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Screening of Endophytic Colonizing Bacteria for Cytokinin-Like Compounds: Crude Cell-Free Broth of Endophytic Colonizing Bacteria Is Unsuitable in Cucumber Cotyledon Bioassay

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Abstract: Endophytic microorganism live inter or intra cellularly in most of the plant tissues without causing any disease symptoms. Endophytes are known to produce plant growth promoting compounds. Cytokinin is a group of plant growth regulators (PGRs) and can be exploited in agriculture for both pre-harvest and post-harvest management of leafy vegetables, fruits and cut flowers. The objective of this study is to isolate cultivable endophytic bacteria from local plants and to screen them using cucumber cotyledon bioassay for cytokinin-like compounds. In this study, one hundred fifteen cultivable endophytic colonizing bacteria were isolated from seventy two different plant species. Putative bacterial endophytes were grown separately in Luria Bertani (LB) medium. Cell-free broth was used without dilution in the cucumber cotyledon bioassay. In bioassay, the total chlorophyll content in cucumber cotyledon samples was estimated by spectrophotometry and compared with positive and negative controls. Bioassay results showed that the total chlorophyll content was less in all 115 samples of cotyledons (treated with crude cell-free broth) in comparison to both negative and positive control. Results clearly show that crude cell-free broth of cultivable endophytic bacteria is unsuitable in their screening for cytokinin-like compounds using cucumber cotyledon bioassay.

Key words: Agriculture · Bioassay · Colonizing Bacteria · Cut-flowers · Cytokinin-like compounds · Endophytes · Fruits · Leafy-vegetables

INTRODUCTION

Endophytes are the microbes that live within the host plant tissues without causing any visible disease symptoms. Depending on their nutritional requirements they can live as biotrophic parasites or saprotrophs. They also represent a huge reservoir of microbes that are explored very poorly [1]. It is believed that plants which are able to survive in harsh environment, plants that are used for special purpose such as herbal medicine and plants which show an unusual longevity contains endophytes which produces novel bioactive compounds [2]. The existence of endophytes has been reported in grasses, palms, rainforest plants and some other plants [3]. Endophytes include a variety of bacteria, fungi and actinomycetes. Cultivable endophytic colonizing microbes can be isolated from wild and agricultural crop plants [4-6].

Several endophytic bacteria and fungi are seed borne but others have mechanisms to colonize the plants [6]. Endophytic bacteria in a single host plant are not restricted to a single species but may contain several genera and species [our unpublished research on *Erythrina fusca* Lour]. Little is known about endophytes interaction in their host plant. However, researchers are trying to understand it and this understanding could have a huge implications in agriculture sector for instance, use of synthetic fertilizers for nitrogen, plant growth promotion, sugar metabolism of cash crops, secretion of organic acids, synthesis of auxin and the occurrence of bacteriocins [7]. It has been speculated that the beneficial effects in the host plant are the combined effect of endophytic activities [8]. However, a few of the reported endophytic bacteria are known as plant pathogen (or latent pathogens) residing within host or non host plant without symptoms [9, 10].

To recognize‘true’ endophytic bacteria requires not only the isolation from surface-disinfected tissues but also microscopic evidence to visualize ‘tagged’ bacteria inside plant tissues [11]. The term ‘putative endophyte’ is

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often used for endophytes that are not validated microscopically. True endophytes may also be recognized by their capacity to reinfect disinfected plant seedlings [11].

Low yield, fungal attack, pathogenic microbial attack and biotic or abiotic stress are often the problems faced in agriculture sector both in developing and developed countries. The beneficial properties of endophytes can be exploited to tackle the issues related to cultivated crop plants and to provide further benefit in agricultural sector. Some of the human pathogens, such as *Salmonella* spp., have been reported as endophytes [6].

