COMPATIBILITY STUDIES ON DIFFERENT ENDOPHYTIC BACTERIAL ISOLATES FROM COCOA PLANT

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ABSTRACT – *A compatibility study were conducted to establish the most effective endophytic bacterial consortium for the management of plant growth hormone as biofertilizer for cocoa plants. Four selected endophytic bacterial isolates with potential to be developed as biofertilizer, which has been isolated within tissues of healthy Theobroma cacao L. plants were identified through Microbial Identification System (Biolog™ GEN III MicroPlates). These four isolates were revealed as Bacillus amyloliquefaciens, B. pumilus, B. subtilis, and Pantoea agglomerans. The strains were tested for their mutual compatibility to be developed as mixtures of endophytic bacterial consortia bio-fertilizer. The compatibility interactions were studied by cross streak culture method and were found that several selected endophytic bacteria were highly compatible between each other. All these selected strains were further subjected for the development of endophytic consortia bio-fertilizer.*

***Key words**:* Endophytic bacteria, compatibility, biolog, cocoa, and consortia

INTRODUCTION

Endophytic bacteria colonize internal structures of plant host without causing disease or negative effects. They often produce bioactive substances such as plant growth hormones to increase plant growth (Khan *et al*., 2014). Endophytic bacteria create a core of attention due to increasing demand in bringing down the use of chemical fertilizers and pesticides, as effort in promoting environmental protection (Vale *et al*., 2010). In many cases, the symbiosis between plant and endophyte occurs in such a way that the plant protects and feeds the endophyte, while the endophyte, in return, produces plant growth hormones to enhance plant growth. Agricultural-based problems caused by the long-term use of chemical fertilizer products, not only pollutes environment, but also causes an imbalance in the proportions of various nutrients, the destruction of organic matter in the soil and a decrease in the structural integrity and properties of aggregates, leading to soil compaction, salinization and other problems (Wang *et al*, 2020). Thus bio-fertilizer contains beneficial microbes, and their interaction with plants can promote dynamic turnover and sustainable cocoa seedlings development. By using bio-fertilizer it could reduce the cost of production for inorganic fertilizer and may find broad application and development of bio-fertilizer.

In most cases, effective microbial inoculants consist of a single strain. However, the present research focus has turned to the formation of microbial consortiums that they would perform better than single strains (Woo and Pepe, 2018). Whereas a single application may be successful, combination inoculants have the potential to respond to a wider range of environmental circumstances and have a number of modes of action (Sarma *et al*., 2015). However, significant questions remain unanswered, such as whether single strains or multi-strain combinations are better, and whether strains in a mixture are compatible. The endophytic bacterial consortiums were chosen based on their capacity to activate plant growth hormones individually. A cross streaking method was used to test compatibility or antagonism among the bacterial strains within the proposed consortia. The compatibility activities were examined for zones of inhibition, which indicated the existence of antagonism (Sarkar and Chourasia, 2017). In a population of mixed community microorganisms, Van Hamme *et al*. (2003) discovered antagonism behaviour between microbes. Antagonist microorganisms can restrict the growth of other microbes through a variety of ways, including the generation of toxins, antibiotics, and siderophores (Hibbing *et al*. 2010).

The microorganisms were mixed together in the hopes that the consortium would be more effective in encouraging plant growth, despite the

fact that antagonistic interactions between the microbes in the combination could reduce the predicted results (Sarma *et al*., 2015). It takes a detailed understanding of modes of interaction, bacterial adhesion to seeds, and plant root colonization to design, formulate, and optimize effective bacterial consortium inoculants (Singh *et al*., 2014). Furthermore, antagonistic relationship studies between each microbe should be conducted before the design and application of formulations containing bacterial consortiums since some antagonistic effects may occur in bacterial consortiums associated with plants (Oliveira *et al*., 2009).

At this moment, significant effort is being placed on the utilization of an automated phenotypic or physiological Biolog™ GEN III MicroPlates system, which allows for uniform and repeatable identification of microorganisms. This system is linked to a database and contains a variety of metabolic activity used by microbes. For each gram-negative and gram-positive bacteria species studied, this systemizes chemical sensitivity testing to produce a unique biochemical pattern or "phenotypic fingerprint" (Fernando and Cruz, 2019).

