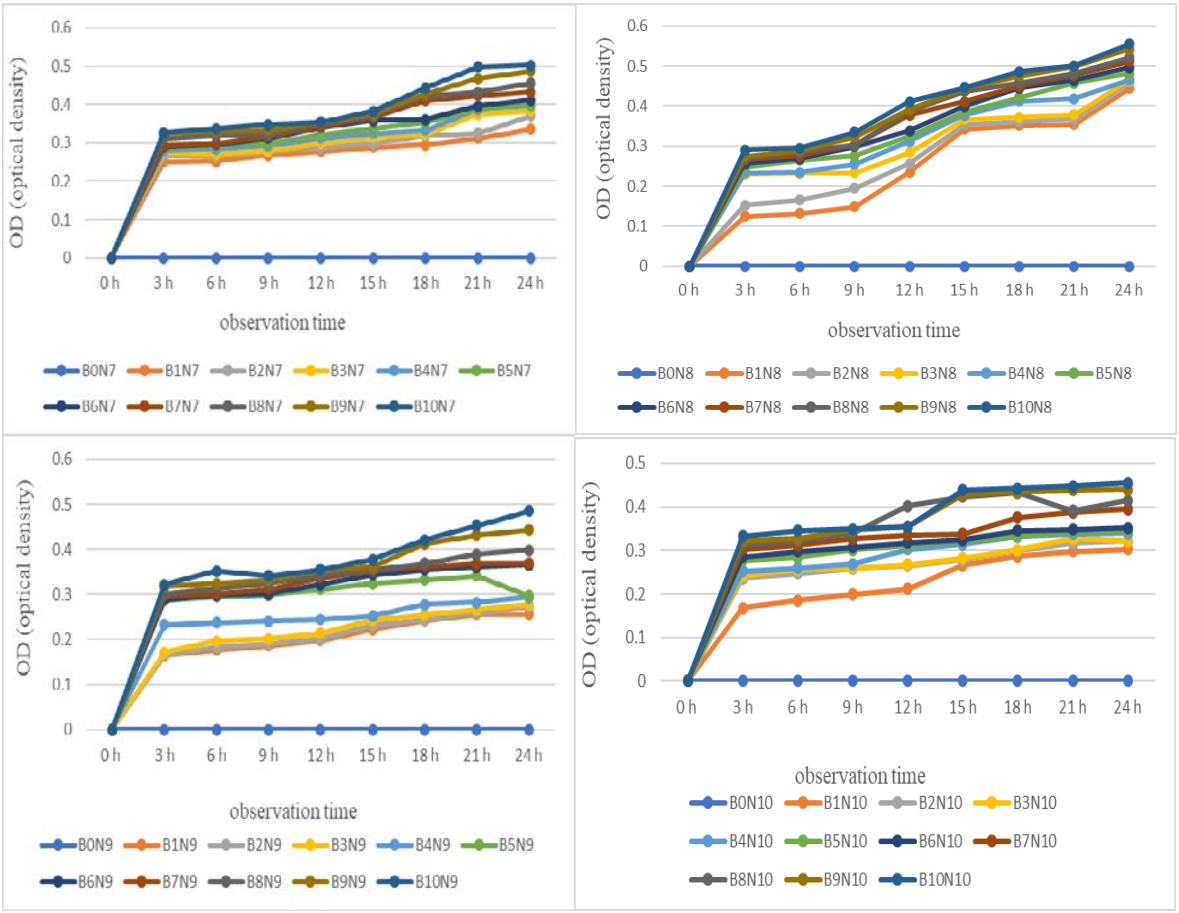
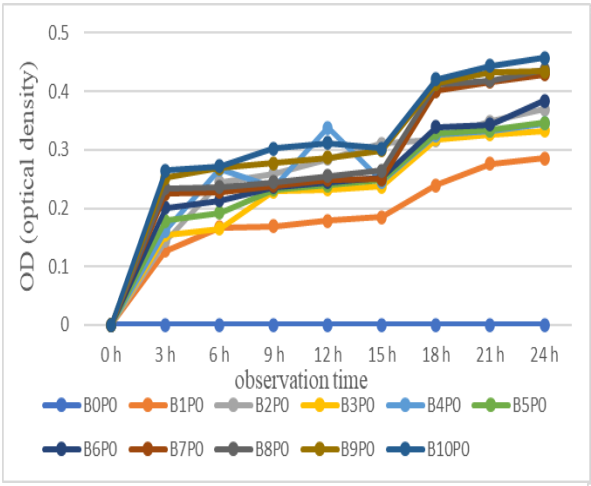
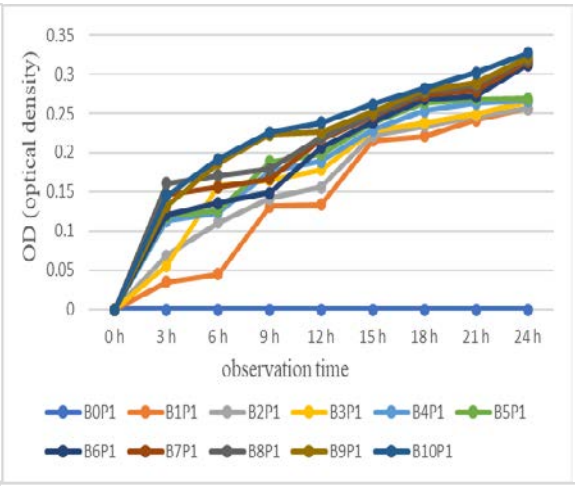


