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ORIGINAL ARTICLE

Understanding colonization and proliferation potential of endophytes and pathogen *in planta* via plating, polymerase chain reaction, and ergosterol assay

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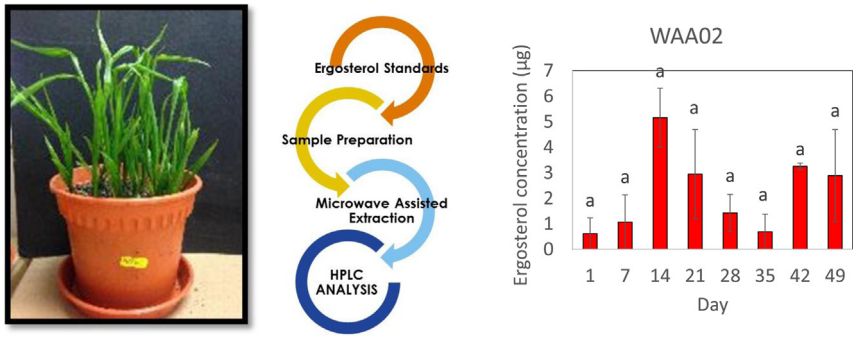
GRAPHICAL ABSTRACT



Colonization and proliferation potential of endophytes and pathogen *in planta* via ergosterol assay and compared to conventional plating and PCR methods.

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ABSTRACT

This study aimed to establish the colonization behavior and proliferation potential of three endophytes and one pathogen *Ganoderma boninense* (Gb) introduced into oil palm ramets (host model). The endophytes selected were *Diaporthe phaseolorum* (WAA02), *Trichoderma asperellum* (T2), and *Penicillium citrinum* (BTF08). Ramets were first inoculated with 100 mL of fungal cells (10^6 cfu mL^{-1}) via soil drenching. For the next 7 days, ramets were sampled and subjected to three different assays to detect and identify fungal colonization, and establish their proliferation potential *in planta*. Plate assay revealed the presence of endophytes in root, stem and leaf tissues within 7 days after inoculation. Polymerase Chain Reaction (PCR) detected and identified the isolates from the plant tissues. The ergosterol assay (via high-performance liquid chromatography, HPLC) confirmed the presence of endophytes and Gb *in planta*. The increase in ergosterol levels throughout 49 days was however insignificant, suggesting that proliferation may be absent or may occur very slowly *in planta*. This study strongly suggests that the selected endophytes could colonize the host upon inoculation, but proliferation occurs at a slower rate, which may subsequently influence the biocontrol expression of endophytes against the pathogen.

Introduction

Endophytes are microorganisms that reside inside the internal tissues of living plants without causing any symptoms to the host plants [1,2]. They can be found in various plants growing in the tropics, temperate regions and in boreal forests [3]. Endophytes are valuable as they produce a variety of bioactive compounds [4]. They are also known to have biocontrol potential against several important plant pathogens [5], either by inducing plant defense mechanisms [6] or by promoting plant growth [7]. The presence of endophytic biocontrol agents (BCAs) in the plants is advantageous as endophytes are protected from adverse soil conditions [8,9]. Several studies have reported the successful use of endophytic BCAs, mainly on vegetable and fruit crops. Chinese cabbage seedlings treated with the endophyte *Heteroconium chaetospira* were resistant to the pathogen *Plasmodiophora brassicae* [1]. Endophytes were also able to protect tomatoes [10], banana [11], barley and beans [12], against their respective pathogens. In addition, the presence of endophytes also improved plant growth. Improved vegetative growth was observed in maize, tobacco and parsley treated with endophytic *Pirifomospora indica* [13], as well as pigeon-peas and bananas treated with non-pathogenic *Fusarium* isolates [11,14]. Improved plant growth leads to robust plants which are less susceptible to pathogen infection. Endophytic BCAs have also been tested on oil palm to control *Ganoderma boninense* (Gb) and these include endophytic bacteria *Burkholderia cepacia* and *Pseudomonas aeruginosa* [5] and species of the mycoparasitic *Trichoderma* sp. [15–17].

Application of endophytic BCAs was however, less effective than chemicals in controlling diseases [18]. Several factors contribute to this, with nonconductive soil conditions (abiotic and biotic factors) as the primary cause of concern. Soil factors are hypothesized to have inhibited the growth of BCAs, leading to poor (or absence of) disease control by BCAs [19]. It was further explained that the survival of introduced BCAs may have been impeded by the intense competition by indigenous microbiota in the soil, or by the poor physicochemical soil conditions [20,21]. In this study, we propose that the colonization behavior and proliferation potential of endophytes *in planta* may be a contributing factor influencing their subsequent biocontrol activity. The ability of endophytes to colonize plant tissues successfully is essential for controlling plant diseases and providing benefits to plants. Their ability to proliferate indicates how readily endophytes are able to adapt and grow inside the plants. This hypothesis is novel, and suggests that the manner endophytes colonize, grow and proliferate in host tissues is important to their subsequent effectiveness as BCAs. The colonization and proliferation potential of endophytes *in planta* could be determinative factors that subsequently impact disease suppression.