Therefore, the present study will address research findings on the evolution of endophytic bacterial consortia and the issue of component compatibility and identification in order to provide useful information for the development of successful endophytic bacterial plant growth hormones consortia for long-term cocoa plantation or others agricultural applications in future.

MATERIALS AND METHODS

Bacterial strains and culture medium

The cultures of four endophytic bacterial strains producing plant growth hormones have been isolated previously within tissues of healthy *Theobroma cacao L.* were obtained from the Microbiology Laboratory of Biotechnology Division, Malaysian Cocoa Board. Stock culture of each strain were maintained at -80^{o}C in nutrient broth with 15% glycerol (Shin *et al*., 2007). The working cultures were established by transferring from stock cultures onto nutrient agar (NA) in Petri dishes and incubated for 24 h at 28^{o}C.

Cross-streak tests between strains

The cross-streaking culture method was used to test the compatibility among the selected endophytic bacterial strains before consortium preparation. The four endophytic bacteria strains were streaked vertically and horizontally on NA plate and incubated for 24 h at 28^{o}C. The results were then obtained photographic documentation of agar plates, including those showing colony lines and inhibition zones that appeared at the intersection of the paired strains.

Phenotypic profile and identification of bacterial strains using Biolog™ GEN III MicroPlates

The cultures of four endophytic bacterial strains were identified and characterized by the Biolog GEN III system (Biolog Inc. Hayward, CA, USA), following the manufacturer's instructions. Bacterial colonies were transferred to inoculating fluid A (IFA) with a sterile cotton swab to generate bacterial cell suspensions. The transmittance of bacterial cell suspensions was adjusted between 90% and 98% using a turbidimeter (Biolog™). A mount of 100 µL of the cell suspension was dispensed into each well of GEN III MicroPlates™. The absorbance of each well of the inoculated microplates were read at 590 nm on a Biolog MicroStation™, at every 24 h for six days. The analysis was carried out triplicates for each strain, and the data were analysed based on extensive species library in the Biolog™ GEN III database.

RESULTS AND DISCUSSIONS

The cross-streaking culture method that was used to test compatibility among the endophytic bacteria, allowed us to observe signs of growth inhibition (if any), which would indicate the presence of antagonism between organisms as shown in Figure. 1. Plate 1 showed antagonistic activities between strain A and B, meanwhile plate 4 confirmed incompatibility or antagonistic activities between strain A and B. The incompatibility activity occurred due to growth competition between each bacterium, resulting in the antagonistic activities (Santiago *et al*., 2017). Studied by Hibbing *et al*., (2010) suggested that incompatibility between microorganisms also can inhibited the growth of other microbes through a variety of ways, including the generation of toxins, antibiotics, and siderophores.

The observation on plate 1, shows slight antagonistic activity between strain C and D. However, there was no sign of growth inhibition between C and D as seen on plate 4, indicating non

or very little antagonistic activities between them. Based on the results obtained, strains A and B will not be considered as potential for consortium development due to signs of strong antagonistic activity between them. On the other hand, strain C and D shows potential to be developed for consortium preparation.