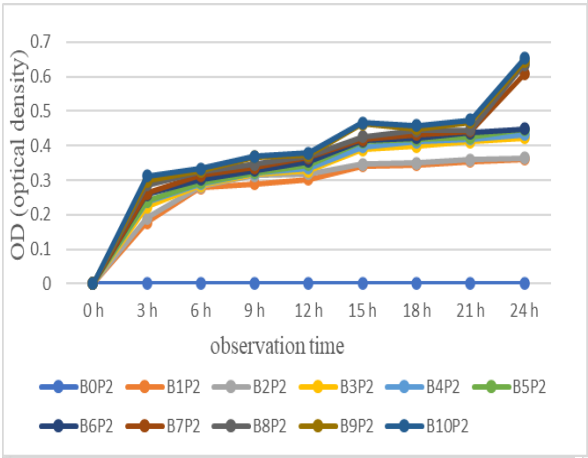
Figure 1. Bacterial growth at different NaCl concentrations. B0, B1, B2, B3, B4, B5, B6, B7, B8, B9, B10 were the types of bacteria tested. N0: NaCl 0 %; N4: NaCl 4 %; N5: NaCl 5 %; N6: NaCl 6 %; N7: NaCl 7 %; N8: NaCl 8 %; N9: NaCl 9 %; N10: NaCl 10 %

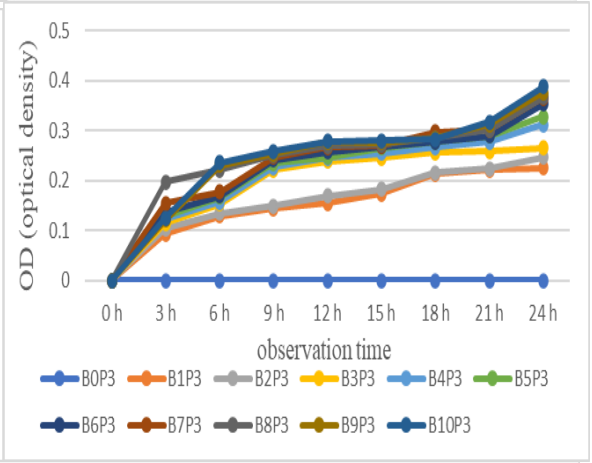
Growth of ten bacteria on media treated with PEG to test whether the ten bacteria could grow in unfavorable environmental conditions, especially water stress. All bacteria were able to grow on PEG-treated media from a concentration of 1 % to a concentration of 10 %, although there is a group of bacteria that consistently shows an upward growth trend and is always on top. These bacteria are B5, B6, B7, B8, B9, B10 (Figure 2). Drought or dry land is a major environmental problem that is currently emerging. This is the most formidable challenge for most countries in the world, apart from pest and disease attacks, because it causes a decrease in plant growth and yield. Therefore, it is necessary to find ways to increase plant growth and production under drought stress conditions. Microorganisms have the opportunity to overcome this. Arzanesh *et al.* (2011) analyzed the presence of siderophores and their relationship to drought resistance of the bacteria, and found that strains producing higher levels of siderophores were associated with excellent host plant resistance to drought. Siderophores are used by bacteria as one of the most important microbial survival strategies because they form complexes with Fe and increase its solubility and uptake under conditions of insufficient availability of iron (Rajkumar *et al*., 2017). Under stress conditions, *Pseudomo*nas sp., produces higher Exopolysaccharides (EPS) levels than conditions without stress. EPS formation in bacteria occurs as a reaction to stress (Ali *et al*., 2014). The ability to produce EPS by bacterial cells is used as a criterion for drought tolerance in bacteria (Sandhya *et al.*, 2009).