To test this hypothesis, the colonization and proliferation potential of endophytes was compared to the oil palm pathogen (*Ganoderma boninense*, Gb) and studied using a model host plant (oil palm). The endophytes (WAA02, T2, BTF08) selected were known BCAs that are antagonistic toward Gb [22] and *Fusarium oxysporum* f. sp. *cubense* race 4 (FocR4) [11]. The colonization and proliferation potential of endophytes was compared to Gb as both endophytes and pathogen compete for similar niche. Gb is a pathogen rampant in the tropics, but the infection and colonization of oil palm by Gb are poorly understood [23]. Gb is known to be able to colonize young oil palm tissues, but only cause disease symptoms at a later growth stage, suggesting that Gb remained a successful colonizer of host tissues for a relatively long period of time [24,25]. In this study, assessments were carried out using three approaches. Plating and PCR were first conducted to demonstrate that endophytes and pathogen are able to enter into plant tissues. PCR further identified the correct species of endophyte (and pathogen) present in the tissues. Growth of endophytes and pathogen was then assessed via ergosterol quantification assay. Ergosterol assay was adopted in this study as it has been widely used as an indicator to estimate fungal biomass in various environments such as air [26], food [27,28], leaf litter [29], mycorrhizal roots [30] and soil [31].

Strong correlations between ergosterol content and fungal biomass revealed the specificity of ergosterol as indicator of fungi [32]. Ergosterol assay also detects presence of fungi in plant tissues effectively as ergosterol is present in fungal cell membranes while absent (or is a minor constituent of cell walls) in higher plants [33]. Ergosterol quantification is more useful than the direct microscopic count, fluorescence microscopy, leaf clearing or staining method, as these methods often lead to under- or over-estimation of fungal biomass due to conversion factors [34]. Ergosterol is also a better biomarker compared to chitin and ATP, due to its specific association with fungi [24]. Ergosterol assay has recently been used to detect Basal Stem Rot (BSR) disease caused by *Ganoderma boninense* in oil palm [35].

In short, this study attempts to understand the colonization behavior and proliferation potential of introduced endophytes *in planta*. This study is important as efficient colonization and proliferation potential may be a contributing factor to their subsequent biocontrol activity, leading to successful control of plant diseases.

Material and methods

Fungal isolates

The endophytic isolates (WAA02, T2, BTF08) were isolated from stem tissues of *Portulaca* weed (WAA02) and *Musa* spp. (BTF08, T2) [8], and have shown moderate antifungal activity toward *G. boninense* (Gb) with percentage of inhibition of radial growth of 39.64, 47.75 and 13.51%, respectively [22]. The pathogen *Ganoderma boninense* (Gb) was obtained from Professor Dr. Sariah Meon from University Putra Malaysia. All isolates were cultured and maintained on Potato Dextrose Agar (PDA) (Merck, New Jersey, USA) (incubated for 7 days, at 28 ± 2 °C). The endophytes WAA02, T2 and BTF08 were subjected to DNA sequencing and the sequences of these isolates were deposited in the National Center for Biotechnology Information (NCBI) (Maryland, USA) database with the respective accession numbers assigned: *Diaporthe phaseolorum* (KT964567), *Trichoderma asperellum* (KT964564) and *Penicillium citrinum* (KT964566). The standard curve for each isolate was constructed to determine the amount of fungal inoculum used to inoculate ramets in subsequent experiments [8]. The standard curve for each isolate was constructed using 14-day-old cultures cultivated in Potato Dextrose Broth (PDB) (Merck, New Jersey, USA). Fungal mycelium was first established in PDB and incubated for 14 days at 28 ± 2 °C, filtered, added into sterile distilled water (SDW), and homogenized into broth culture using a handheld LabGEN 125 homogenizer (Cole-Parmer, Illinois, USA). The broth culture was then diluted to 1:2, 1:4, 1:6, 1:8, 1:10, 1:12 and 0.1 mL of the contents from each dilution was pipetted and plated on PDA plates (supplemented with 0.01 g l^{-1} of Rose Bengal (Acros Organics, Fisher Scientific, USA)). The absorbance reading for each dilution was also read at 600 nm using TECAN® Infinite M200 Multi Detection Microplate Reader Part (Männedorf, Switzerland). The inoculated plates were incubated for 7 days at 28 ± 2 °C. Colonies formed on the plates were then enumerated, and the absorbance values and colony forming units (cfu mL^{-1}) were estimated from the standard curve. The inoculum for each isolate is adjusted to 6 $\log_{10}$ cfu mL^{-1}.