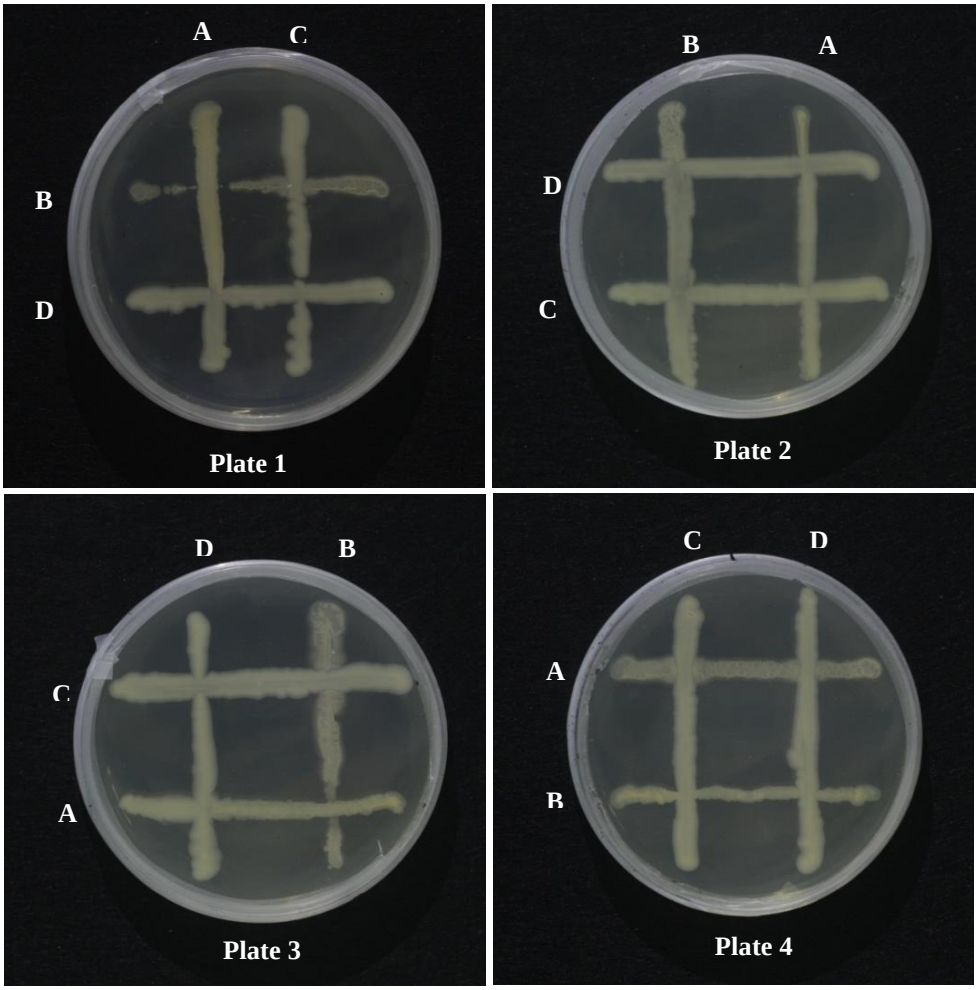

Figure.1 Determination of compatibility activities among endophytic bacterial strains using cross streaking method.

Cross-streaking between strain A, and C as shown in Plate 2 and Plate 4, clearly showed no signs of growth inhibition between the two strains. This indicates that there was no antagonistic activity between these two isolates, thus they can be developed as consortium preparation. This is similar to the cross-streak results between strains A and D. These two strains can also be considered to be developed as consortia due to no signs of antagonistic reaction between them as seen in all four Plates 1, 2,3 and 4.

Compatibility between strains B and C also showed promising results as shown in all four Plates 1, 2, 3 and 4. There were no signs of growth inhibition between strains B and C observed, thus they too can be considered to be developed as consortia. Cross-streaking between isolates B and D also showed promising results as seen in Plate 2 and 4. There were no signs of growth inhibition seen between strains B and D, putting them as potential isolates for consortium development. Thus, six combinations with little to no antagonistic activities were proposed for consortium development as summarized in Table 1. The successful endophytic bacterial consortium, the cultures mixture formulation must be able to grow in the presence of each other without interfering of other microbes. If the endophytic bacterial in consortium have incompatibility relationships, the mixtures become

unstable, and the predicted functioning of the consortia is not achieved.

Table 1 Combination for the proposed formulation of endophytic bacterial strains consortia.

Strains	A	B	C	D
Combination 1	√	X	√	√
Combination 2	√	X	√	X
Combination 3	√	X	X	√
Combination 4	X	√	√	√
Combination 5	X	√	√	X
Combination 6	X	√	X	√

The physiological profile of endophytic bacterial strains using Biolog™ GEN III MicroPlates selected tests for sugars, amino acids and sensitivity tests revealed variable results of metabolic activity (Table 2). Endophytic bacterial strain A showed several positive results on utilization of selected sugar, followed by endophytic bacterial strain C and strain D. Meanwhile endophytic bacterial strain B has less metabolic activity on the tested selected sugar. Similar results also were observed from physiological profile on selected amino acids utilization. Endophytic bacterial strain A showed several positive results on utilization of selected amino acids compared to strain B, C and D.

Sensitivity to pH (5 and 6 respectively) as well as salinity (1, 4 and 8% NaCl) of the endophytic bacterial strains were also determined using Biolog™ GEN III MicroPlates. All endophytic bacterial strains A, B, C and D were able to grow at pH 6, however strain B grow moderately on pH 5 compared to strain A, C and D. Similarly, endophytic bacterial strain A, C and D showed capability to grow at both low and high salinity (1, 4 and 8% NaCl). Meanwhile endophytic bacterial strain B grew well at 1% NaCl, moderately at 4% NaCl but unable to grow at 8% NaCl.