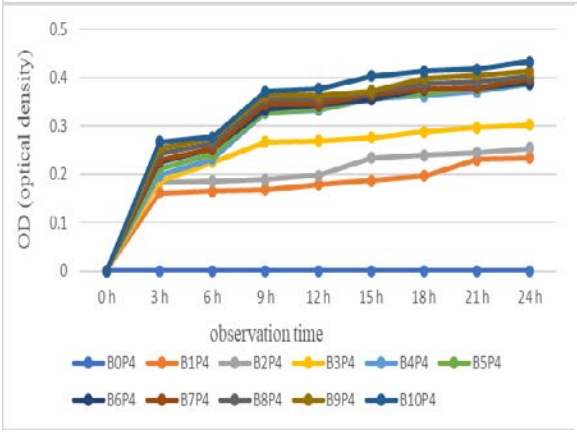
Incorporation of microorganisms as active and vital components in agricultural systems is imperial to stimulate drought tolerance. Cohen *et al.*, 2015 and Kang *et al.*, 2014, stated that among microorganisms, bacteria might promote plant growth by producing essential phytohormones and mineral solubilization. In addition, the bacteria may enhance the antagonistic effect against the pathogen. In plants grown under extreme stress conditions, inoculation of Plant Growth Promoting Bacteria (PGPB) improved stress tolerance, at least partly, by increasing root length and allowing better access to water (Enebe and Babalola, 2018).