Colonization potential of endophytes and pathogen determined via plate assay and PCR detection

Tissue-cultured oil palm ramets were gratefully supplied by Applied Agricultural Resources (AAR) (Selangor, Malaysia). The ramets were of 13–15 cm in height, and of 3–4 leaf stage. Ramets were transplanted into pots containing 1 kg of sterilized soil mixture (2:1 ratio of black soil: burnt soil). Inoculation was performed separately by soil-drenching with 100 mL of inoculum (6 $\log_{10}$ cfu mL^{-1}) according to the following treatments: W (+isolate WAA02), T (+isolate T2), B (+isolate BTF08), C (+control containing SDW and G (+pathogenic isolate Gb). A total of 12 ramets per treatment were prepared and at each sampling interval (day 1, 3, 5 and 7), 3 ramets were sampled (triplicates) per treatment for analysis. All ramets were incubated in semi-controlled conditions (shaded-greenhouse) for 7 days with conditions of approximately 28 ± 2 °C and a photoperiod of 12 h.

On the 1st, 3rd, 5th and 7th days after inoculation, ramets were sampled and washed under running tap-water for 15 min. The ramets were cut and divided into root, stem and leaf tissues. The leaf tissues were then cut into 1 cm × 1 cm segments whereas the stem and root tissues were cut randomly to a length of 1 cm each. The tissues were then subjected to triple sterilization, beginning with 40% household bleach for 5 min and subsequently into 50, 70, 90 and 100% ethanol for 2 min (each immersion). A quick rinse in sterilized distilled water was performed and repeated thrice prior to injuring (by making incisions to the surface of the tissues) the outer layer of the ramet tissues using sterilized scalpel [11]. Injured sites function as outlets for endophytes to grow out from the tissues. Each piece of the injured tissue segment was then placed on PDA supplemented with Rose Bengal (0.033 g/L) (Acros Organics, Fisher Scientific, USA) and incubated at 28 ± 2 °C for 7–14 days. Growth of fungal mycelium from injured sites indicated growth of endophytes. In addition, the remaining tissues from each part of the ramet were used for DNA extraction and Polymerase Chain Reaction (PCR). Briefly, genomic DNA was extracted from 100 mg of tissues collected from each ramet part (root, stem and leaf, respectively) using the GF-1 Plant DNA Extraction Kit-50 preps, as described by the manufacturer (Vivantis®, California, USA). The genomic DNA was amplified using universal primers ITS1 (5′-TCCGTAGGT GAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGA TATGC-3′) under the following reaction conditions: amplification process was initiated by pre-heating of 1 min at 95 °C, followed by 34 cycles of denaturation at 95 °C, 30 s, annealing at 60 °C, 40 s, extension at 72 °C, 90 s and a final extension at 72 °C, 5 min [36]. Subsequently, the PCR products were purified using the Wizard® SV Gel and PCR Clean-Up System (Promega®, Wisconsin, USA) and outsourced to First Base® Technologies (Malaysia) for sequencing. The sequence results were compared to the database from NCBI using Basic Local Alignment Search Tool (BLAST) search (http://blast.ncbi.nlm.nih.gov/BLAST.cgi).

Correlation between ergosterol content and fungal mycelium biomass

This experiment determined the correlation between ergosterol content and fungal biomass, to reflect that ergosterol content

could be used to estimate the fungal biomass (mycelium weight). To achieve this, isolates were inoculated into 200 mL PDB (Merck, New Jersey, USA) and incubated in static batch culture manner for 14 days at 28 ± 2 °C. After 14 days, fungal mycelium was filtered with miracloth (Merck, New Jersey, USA), rinsed with sterile distilled water (SDW) and the mycelium was frozen overnight. The next day, frozen fungal mycelium was macerated in liquid nitrogen using a pestle and mortar until fine powder was obtained. The powdered mycelium was weighed to 0.5, 1.0, 1.5 and 2.0 g and each specific weight was then used for ergosterol detection using microwave-assisted extraction and subjected to High Performance Liquid Chromatography (HPLC) analysis as described below. Correlation was then determined between ergosterol concentrations and their respective biomass of the fungal mycelium.

