ORIGINAL ARTICLE

Prevalence of Endophytes Antagonistic Towards Fusarium Oxysporum F. Sp. Cubense Race 4 in Various Plants

A.S.Y. Ting, S.W. Mah and C.S. Tee

Faculty of Engineering and Science, Universiti Tunku Abdul Rahman, Jalan Genting Kelang, Setapak 53300, Kuala Lumpur, Malaysia.


ABSTRACT

This study was conducted to determine the diversity of beneficial endophytes from various plant species, associated with their prevalence according to plant type, plant group and their environmental setting. Isolation was performed using stem and leaf tissues, and the isolates obtained were subsequently tested for antagonistic activity towards the fungal pathogen Fusarium oxysporum f. sp. cubense race 4. Our results revealed that antagonistic endophytes were mostly fungal endophytes and they were found primarily in weeds and medicinal plant samples. Plants from rural landscapes were observed to host more antagonistic endophytes compared to plants from urban landscapes. As such, we conclude that endophytes with potential as biocontrol agents for Fusarium wilt management are prevalent in plants that exist in the rural areas, and that sourcing from weeds or medicinal plants would provide a higher recovery rate of antagonistic endophytes.

Key words: beneficial endophytes, endophyte diversity, medicinal plants, recovery rate, weeds

Introduction

Endophytic microorganisms are organisms that reside, or exist at least during part of their life cycle, in the tissues of living plants asymptomatically (Wilson, 1995; Clay & Schardl, 2002). They are abundant in host plants, especially in the crown, stem and leaf tissues. Each individual plant is host to one or more endophytes; therefore there is vast opportunity to find new and interesting endophytic microorganisms among myriads of plants in different ecosystems.

The interest in endophytes first began when fungal endophytes in grasses were found to produce alkaloids which are toxic to herbivores (Bacon et al., 1977) and later in repelling insects (Rowan et al., 1994). This provided early indications of the possible bioactivity of endophytes which could be further explored for applications as agricultural bioagents. As bioagents, endophytes are valued not only for their potential in conferring resistance to pests and pathogens (Kimmons et al., 1990; Siegel & Latch, 1991; Stovall & Clay, 1991; Gwinn & Gavvin, 1992; Mahmood et al., 1993), but for their beneficial association with the host plant as well, such as enhanced nutrient uptake to improve growth (Clay et al., 1989; Rice et al., 1990; Ting et al., 2008), and improved tolerance to stress factors (Lewis et al., 1997; Cheplick et al., 2000; Malinowski & Belesky, 2000). In the recent years, investigations into the natural products produced by endophytes have further redefined their usage as agrochemical agents (Findlay et al., 1997; Strobel et al., 1999) and increased the explorations for beneficial endophytes.

Attempts to source for beneficial endophytes for bioactivity screening are however, often hampered by the poor understanding of the association and distribution of endophytes with host plants and consequently, the selection of target plants as sources for endophytes. Currently, very little information is available to fully

Corresponding Author: A.S.Y. Ting, Faculty of Engineering and Science, Universiti Tunku Abdul Rahman, Jalan Genting Kelang, Setapak 53300, Kuala Lumpur, Malaysia.
Phone No.: 603-41079802; Fax No.: 603-41079803
E-mail: adelsuyien@yahoo.com; tingsy@utar.edu.my
comprehend the distribution of endophytes in the various host species, although influences from biological, ecological and geographic factors are known to shape the endophyte community (Strobel & Daisy, 2003), and subsequently their general metabolism and bioactivity.

In our study, we investigated the recovery potential of endophytes from various host species. Our plant samples were obtained from the urban and rural landscape to isolate endophytes with novel strategies for survival in these different environmental settings (Strobel et al., 1999). Some of our plant samples are medicinal plants which have ethno-botanical history (Strobel et al., 1993; Castillo et al., 2002), while other host varieties such as fruit trees, weeds and ornamentals were also selected to obtain a greater diversity of endophytes. The endophytes were then screened for their biocontrol properties against Fusarium oxysporum f.sp. cubense race 4 (FocR4) the causal agent of Fusarium wilt of banana. Results were then analyzed and the prevalence of antagonistic endophytes in various host species is reported in this paper. The results here provide a preliminary understanding of the association of endophytes with their host plants and serve as a platform to strategize the isolation procedure from host plants to increase endophyte biodiversity.