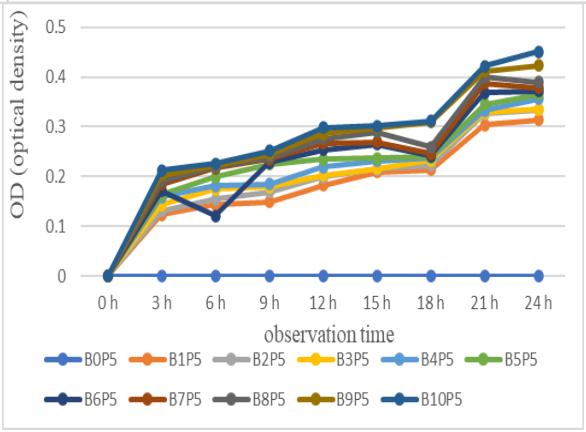












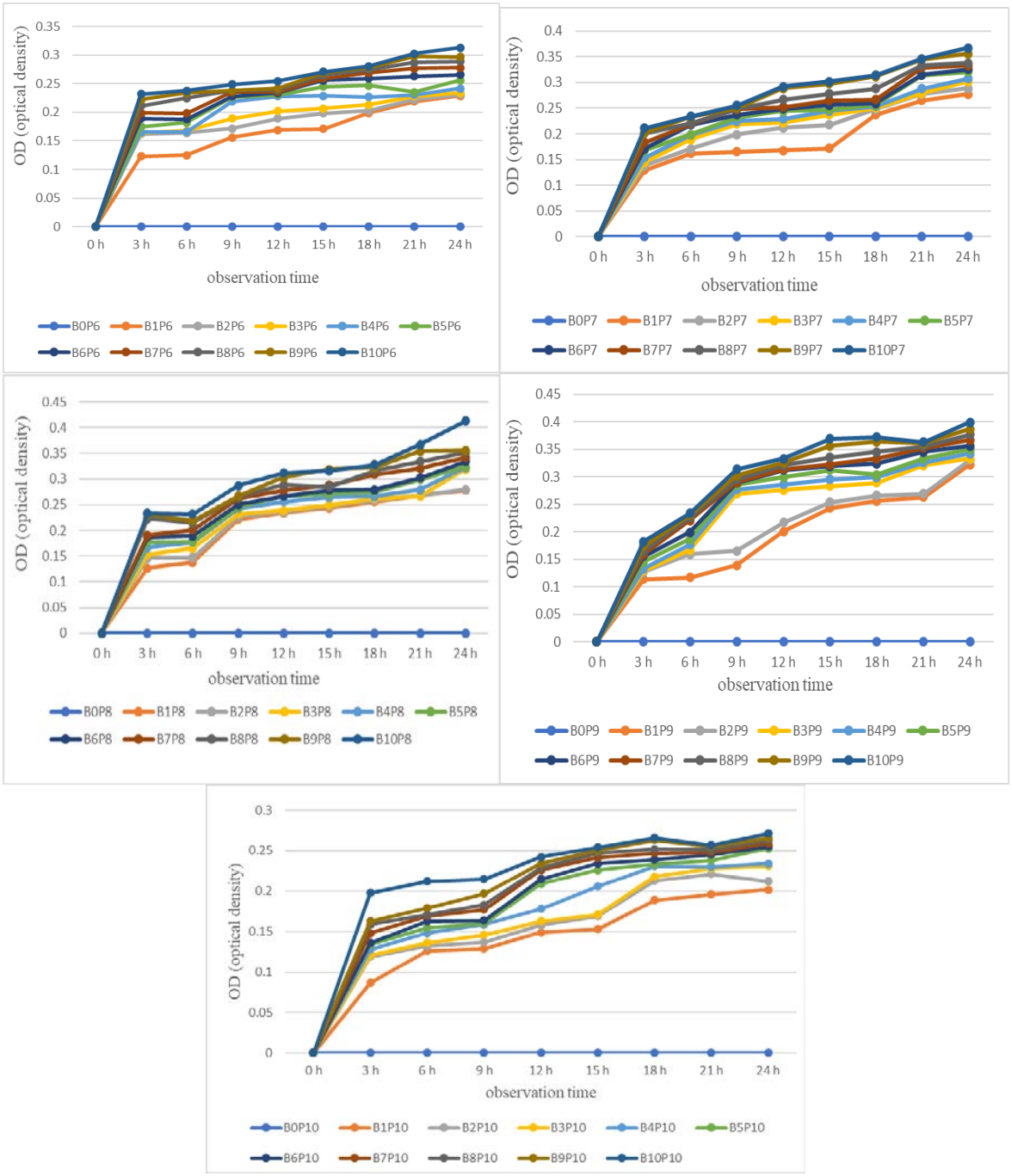


Figure 2. Bacteria growth trend on media with PEG in different concentrations. Name of bacteria: B0, B1, B2, B3, B4, B5, B6, B7, B8, B9, B10. P0: PEG 0 %; P1: PEG 1 %; P2: PEG 2 %; P3: PEG 3 %; P4: PEG 4 %; P5: PEG 5 %; P6: PEG 6 %; P7: PEG 7 %; P8: PEG 8%; P9: PEG 9 %; P10: PEG 10 %.

Ten isolates were identified as IAA-producing strains because the OD of all bacteria at 1 h to 24 h of observation showed an upward trend from (20 to 100) tryptophan concentrations. The top five bacteria are B6, B7, B8, B9, and B10. The five bacteria had the highest OD among the ten bacteria tested (Figure 3). IAA production by bacteria can vary between different species and strains besides being influenced by culture conditions, growth stages and substrate availability (Mohite, 2013). The technique for detecting IAA using the Van Urk Salkowski reagent is an essential option for qualitative and semi-qualitative determinations that guarantee the presence of hormones in bacterial culture supernatants or liquid formulations of biological inoculants. The amount of IAA produced by bacteria was within the detection limit of the Salkowski reagent (Ehmann, 1997). The results showed that the higher is the tryptophan concentration, the higher is the OD value. Indole production increased with increasing tryptophan concentration in testing Fluorescent Pseudomonas isolates for their ability to produce indole acetic acid in pure culture without the presence of and L-tryptophan (Karnwal, 2009).