Proliferation potential of endophytes and pathogen via ergosterol quantification

Similarly, treatments W, T, B, G and C (control) were prepared and incubated under similar conditions as previously described. Non-inoculated ramets served as control. There were 8 ramets assigned to each treatment where each ramet represented each harvest day. Three inoculated ramets were sampled per treatment as triplicates, at days 1, 7, 14, 21, 28, 35, 42 and 49 throughout the 49 days incubation period. Whole ramet (approximately 1 g ± 0.1 g) was sampled and macerated in liquid nitrogen with a pestle and mortar, until fine powder was achieved. The powdered samples were then subjected to microwave-assisted extraction, followed by ergosterol quantification using HPLC. For microwave-assisted ergosterol extraction [37], the powdered sample was transferred to a Pyrex test tube with a Teflon screw cap (16 mm × 150 mm). Four mL of methanol (Merck, New Jersey, USA) and 1.0 mL of 2 M sodium hydroxide (Sigma-Aldrich, Missouri, USA) were then added and the mixture heated with a conventional microwave (Panasonic, Osaka, Japan) at 70 °C for 15 s. The mixture was then allowed to cool for 30 s and neutralized with 2 M hydrochloric acid (Sigma-Aldrich, Missouri, USA). The neutralized mixture was extracted three times with 2 mL of HPLC grade pentane (Merck, New Jersey, USA). Pentane was then evaporated (via water bath at 35 °C, overnight) and the extracts dissolved in 500 µL methanol and filtered through a 0.22 µm PTFE membrane syringe filter (Fisher Scientific, New Hampshire, USA). The filtered samples were then quantified using HPLC with the following conditions: 100% HPLC grade methanol for mobile phase, a Chromolith 2.0 µ C18 reverse-phase column (Merck, New Jersey, USA), flow rate of the mobile phase was 1.0 mL min^{-1} and the wavelength for the diode array detector (Agilent Technologies, California, USA) was 282 nm. Injection volume was determined at 20 µL per sample and the average ergosterol retention time was approximately 5.1 min [38]. Quantification of ergosterol was determined by comparing peak areas against pure ergosterol standard (> 95.0% HPLC pure) (Sigma-Aldrich, Missouri, USA) which was constructed with 25–300 µg pure ergosterol (standard calibration curve) for each run. To determine fungal growth in a whole ramet, fungal mycelium weight for each treatment was also estimated using calibration curve of ergosterol against fungal biomass.

Statistical analysis

The data were statistically analyzed using the software Statistical Package for the Social Sciences (SPSS) version 20.0. One way ANOVA with the help of Tukey's studentized range test ($HSD_{(0.05)}$) was applied to analyze all the data collected. Pearson correlation was used to analyze the correlation between ergosterol concentration and mycelium weight. Differences were considered significant at $P < 0.05$.

Results

Colonization potential of endophytes and pathogen in planta via plate assay and PCR detection

Endophytes were detected in all plated tissue segments (roots, stems, leaves) 7 days after inoculation, demonstrating that all isolates were able to colonize the host plant (oil palm ramets) by the first 7 days. The morphologies of the fungal colonies from plated tissue segments were similar to fungal colonies cultured on PDA (pure cultures). The detection of one single band observed from agarose gel electrophoresis and BLAST results after DNA sequencing revealed that isolates recovered from the plant tissue sections were indeed the introduced endophytes (Table 1). No other species other than the introduced (inoculated) species was recovered from the inoculated ramets. This confirmed that ramets were solely colonized by the introduced isolates.

Correlation between ergosterol content and biomass of fungal mycelium

Positive correlation between mycelium weight (biomass) and ergosterol concentration was observed for isolates BTF08

Table 1 Detection and identification of endophytes and Gb in root, stem and leaf tissues of oil palm ramets via plate assay (culture on growth medium) and PCR method. The exemplary data here are excerpted from readings on 7th day after inoculation.

Plant part	Growth	PCR method				
		G	W	T	B	C
Root	+	*G. boninense*	*D. phaseolorum*	*T. asperellum*	*P. citrinum*	No band was detected from agarose gel
Stem	+	*G. boninense*	*D. phaseolorum*	*T. asperellum*	*P. citrinum*	No band was detected from agarose gel
Leaf	+	*G. boninense*	*D. phaseolorum*	*T. asperellum*	*P. citrinum*	No band was detected from agarose gel

Note: – "+": present; "–": absent. G: Gb-inoculated ramets; W: WAA02-inoculated ramets; T: T2-inoculated ramets; B: BTF08-inoculated ramets; C: un-inoculated ramets.

(r = 1), WAA02 (r = 0.959), Gb (r = 0.946) and T2 (r = 0.887) (Fig. 1). Ergosterol quantification was determined using the standard curve (y = 7.9424x, R^2 = 0.9956) of HPLC response (peak area) against ergosterol concentrations (μg mL^{-1}) (Fig. 2). A strong, positive correlation suggested that ergosterol levels are good indicators of fungal biomass. Isolate BTF08 was found to have relatively higher ergosterol concentration followed by isolates T2, Gb and WAA02 with 66.4, 39.3, 22.6 and 13.4 μg mL^{-1} at 2.0 g fungal mycelium weight (Fig. 1). Pearson correlation data and their corresponding 2-tailed significant values and N values are provided in the Supplementary Data.