The research findings reported by Saravanan *et al*. [12] gives several indications that endophytes promotes plant growth by more than a few independent mechanisms besides nitrogen fixation, including synthesis of phytohormones and increased uptake of nutrients. Cytokinin is a group of plant growth hormones [13]. One of its biological functions is stimulation of the chlorophyll synthesis by converting etioplasts into chloroplasts [14]. This biological activity of cytokinin has many pre-harvest and post-harvest applications in agriculture especially to prolong the shelf-life of leafy vegetables and cut flowers. The cultivable endophytic colonizing bacteria (bacterial endophytes) can be screened for cytokinin-like compounds. Once identified through screening, the identified cytokine-like compound producing cultivable endophytic colonizing bacteria can be exploited for their several applications in agriculture such as prolonging the shelf-life of the vegetables, cut-flowers and fruits. The objectives of this study are to isolate cultivable endophytic colonizing bacteria from various local plants and to screen these isolates for cytokinin-like compounds using cucumber cotyledon greening bioassay (CCGB) [14]. This paper reports the screening results for 115 putative endophytes and suitability of the cell-free broth of endophytic isolates in cucumber cotyledon bioassay.

MATERIALS AND METHODS

Plant Materials: Plant leaf samples were collected from Kedah (AIMST University, Bandar Laguna Merbok, Semeling, Tupah area) and Botanical Garden of Penang, Malaysia. Healthy plant leaf samples were selected and collected in order to avoid contamination by plant pathogens. Collected plant leaf samples were brought to the laboratory within 24 hours after sample collection following guidelines of Monnanda *et al.* [3]. Plant leaves were used in this study although plant stems, roots, flowers, twigs, fruits and barks also can be used to isolate endophytes.

Surface Decontamination of Collected Leaves Samples: At the beginning, leaf samples were washed thoroughly under plenty of running tap water. This is important to remove adhering soil particles and the majority of microbial surface epiphytes and microbes. The surface decontamination method described by Fisher *et al.* [4] was used with some minor modification. The samples were taken into laminar hood for surface sterilization with 70 % ethanol for 30 seconds and then treated with sodium hypochlorite (5 %) for 3 minutes. Finally, leaf samples were rinsed thrice with autoclaved distilled water. Surface decontaminated leaves were cut into pieces of about 1 cm² using sterile surgical blade. Leaf pieces were aseptically transferred into Petri dishes containing Luria Bertani (LB) agar medium and then incubated at 37 °C for 18 to 20 hours in dark.

Isolation of Endophytes: Well grown colonies of cultivable putative endophytes were identified and differentiated based on morphology on LB medium. Pure culture of each putative endophyte was cultivated separately in universal bottles containing 10 ml LB medium. The culture cultivation was carried out at 37°C, 160 rpm for 18 hours [11]. Glycerol stocks were prepared and kept at -80 °C to preserve the putative endophytic isolates for future research. Broth from each universal bottle was centrifuged at 4000 rpm for ten minutes and cell-free supernatant (cell-free broth) was used in CCGB.

Cucumber Cotyledon Bioassay: To conduct cucumber cotyledon bioassay, the cucumber (*Cucumis sativus* L.) seeds were purchased from ABC Flowerland nursery, Sungai Petani, Kedah, Malaysia. Seeds were germinated on tissue paper saturated with autoclaved distilled water in the plastic tray. For germination, seeds were incubated at room temperature in dark for 7 days following the guidelines reported by Fletcher *et al.* [14]. Cotyledons were excised from cucumber seedlings (7 day old) that were grown in the dark condition. By weighing, cotyledons were added in Petri dishes containing the crude (100 %) cell-free broth of respective endophytic bacterial isolate. A negative control with sterile distilled water alone and a positive control with commercially available synthetic cytokinin 6-Benzylaminopurine (BAP) was used in the assay for comparison following guidelines given by Fletcher *et al*. [14]. The concentration of BAP in positive control was 25 ppm.

Extraction and Quantitative Estimation of Chlorophyll Content: Cucumber cotyledon samples along with positive and negative control were incubated

under fluorescent tube light for 3.5 hours at 22°C ±2. After the incubation, the cotyledons were collected and ground with 80 % acetone with mortar and pestle. The chlorophyll extracts were collected and then centrifuged at 4000 rpm for ten minutes. The derived supernatant was analyzed for total amount of chlorophyll estimation using spectrometer (λ 663nm and 645nm) [14].