**Materials and methods**

**Isolation of Endophytes from Host Plants:**

The plant samples selected were asymptomatic plants found at various locations from the urban and rural landscapes in Kuala Lumpur, Perak and Selangor. Urban landscapes include house gardens and parks in townships while rural landscapes include mostly secondary jungles and forest reserve parks. Three leaflets were picked randomly from fruit trees, medicinal shrubs, weeds or ornamental plants, and processed for isolation. For plants with no leaves such as *Aloe vera*, the stem tissues were used instead. The leaf (or stem) tissues were then cut into smaller pieces measuring 3 x 5 cm². The tissue sections were surface sterilized with 20% Clorox® for 5 min, followed by immersion in 70% ethanol for 2 min, and subsequently a quick rinse in sterile distilled water. The tissue sections were then cut longitudinally and plated on nutrient agar (NA) (R&M), potato dextrose agar (PDA) (Merck) and Actinomycetes selective agar (R&M). The plates were incubated at room temperature (27±3°C) for 48 h, 7 days and 10 days to observe for bacterial, fungal and Actinomycetes, respectively. Colonies growing from the tissues were isolated and pure cultures established.

The numbers of endophytes isolated were recorded according to the three main categories; based on different plant types, their grouping into monocotyledonous and dicotyledonous plant groups, and from different landscapes (environmental settings). Due to the unequal number of plants sampled, the recovery rate of endophytes from each plant type (endophyte/plant type) was estimated instead to determine the prevalence of endophytes in each plant type. The following formula was used to assess the prevalence of endophytes in all the plant types (weeds, medicinal, ornamentals and fruit tree samples), with the following example for weed plants:

\[
\text{Prevalence of endophytes in weeds} = \frac{\text{total endophytes isolated from all weed plant samples}}{\text{total number of weed plant samples}}
\]

For plant groups (monocotyledonous or dicotyledonous) and endophytes sampled from different environmental settings (urban or rural landscapes), the following two formulas were used for calculation:

\[
\text{Prevalence of endophytes in monocotyledonous plants} = \frac{\text{total endophytes isolated from all monocotyledonous plant samples}}{\text{total number of monocotyledonous plant samples}}
\]

\[
\text{Prevalence of endophytes in urban landscape} = \frac{\text{total endophytes isolated from all plant samples in urban area}}{\text{total number of all plant samples in urban area}}
\]

**Screening for Biocontrol Activity:**

The antagonistic potential of the endophytes was detected using the dual-culture assay. For this assay, the mycelial plug of 7-day old *Fusarium oxysporum* f.sp. *cubense* race 4 (FocR4) and fungal endophyte were co-inoculated onto a 9 cm-diameter PDA plate with equal distance of 3 cm apart from each other and from the
periphery of the plate. The procedure was repeated for bacterial endophytes by substituting agar plugs of fungal endophytes with bacterial streaks. For control, agar plug and sterile distilled water were used to replace mycelial plug of fungal endophyte and bacterial streaks, respectively. Three replicates were performed for each tested endophyte. Incubated plates were incubated for 7 days at room temperature and the radial growth of FocR4 was measured and expressed as percentage inhibition of radial growth (PIRG), whereby \( R_i \): radial growth of FocR4 in control plates, \( R_e \): radial growth of FocR4 in plates with endophytes, and calculated as follows:

\[
\text{PIRG (\%)} = \left[ \frac{(R_i - R_e)}{R_i} \right] \times 100\%
\]

The numbers of antagonistic isolates were then recorded for each category as total number of antagonistic endophytes isolated from different plant types, from monocotyledonous and dicotyledonous plant groups, and from different landscapes. To estimate the prevalence of antagonistic endophytes in each plant type (antagonistic endophyte/plant type), or plant group or landscape, the previous formulas were applied, substituting the total endophytes isolated with the total number of antagonistic endophytes. In addition, the percentage of antagonistic endophyte (%) recovered from each plant type (or plant group or landscape) was also calculated by multiplying 100% to the values obtained using the previous formulas stated.

**Results and Discussion**

**Endophytes from Various Plant Types:**

A total of 54 plants were sampled for endophyte isolation. However, since only plants that were geographically accessible were collected for this study, this resulted in the unequal sample size for each plant type; fruit trees (52%), ornamentals (37%), weeds (7%) and medicinal plants (4%). Thirty five of the total plants sampled belonged to the monocotyledonous plant group contributed mainly by the banana plants (fruit trees), while 19 of the plant samples, especially ornamentals, belonged to the dicotyledonous plant group.