Proliferation potential of endophytes and pathogen in planta

Ergosterol was detected in endophyte- and pathogen-inoculated ramets but the concentrations did not increase significantly throughout the 49 days of incubation (Fig. 3). Ergosterol was detected in G-ramets on all days except days 28, 35 and 49 (Fig. 3a) whereas ergosterol was detected in W-ramets during every sampling intermittent (Fig. 3b). For T-ramets, ergosterol was detected on all days except days 28 and 35 (Fig. 3c) while for B-ramets, ergosterol detection was positive on days 7, 14, 21 and 42 (Fig. 3d). Nevertheless, these ergosterol levels showed no significant increase over time, suggesting that the endophytic isolates WAA02, T2, BTF08 and pathogenic isolate Gb were present in the seedlings but were not be able to proliferate inside the ramets (internal tissues). This was further concurred by the insignificant P-value of 0.150, 0.079, 0.545, and 0.734 obtained, respectively, using Tukey's HSD test. Further data on N (sample size used to generate data at each time point), mean values, standard error and significance (P values) is presented in Supplementary Data. Ergosterol was absent in non-inoculated ramets (treatment C) throughout the 49 days (data not shown).

For isolate WAA02, 2.26 ± 0.45 μg mL^{-1} of ergosterol was detected after 49 days incubation (Fig. 4). This ergosterol concentration was equivalent to approximately 0.29 ± 0.06 g of fungal mycelium, derived from the standard curve of HPLC responses (peak area) of mycelium weight (g) against concentration of ergosterol (μg mL^{-1}). The ergosterol and biomass equivalent for WAA02 was significantly higher than most

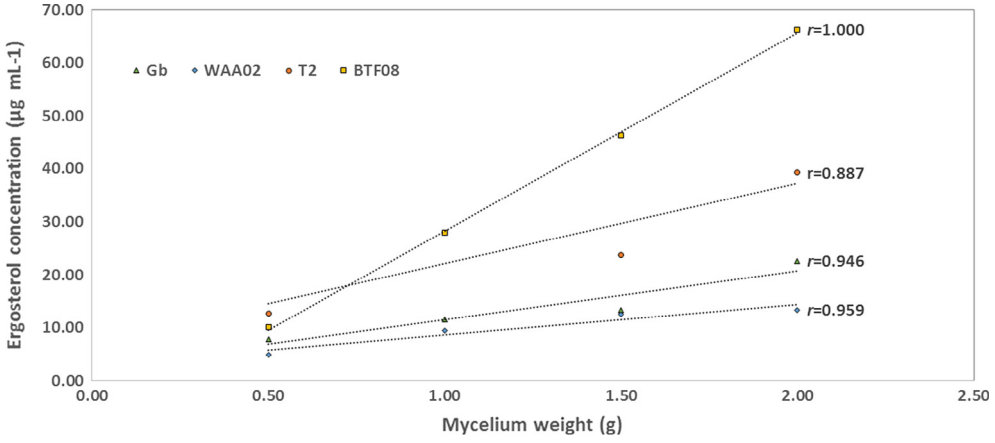

Fig. 1 Correlation (r) between ergosterol concentration (μg mL^{-1}) and fungal mycelium weight (g) for Gb, WAA02, T2 and BTF08.