RESULTS

One hundred and fifteen putative cultivable endophytic colonizing bacterial isolates were isolated from leaf samples of seventy two (72) different plant species collected from northern part of Peninsular Malaysia (Fig. 1). The names of the plant species from which leaf samples were collected for isolation of cultivable endophytic colonizing bacterial isolates are shown in Table 1. Most of the surface decontaminated plant leaves samples gave 1 or 2 different putative cultivable endophytes and some had even 3 or 4 types of putative endophytes.

The crude cell-free broth of each isolated putative endophytic bacterial isolates was tested using CCGB to identify cytokinin-like compound producing isolates. All the putative cultivable endophytic isolates showed no positive result in CCGB. The results of CCGB are depicted in Table 1. The total amount of chlorophyll content in cucumber cotyledons which were exposed to cell-free broth of isolates was less than the negative control.

Table 1: Plant species from which leaves samples were collected and used for the isolation of cultivable endophytic colonizing bacterial isolates

Plant species name	Collected from (A/B/S/T/BGP[!])	Total isolates[#]	Isolate Code	CCGB[?] Result (–/+)
Abelmoschus esculentus (L.) Moench	B	2	Aee1, Aee2	–
Allamanda cathartica Linn.	B	1	Ace1	–
Allium cepa Linn.	B	1	Acepe1	–
Alocasia lowia Var.	A	2	Ale1, Ale 2	–
Amherstia nobilis Wallich.	BGP	2	Ane1, Ane2	–
Anacardium occidentale Linn.	T	1	Aoe1	–
Androgarphis paniculata Burm.f.	S	3	Ape1, Ape2,Ape3	–
Annona reticulate Linn.	B	1	Are1	–
Arfeuilla arborescens Linn.	BGP	1	Aae1	–
Artocarpus champeden Spreng.	T	2	Achae1, Achae2	–
Artocarpus heterophyllus Lam.	T	1	Ahe1	–
Averrhoa bilimbi Linn.	T	1	Abe1	–
Azadirachta indica A.juss.	S	2	Aie1, Aie2	–
Bambusa vulgaris Schrad.	T	2	Bve1, Bve2	–
Bougainvillea buttiana Hort.	A	2	Bbe1, Bbe2	–
Bougainvillea peruviana Humb.Bonpl.	A	2	Bpe1, Bpe2	–
Bougainvillea spectabilis Willd.	A	2	Bse1, Bse2	–
Calistemon pallidus Bonpl. DC.	BGP	1	Cpale1	–
Capsicum annuum Linn.	B	1	Cae1	–
Carica papaya Linn.	B	1	Cpe1	–
Ceiba pentandra Gaertn.	T	1	Cpene1	–
Citrus hystrix DC.	B	2	Che1, Che2	–
Citrus maxima Merr.	B	1	Cme1	–
Citrus medica Linn.	B	1	Cmede1	–
Clitoria ternatea Linn.	B	1	Cte1	–
Cocos nucifera Linn.	B	1	Cne1	–
Costus speciosus J.König.Smith	A	1	Cse1	–
Costus woodsonii Maas.	A	2	Cwe1, Cwe2	–
Couropita guianeis Aubl.	BGP	1	Cge1	–
Crysanthemum indicum Linn.	S	2	Cie1, Cie2	–
Curcuma domestica Valeton.	S	1	Cde1	–
Cymbopogon citratus Stapf.	B	2	Cce1, Cce2	–
Cyrtostachys renda Linn.	BGP	2	Cre1, Cre2	–
Dendrobium victoria-reginae Raf.	B	1	Dve1	–
Diospyros embryopteris Pers.	BGP	2	Dee1, Dee2	–
Durio grandiflorus Mast.	T	1	Dge1	–
Durio zibethinus Linn.	T	2	Dze1, 2	–
Elaeis guineensis Jacq.	T	2	Ege1, 2	–
Garcinia mangostana Linn.	BGP	1	Gme1	–
Grewia paniculata Linn.	BGP	2	Gpe1, Gpe2	–
Hevea brasiliensis Müll.Arg.	T	2	Hbe1, Hbe2	–
Hibiscus rosa-sinensis Linn.	A	1	Hrce1	–
Hibiscus rosa-sinensis Linn.	A	3	Hre1, Hre2, Hre3	–
Hibiscus sabdariffa Linn.	A	1	Hse1	–