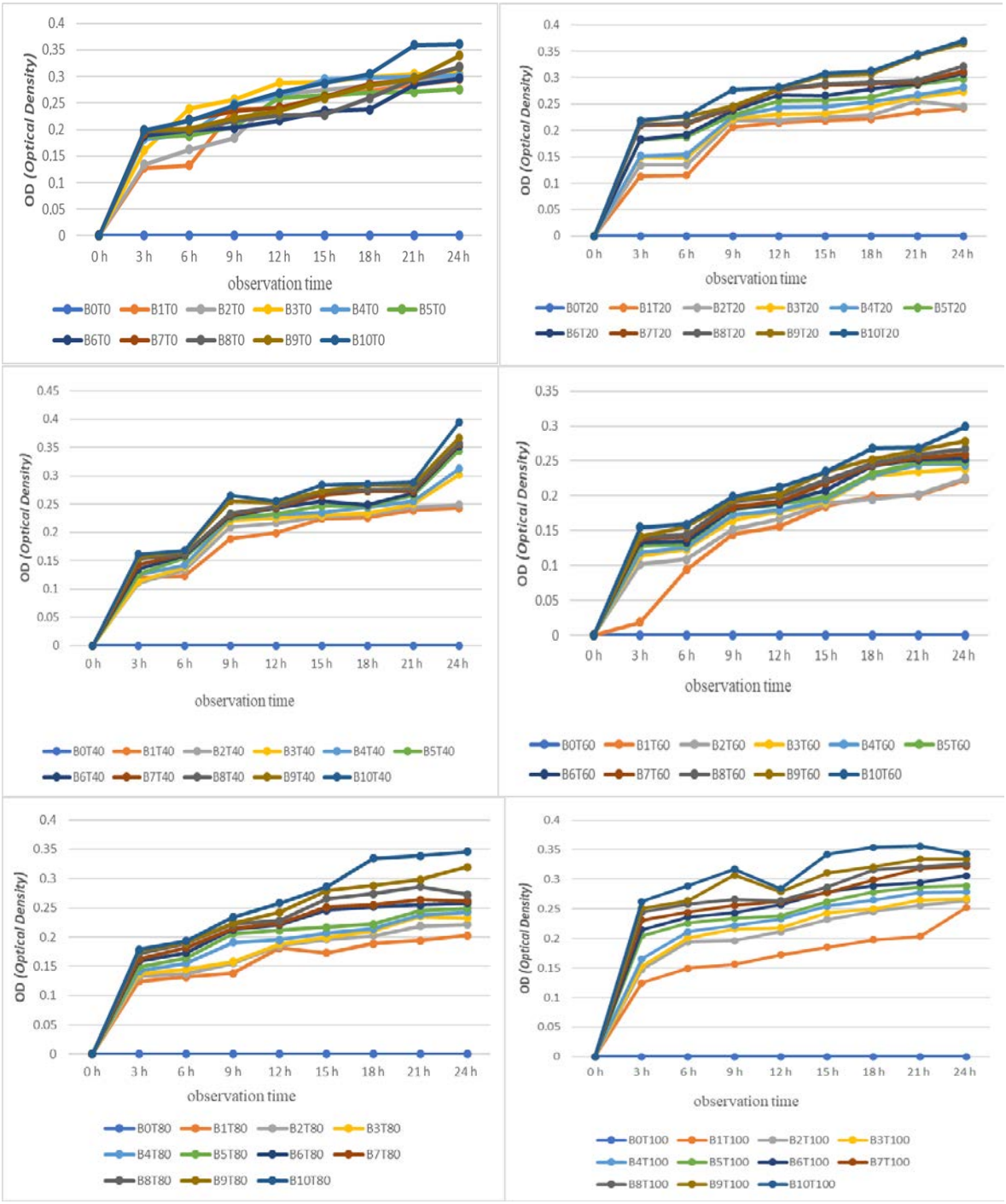


Figure 3. Bacteria growth trend on media with Tryptophan in different concentrations. Name of bacteria: B0, B1, B2, B3, B4, B5, B6, B7, B8, B9, B10. T0: Tryptophan 0 %; T20: Tryptophan 20 %; T40: Tryptophan 40 %; T60: Tryptophan 60 %; T80: Tryptophan 80 %; T100: Tryptophan 100 %

This study needs to relate soil organic matter content for future research directions to validate the conclusions. PGPR requires standard soil organic content to be efficient and effective (Adinurani *et al*., 2021; Ekawati, 2019; Goenadi *et al*. 2021; Medina and Azcón, 2012; Muhammad *et al*. 2021). It takes real action and collaboration between various stakeholders so that all organic waste —agricultural waste, livestock waste, kitchen waste, leftover food, and human excrement from pit latrines and septic tanks (Abdullah *et al*. 2020, Anukam and Nyamukamba, 2022; Manzoor *et al*., 2020; Prasetyo *et al*., 2022a and b, Setyobudi *et al*. 2021, Somorin, 2020; Susanto *et al*., 2020) can return to agricultural lands to maintain and increase soil fertility.

4. Conclusions

Based on the research on the ability to test drought stress, salinity, and potential IAA (Indole Acetic Acid) on bacterial isolates of dry land annual crops, the results are as follows:

(i) Various types of bacteria are produced from the purification process; the success of this bacterial

suspension is produced from the annual Sonokeeling, Shrubs, and Coconut plants.

(ii) Bacteria tested for drought and salinity can survive in unfavorable conditions. The high absorbance value at each observation time interval proves that the bacteria can live and have the potential to be used as biological fertilizers or PGPR.

(iii) The best bacteria that can survive in the saline conditions were B7, B8, B9, B10, while the potential as plant growth hormone bacteria are B6, B7, B8, B9, B10.

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