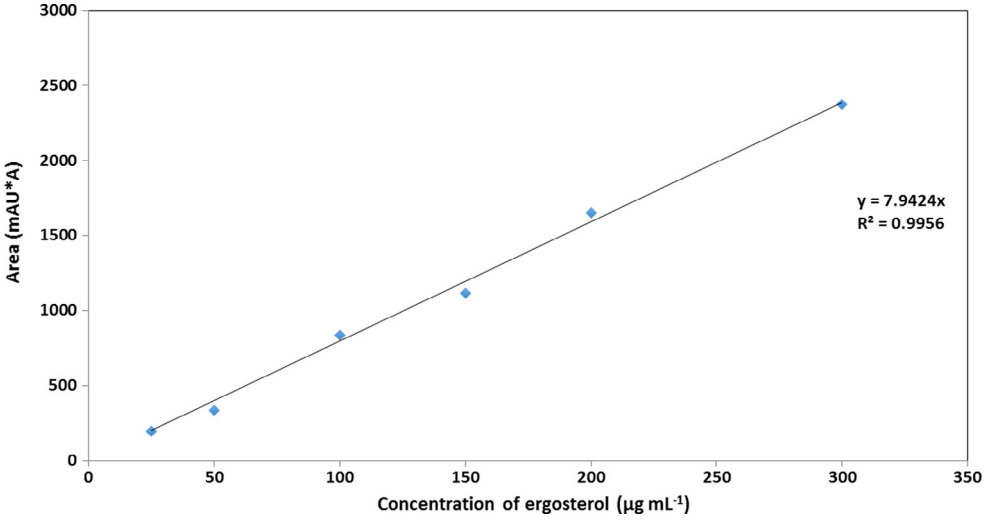

Fig. 2 Standard curve of HPLC response (peak area) against ergosterol concentrations (μg). Data represent means of three replicates.

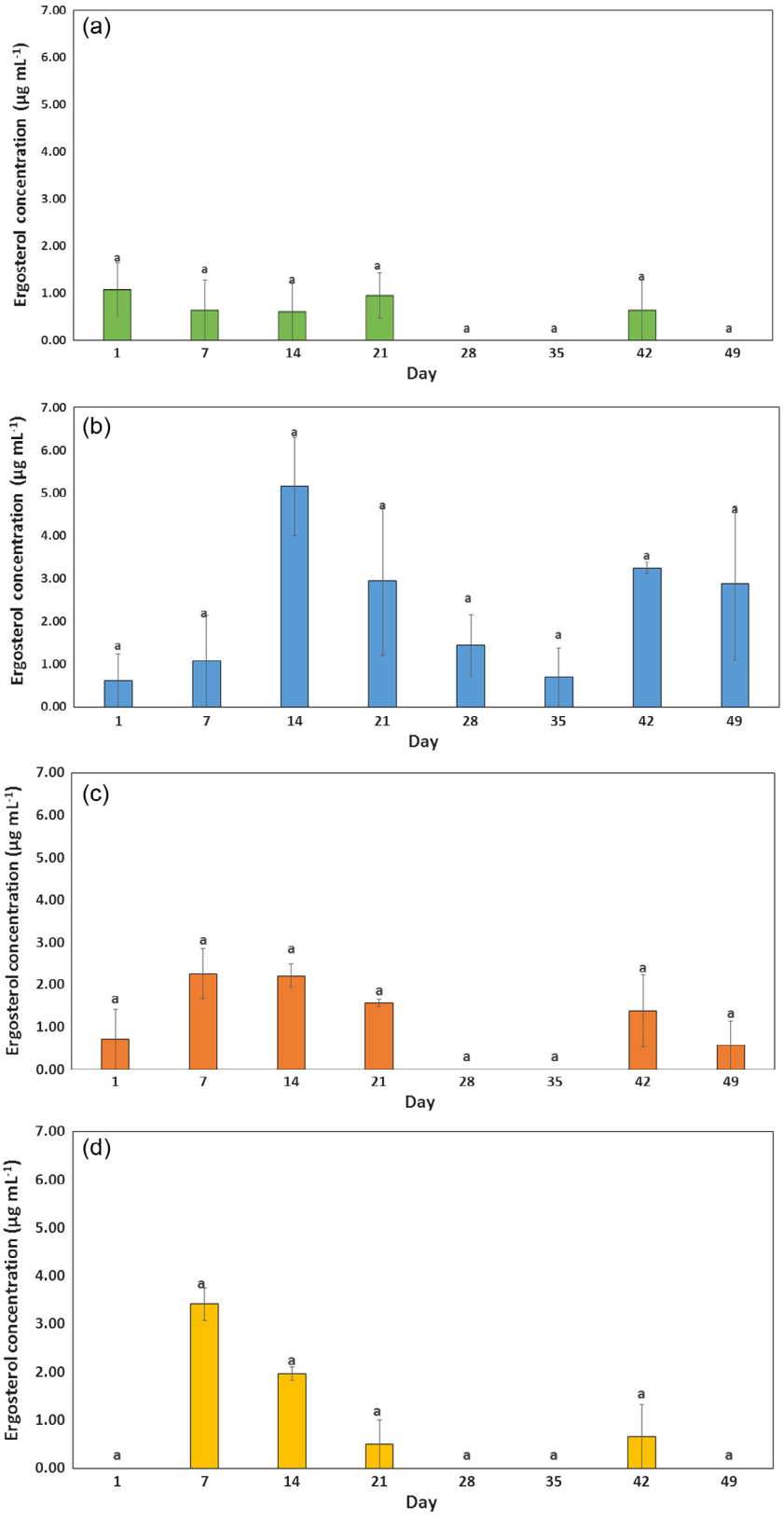

Fig. 3 Ergosterol concentration (µg mL^{-1}) and estimated fungal mycelium weight (g) in ramets that were inoculated with [a] G, [b] W, [c] T and [d] B detected at every time interval during 49 days incubation period (G = Gb-inoculated ramets, W = WAA02-inoculated ramets, T = T2-inoculated ramets, B = BTF08-inoculated ramets). Bars represent means ± SE (standard error) of triplicate treatments. Means with different letters are significantly different at $P < 0.05$, $n = 3$ using Tukey's HSD test.