Table 1: Continued

Hymenocallis liriosme Raf.	A	2	Hle1, Hle2	–
Imperata cylindrica Linn.Beauv.	S	1	Ice1	–
Ipomoea indica Burm.	B	1	Iie1	–
Jasminum officinale Linn.	B	2	Joe1, Joe2	–
Lawsonia inermis Linn.	B	1	Lie1	–
Mangifera indica Linn.	S	4	Mie1, Mie2, Mie3, Mie4	–
Manihot esculenta Crantz.	B	2	Mee1, Mee2	–
Mimusops elengi Linn.	BGP	2	Melee1, Melee2	–
Moringa oleifera Linn.	B	1	Moe1	–
Murraya koenigii Linn.	S	1	Mke1	–
Musa acuminate Colla.	B	2	Mae1, Mae2	–
Nelumbo nucifera Gaertn.	T	2	Nne1, Nne2	–
Nephelium lappaceum Linn.	T	1	Nle1	–
Ocimum sanctum Linn.	T	2	Ose1, Ose2	–
Orthosiphon staminae Benth.	S	2	Ostae1, Ostae2	–
Oryza sativa Linn.	B	2	Ose1, Ose2	–
Pandanus amaryllifolius Roxb.	B	1	Pamae1	–
Parmentiarea ceritera Linn.	BGP	2	Pce1, Pce2	–
Plectranthus amboinicus Lour.	B	3	Pae1, Pae2, Pae3	–
Plumeria obtuse Linn.	A	1	Poe1	–
Punica granatum Linn	B	1	Pge1	–
Saccharum officinarum Linn.	B	2	Soe1, Soe2	–
Sansevieria trifasciata Prain.	A	2	Ste1, Ste2	–
Solanum melongena Linn.	B	3	Sme1, Sme2, Sme3	–
Syzygium aqueum Burman f.	B	1	Sae1	–
Syzygium malaccense Merr.Perry	T	2	Smale1, Smale2	–
Rosa damascena Mill.	B	1	Rde1	–
Zingiber officinale Roscoe.	B	1	Zoe1	–

[a]A, AIMST University area; B, Bandar Laguna Merbok; BGP, Botanical Garden of Penang; S, Semeling; and T, Tupah area. [#]indicates the number of putative cultivable endophytic isolates isolated from leaves samples of respective plant species. [?]Cucumber cotyledon greening bioassay [14].

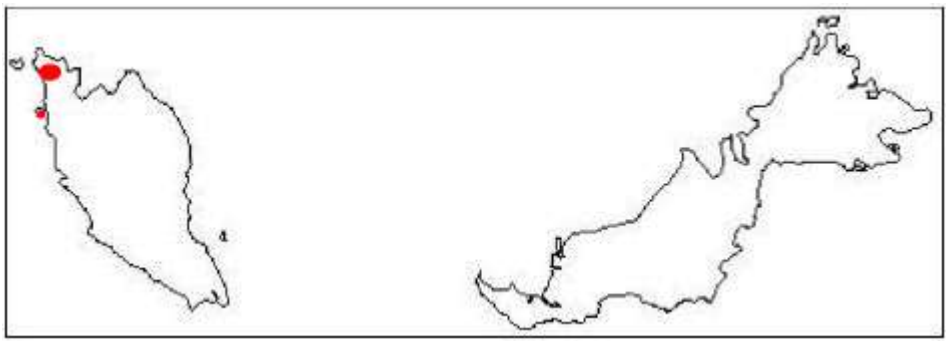

Fig. 1: Collection of plant material samples (leaves) from northern part of Peninsular Malaysia. The areas from where plant material samples were collected are shown in red color (blank outline map of Malaysia was obtained from digital library of maps available at http://geography.about.com/library/blank/blxmalaysia.htm, verified on June 3, 2010).