From the isolation exercise, the endophytes recovered from the plant samples were mainly bacterial endophytes, with 55 isolates (63%) compared to 32 fungal endophytes (37%) isolated. The recovery of endophytes was highest in medicinal plants with 3.0 isolates/plant, followed by recovery from weeds with 2.3 isolates/plant, from ornamentals with 1.8 isolates/plant and from fruit trees with 1.3 isolates/plant (Figure 1). From this, it is clear that endophytes were more prevalent in our medicinal and weed plant samples and was least prevalent in fruit trees. Our results also revealed that a slightly higher recovery rate of endophytes were achieved in plants from the urban landscapes compared to plants originating from rural landscapes, with 1.7 and 1.5 endophytes/plant recovered, respectively (Figure 1). Plant samples from the dicotyledonous plant group also produced a slightly higher endophyte/plant recovery rate with 1.8 endophyte/plant recovered compared to 1.5 endophyte/plant in monocotyledonous plants (Figure 1).

**Antagonistic Activity of Endophytes:**

The antagonistic activity of the endophytes was evident in the plate assay. Bacterial endophytes produced inhibitory metabolites that appeared to have diffused into the agar and inhibited the growth of the FocR4 colony, resulting in the formation of inhibition zone between the colony of FocR4 and the bacterial streak (Figure 2A). Fungal endophytes on the other hand, inhibit the growth of FocR4 by overgrowing on the colony of FocR4 (Figure 2B).

From a total of 87 endophytes tested, only 30 endophytic isolates showed antagonistic effect towards FocR4. Of the 30 isolates, seven were bacterial isolates while 23 were fungal isolates. This resulted in a higher percentage (72%) of fungal endophytes that was antagonistic towards FocR4 compared to only 13% of the total bacterial endophytes isolated. Sixteen of the 20 antagonistic endophytes were strong antagonists, with PIRG values above 50%, from 60% to 87%. The PIRG values produced by the bacterial endophytes (ICB01, AVB02, AVA02, BTB22, CRA01, HRB01, LCB01) were between 67% and 78%. They were mostly isolated from ornamental plants (ICB01, CRA01, HRB01), medicinal plants (AVB02, AVA02) and fruit (BTB22) and weed (LCB01) plants. Four of the nine fungal endophytes (ALF01, BTB08, BTB15, BTB21) also produced inhibitory metabolites as their main mode of inhibition, resulting in PIRG values of 55% to 75%. The other five fungal isolates (WAA03, WAA02, MIF01, BTB05, BTB07) were fast colonizers, inhibiting the growth of FocR4 by growing and colonizing the agar plate rapidly. These five fungal endophytes recorded PIRG values between 79% and 87%. Most of the fungal endophytes were isolated from the banana plants (BTB05, BTB07, BTB08, BTB15, BTB21), followed by weeds (WAA03, WAA02, MIF01) and ornamental plants (ALF01).
Prevalence of Antagonistic Endophytes in Various Plant Types:

When analyzed based on their host association, we observed that the highest recovery rate of antagonistic endophytes were from medicinal and weed plant samples, each with a recovery rate of 1.5 antagonistic endophyte/plant (Figure 3). Weed plant samples were however, able to host more antagonistic endophytes as 66.7% of the total endophytes isolated were antagonistic, compared to only 50.0% from medicinal plants (Figure 4). Ornamental plants were the least suitable source of plants to isolate for antagonistic endophytes as the percentage of antagonistic endophytes recovered from ornamental plants was the lowest at 27.8% (Figure 4).

Antagonistic endophytes were also observed to be more prevalent in dicotyledonous plant samples. Dicotyledonous plants not only have higher total endophyte recovery (1.8 isolate/plant) (Figure 1) but more antagonistic endophytes (0.95 antagonistic endophyte/plant) as well compared to monocotyledonous plants (Figure 1, Figure 3). Furthermore, 90.0% of the total endophytes isolated from dicotyledonous plants were antagonistic towards FocR4, compared to only 35% from monocotyledonous plants (Figure 4). In addition, comparisons on the antagonistic endophytes derived from plant samples from the rural and urban landscapes showed that plants from the rural environmental settings have more antagonistic endophytes (Figure 3) although they host lesser number of endophytes compared to plants from the urban settings (Figure 1). Only 44% of the endophytes from urban landscapes were antagonistic, compared to rural landscapes with 49% (Figure 4).