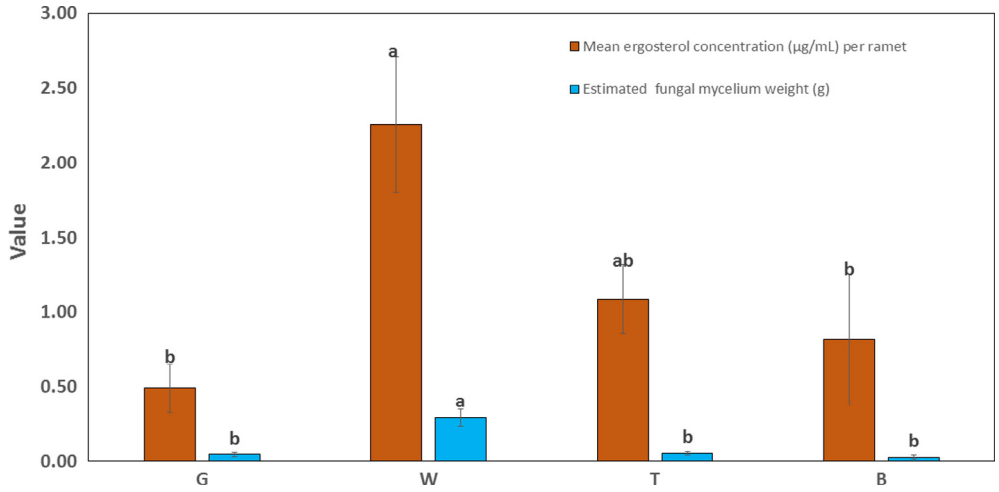

Fig. 4 Mean ergosterol concentration (µg mL^{-1}) per plant tissues and mean estimated fungal mycelium weight (g) of G, W, T and B derived from inoculated oil palm ramets after 49 days incubation (G = Gb-inoculated ramets, W = WAA02-inoculated ramets, T = T2-inoculated ramets, B = BTF08-inoculated ramets). Bars represent means ± SE (error bar) of triplicate treatments. Means with different letters are significantly different at $P < 0.05$, $n = 24$ using Tukey's HSD test.

isolates, especially Gb. Proliferation potential of Gb was very poor in the plant tissues, evident by the significantly lower ergosterol content of 0.49 ± 0.16 µg mL^{-1}, in which an estimate of 0.05 ± 0.02 g fungal biomass was obtained (Fig. 4). For isolates T2 and BTF08, their proliferation potential was similar, with ergosterol content of 1.09 ± 0.23 µg mL^{-1} and 0.82 ± 0.44 µg mL^{-1} in T2 and BTF08, equivalent to mycelium weight of 0.05 ± 0.01 g and 0.03 ± 0.01 g, in 1 g of ramets, respectively (Fig. 4). Although weekly observations revealed that the growth of endophytes *in planta* was gradual (Fig. 3) and with insignificant *P*-value ranged from 0.085 to 1.000 using Tukey's HSD test, endophytes did remain *in planta* and may proliferate gradually. Further data on N (sample size used to generate data at each time point), mean values, standard error and significance (*P* values) are presented in Supplementary Data.

Discussion

Isolates WAA02, T2, BTF08 and Gb were successfully reisolated from all plant tissues (roots, stems and leaves). This confirms the ability of introduced endophytes to colonize host plants effectively [39]. The DNA sequencing results further validated that isolates recovered from the plant tissue sections were indeed the introduced endophytes using BLAST. The plating and PCR analysis detected the presence of isolates in the plant tissues, but does not provide information on the biomass abundance *in planta*. This has to be determined via quantitative PCR (qPCR) or estimation based on ergosterol content. Nevertheless, PCR was able to determine the type of isolate colonizing the tissues and is a more reliable method compared to the time-consuming process of microscopic identification. Microscopic identification is also limited by the morphology of fruiting structures that are difficult to determine and distinguish [40].