A positive control, negative control and a sample (a representative) are shown form a CCGB in Figure 2. The cell-free broth of lead isolate/s should produce total amount of chlorophyll more than the amount of total chlorophyll that is produced by negative control. If the respective putative endophytic isolates are not producing cytokinin-like compounds then total amount of chlorophyll should be at least equivalent to total amount of chlorophyll in negative control. However, total amount of chlorophyll per gram fresh weight of cotyledons was lower than negative control when treated with cell-free both of putative cultivable endophytic colonizing bacterial isolates. It suggests that the conversion rate of etioplasts into chloroplasts in the cotyledons that were treated with cell-free broth was lower than in negative control.

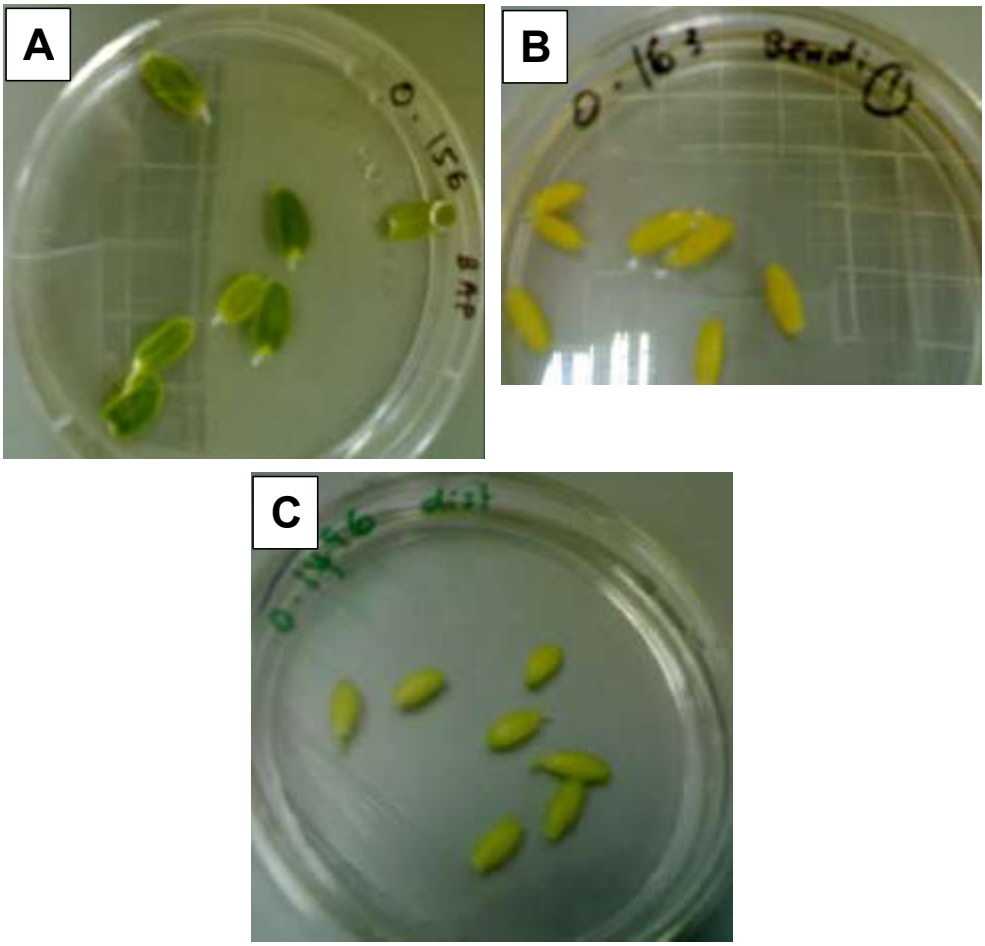

Fig. 2: Cucumber cotyledon greening bioassay. A, positive control; B, a sample [cotyledons treated with cell-free broth of *Aee*1 isolate from *Abelmoschus esculentus* (L.) Moench]; C, negative control.

DISCUSSION

The number of cultivable endophytes can vary from plant to plant and it can be attributed to host genotypes, host developmental stage, inoculum density of endophyte/s, environmental conditions and many more other factors [5, 15]. One of the reasons could be colonizing factor of endophytes, because endophytes generally colonize in various part of a plant tissues such as leaves, twigs, barks, stems, roots, flowers, fruits etc. and not all bacterial endophytes can grow on the LB medium. In this study, endophytes has been isolated from leaves alone, though there are possibilities that other types of endophytes might be residing in some other organ tissues of the respective plants.