These utilization metabolite profiles of substrates and chemical sensitivity tests allowed for identification of selected endophytic bacterial using Biolog™ GEN III MicroPlates (Table 3). Endophytic bacterial strain A was identified as *Bacillus subtilis*, while strain B, C and D were identified as *Bacillus amyloliquefaciens*, *Pantoea agglomerans* and *Bacillus pumilus* with similarity index of 0.6, 0.7, 0.6 and 0.7, respectively. Identification of selected endophytic bacterial isolates using Biolog™ GEN III MicroPlates showed high accuracy index scored of above 0.6. The identification results gave a broad valuable pattern on utilization metabolite profiles of substrates and chemical sensitivity tests for each endophytic bacterial strain. However, second identification assay were needed based on 16S rRNA gene sequencing which provide 90.6% correct identification to conclude the results compared to 68.3% accuracy based on Biolog System assay (Morgan *et al*., 2009). The identification methods between Biolog System and 16S rRNA gene sequencing have their own advantages and shortage, but several researchers have recommended that these two assayed should be simultaneously used to achieve a correct taxonomy of the strains (Paolis and Lippi, 2008). In the future, 16S rRNA gene sequencing should be used to confirm the identities of these beneficial endophytic bacteria that have been shown to produce plant growth hormones.

Table 2 Physiological profile of endophytic bacterial strains using Biolog™ GEN III MicroPlates selected tests for sugars, amino acids and sensitivity tests

Biolog™ GEN III MicroPlates	Endophytic bacterial strains			
	A	B	C	D
SUGARS				
D-Maltose	+	-/+	-	-/+
D-Mannose	+	-	+	-/+
D-Galactose	-/+	-	-/+	-
D-Trehalose	+	-	+	-/+

Sucrose	+	-	+	+
α-D-Lactose	-/+	-	-	-
D-Turanose	+	-	-	-/+
D-Cellobiose	+	-	+	+
β-Gentiobiose	+	-	+	-/+
D-Melibiose	-/+	-	-/+	-/+
Stachyose	-/+	-	-/+	-/+
D-Raffinose	-/+	-	+	-/+
D-Glucose	+	-/+	+	+
D-Fructose	+	-/+	+	+
D-Fucose	+	-/+	-	-
L-Fucose	-/+	-/+	-	-
L-Rhamnose	-/+	-	-	-
AMINO ACIDS				
D-Serine	-	-/+	-	-
L-Arginine	+	-	-/+	-/+
Gly-Pro	-/+	-	-/+	-
L-Alanine	+	-/+	+	+
L-Aspartic Acid	+	-/+	+	+
L-Histidine	-	-	-	-/+
L-Glutamic Acid	+	-/+	+	+
L-Pyroglutamic Acid	+	-	-/+	-
L-Serine	+	-	-/+	-
SENSITIVITY TESTS				
pH 5	+	-/+	+	+
pH 6	+	+	+	+
1% NaCl	+	+	+	+
4% NaCl	+	-/+	+	+
8% NaCl	+	-	+	+

Table 3 Identification of endophytic bacterial strains based on Biolog™ GEN III MicroPlates

Endophytic bacterial strains	Similarity Index	Identity
A	0.6	*Bacillus subtilis*
B	0.7	*Bacillus amyloliquefaciens*
C	0.6	*Pantoea agglomerans*
D	0.7	*Bacillus pumilus*

CONCLUSIONS

Preparation of a successful consortium formulation requires the mixture of endophytic bacterial strains in the consortium to grow in the presence of each other without incompatibility or antagonistic activities. Incompatibility between mixture strains A and B of consortia indicated the instability of the growth and expected functioning of the consortia formulation is not achieved. The results of the present study suggest that the compatibility of strains A, C and D in combined inoculations is important for promoting plant growth. Thus, six combinations with little to no antagonistic activities were proposed for consortium development. Endophytic bacterial strain A was identified as *Bacillus subtilis*, strain B as *Bacillus amyloliquefaciens*, strain C *as Pantoea agglomerans* and strain D as *Bacillus pumilus* using Biolog™ GEN III MicroPlates. Formulation consortia of these endophytic bacterial strain with each compatible strains may provide useful information for the development of successful endophytic bacterial plant growth hormones consortia for management of cocoa plantation fertilizer.

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