Detection of ergosterol in plant tissues was relatively inconsistent. Ergosterol detected from the day of inoculation may be attributed to mycelium fragments that presumably entered the host tissues via the xylem tissues. The irregularities in ergosterol detection for some sampling points (e.g. on 28th, 35th and 42nd day) may be attributed to the possibility that the biomass in tissues may have been diluted when tissues without biomass were pooled for assay, and vice versa. As such, this may have contributed to the inconsistent levels of ergosterol. All been said, the levels were insignificantly different from one another. It was also unexpected that ergosterol concentrations did not increase significantly throughout the 49 days incubation period for all inoculated seedlings. This contradicted to the study by Mohd As'wad et al. [38]. This suggested that all isolates did not proliferate significantly inside plant tissues. Our results were similar to other studies where fluctuations in ergosterol concentrations were observed throughout the experimental period [40]. They associated fluctuations in ergosterol concentrations to fungal physiology inside plant tissues or the variation in number of viable and non-viable spores [40]. It was proposed that ergosterol degrades when spore state changes from viable to non-viable spores. We therefore, postulate that each of the ramets in our study may have different amount of viable and non-viable spores, resulting in inconsistent ergosterol concentrations. This observation also highlighted the fact that proliferation ability of endophytes may be one influential factor determining biocontrol efficiency of endophytes in the field. We suggest that to overcome this limitation and determine proliferation ability of these selected isolates, quantitative PCR (qPCR) can be used as gene copy per genome will not be varied and affected by environmental conditions.

The proliferation potential of isolate WAA02 was greater than BTF08, T2 and Gb as WAA02 is a fast-growing isolate, and produces abundant hypha for colonization. It is suggested that perhaps the abundance of hypha present may have led to higher ergosterol levels, as ergosterol is a primary sterol found in the cell membrane of fungi [41–43]. The cell membrane acts as a barrier between an organelle and its environment, and also serving as a matrix for the association of proteins with lipids [44]. It was therefore expected that ergosterol would be detected for all isolates in this study, and that the ergosterol levels can be used as a measure of the proliferation potential

of endophytes *in planta*. This observation agrees with many studies [9,32,38], but this is the first reporting for T2 (*T. asperellum*) and BTF08 (*P. citrinum*). On the contrary, the slow-growing nature of BTF08 and Gb may have resulted in poorer proliferation rate (lower ergosterol content). These isolates may not grow and proliferate as well as WAA02 *in planta*. The poor growth of Gb *in planta* suggested that endophytes could be introduced prior to contact with Gb, and Gb may be excluded via competitive exclusion for space and nutrients [45,46].

Results from the ergosterol and fungal biomass analysis, have also suggested that different fungal species may have different ergosterol concentrations due to their sporulation and mycelium structure. When analyzed using the same biomass (2 g), various ergosterol concentrations were derived. This is presumably due to the nature of fungi, having both free and esterified forms of ergosterol, which varies in ratio among different fungal species [27,47]. The free-forms are localized in cell membranes, while the esters are found in cytosolic lipid particles [44,48]. Ratios of these two forms have been known to serve as indicators to differentiate fungal species [48,49]. Nevertheless, in this study, the ratios were not further analyzed and the total ergosterol concentration is used instead to estimate proliferation of fungal isolates *in planta*. Overall, ergosterol is well known to be common in fungi and the detection method of ergosterol is established. In this study, we also used ergosterol to study the progressive growth and possible proliferation of endophytes in plants throughout a 49 day period, rather than to just quantify the amount of fungi in the samples. Thus, we present a new application of a well-established technique in this study. In future, proliferation ability of endophytes can be fully described using ergosterol assay and qPCR.

Conclusions

Colonization and proliferation potential of introduced endophytes and pathogen in a host plant (oil palm) was established via plate assay, PCR and ergosterol quantification. Endophytic isolates (WAA02, T2, BT0F8) were found to have similar colonization potential with pathogen, colonizing roots to leaves within 7 days of inoculation. Isolate WAA02 has better proliferation potential due to the higher ergosterol concentration and fungal biomass recovered. The increase in ergosterol levels throughout 49 days was however insignificant, suggesting that proliferation may be absent or may occur very slowly *in planta*. This study strongly suggests that the selected endophytes could colonize the host upon inoculation, but proliferation occurs at a slower rate. This supports the hypothesis that colonization and proliferation potential may influence the biocontrol expression of endophytic BCAs. Investigation on extent of colonization by endophytic isolates via quantitative real time PCR (qPCR) and endophytic factors that influence colonization behavior can be conducted in future.

Author contribution statement

ASYT and SR conceived and designed this research project. YYC conducted the experiments. ASYT, SR and YYC analyzed the data. YYC and ASYT wrote this manuscript. All authors read and approved the manuscript.

Conflict of Interest

The authors have declared no conflict of interest with any parties which may arise from this publication.

Compliance with Ethics Requirements

This study does not contain any studies with human participants which requires informed consent from respective individuals.

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