Cytokinin is a group of phytohormone that plays an important role in regulating plants growth and development, including cell division, cell enlargement, chloroplast development, senescence and cell differentiation [16]. Naturally occurring cytokinins are N6-substituted adenine derivatives [13]. It plays an important role in regulating plant gene expression by selectively increasing or decreasing the abundance of specific mRNAs species [17]. It also stimulates the expression of various plant genes, including those encoding light-harvesting chlorophyll a/b-binding proteins [18]. In addition to higher plants, several bacteria, including *Agrobacterium* are also proven to produce cytokinin [19]. Gall-forming bacteria are also known to produce cytokinin, for instance *Pseudomonas syringae* pv. "Savastanoi" [20], *Pseudomonas solanacearum* [21] and *Pantoea agglomerans* [22]. The nitrogen-fixing symbiotic *Cyanobacterium nostoc* possesses a gene related to cytokinin production [23]. All these above stated facts indicate that there are endophytic bacteria which produce cytokinin or cytokinin-like compounds and could be exploited in agriculture for various applications. To identify cytokinin-like compound

producing endophytes CCGB was used in this study because in a comparative study, Dumbroff and Brown found that the CCGB was convenient and sensitive and provides a more linear response [14, 24]. Brenner *et al*. also reported that the CCGB provides an accurate method for cytokinin quantification and identification [25]. The amount of total chlorophyll present in the cotyledons helps in interpreting the presence of cytokinin or cytokinin-like functional bioactive compounds.

For the screening of putative endophytic isolates, their cell-free broth was used in CCGB. The total amount of chlorophyll was less in all cotyledon samples that were treated with cell-free broth of isolates. One of the interpretations could be the compounds present in cell-free broth of isolates might have inhibited the conversion process of etioplast to chloroplast in the cucumber cotyledons during the incubation period of 3.5 hours under fluorescent light. The cucumber cotyledons from both the positive and negative controls turned into green in color and produce higher amount of total chlorophyll in comparison to the cotyledons that were treated with cell-free broth samples of isolates.

A similar work was conducted by Stirk *et al.* [26] to determine cytokinin like activity using CCGB to screen microalgae. The CCGB was also used to determine the cytokinin and auxin-like activity of the microalgal strains [27]. However, in their work the pellets of microalgae were used because the cytokinin and auxin-like compounds in microalgae present endogenously whereby that will not be the case in cultivable endophytic colonizing bacterial isolates screened in this study.

The results also suggest that use of crude cell-free broth of cultivable endophytic colonizing bacterial isolates (putative endophytes) do have –ve impact on chlorophyll synthesis. This could be attributed to other components that are present in cell-free broth. Possibly, use of diluted cell-free broth might have produced more desirable results. Apart from using bioassay alone, the cytokinin-like compound detection in cell-free broth could also be done by using thin layer chromatography (TLC) or high performance liquid chromatography (HPLC) techniques [28].

The absence of positive results in screening of putative endophytes by CCGB does not preclude the importance of isolated putative endophytic isolates. This study is only emphasizing on screening of isolates for cytokinin-like functional activity; the screening of endophytic bacterial isolates can be considered for other plant growth hormones, secondary metabolites and other various bioactive compounds that have commercial value in agriculture sector and biotech industry.

CONCLUSION

The present study is successful in isolation of endophytic colonizing bacterial isolates and their screening for cytokinin-like compounds using crude cell-free broth and CCGB. In a nut shell, 115 putative bacterial endophytes have been isolated from 72 different plant species. The screening of 115 putative endophytic isolates using CCGB gave no lead isolates for cytokinin-like compounds. The low amount of total chlorophyll in cucumber cotyledon samples in comparison to negative control indicates that the use of crude cell-free broth of endophytic bacterial isolates in CCGB is not suitable. In the future, isolates can be screened using solvent extracts along with diluted broth in CCGB. Screening of the isolates for plant growth promoting activity using other bioassays needs to be done. Nonetheless, our research findings could serve as the foundation for the further research work on screening of endophytes for cytokinin or cytokinin-like compounds.

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