Discussion:

Results from this study showed that endophytes can be isolated from medicinal plants, weeds, ornamentals and fruit trees, found in both urban and rural settings. From a total of 54 plants sampled, we obtained 87 endophytic isolates and 30 of these endophytes showed antagonistic activity towards our test pathogen (FocR4). We theorized that a greater diversity and a higher number of antagonistic endophytes can be achieved with the increase in number of plants sampled. Therefore, there is much potential in recovering novel endophytes and their metabolites from the 300 000 plant species on earth (Strobel & Daisy, 2003), not just as agricultural bioagents but for their value in medicinal and industrial applications as well. In our study, as part of a strategic plan to screen for bioagents to manage Fusarium wilt, the endophytes were all isolated from leaf and stem tissues. FocR4 is a vascular-inhabiting fungal pathogen, thus endophytes able to colonize the plants from root to stem or leaf tissues, have the advantage as biocontrol agents. In addition, the endophyte recovery is reportedly higher in leaf and stem tissues compared to root tissues (Clay & Schardl, 2002).

The endophytes isolated were predominantly bacterial endophytes. Bacterial endophytes are known to colonize the host plants via the xylem tissues (Bacon & White, 2000) thus are more abundantly and easily isolated from host tissues. Both bacterial and fungal endophytes can co-exist in a single host plant as observed from this study. However, we have better success in recovering multiple bacterial endophytes in a single plant, than at recovering multiple fungal endophytes from a single plant. This observation thus suggested the possible predominance of a single fungal genotype in a host plant. In our study, we associated the predominance factor of our fungal endophytes to their rapid colonization on plate cultures, which presented an intense competition for sites and nutrients. This could have contributed to having only single fungal genotypes recovered from a single plant. Although we did not pursue molecular evidence of genotype dominance, Kover et al. (1997) and Meijer & Leuchtmann (1999) have proven that in some host species, a single fungal genotype exist as a predominant genotype.

As in many previous studies (Siegel & Latch, 1991; Stovall & Clay, 1991), the antagonistic activities of the endophytes isolated from the various host species were established through in vitro assessments. Inhibition by endophytes towards the pathogenic FocR4 is most likely attributed to the antifungal compounds produced. This is because bacterial and fungal endophytes are generally known to produce antifungal compounds which can inhibit a broad spectrum of pathogens (Harrison et al., 1991; Ballio et al., 1994; Miller et al., 1998; Strobel et al., 2001; Stinsom et al., 2003). In our investigations, we also observed that antagonistic endophytes were seldom recovered from the same single plant. Li et al. (1996) explained that usually only one endophyte from a single plant would produce biologically active compounds.

The five fungal endophytes in this study with rapid growth may belong to the category of aggressive saprophytes as theorized by Bacon & White (2000). While the rapid-growth is desirable for effective inhibition of FocR4, it complicates the identification process of the isolates as no spores were produced even on nutrient rich PDA plates. White (1988) stated that the absence of sexual spores in fungal endophytes could also indicate that the fungus remain internally, goes through an asymptomatic life cycle and are likely to be transmitted vertically (White, 1988). Thus our five rapid-growing fungal endophytes may be highly desirable for Fusarium wilt management of bananas as the antagonistic endophytes can be transmitted vertically from the mother plant to the suckers.
Fig. 1: Endophyte recovery rate (endophyte/plant) for plants sampled from the various plant types, landscapes and plant groups. (Note: FT-fruit trees, O-ornamentals, W-weeds, M-medicinal plants, R-rural landscape, U-urban landscape, MC-monocotyledonous, DC-dicotyledonous). Bars indicate standard error of means.

Fig. 2: Growth inhibition produced by bacterial (a) and fungal (b) endophytes toward FocR4 in the plate assay after 7 days incubation. Control plate is shown in (c). (Note: Mycelial plug of FocR4 is inoculated on the left for every plate).

Fig. 3: Antagonistic endophytes (antagonistic endophyte/plant) recovered from plants of various plant types, landscapes and plant groups. (Note: FT-fruit trees, O-ornamentals, W-weeds, M-medicinal plants, R-rural landscape, U-urban landscape, MC-monocotyledonous, DC-dicotyledonous). Bars indicate standard error of means.
Results have shown that the antagonistic endophytes were mostly recovered from weed plants, while ornamental plants host the least number of antagonistic endophytes. More antagonistic endophytes were also recovered from rural landscapes which are subjected to higher selection pressure and more environmental stress compared to urban landscapes. The prevalence of antagonistic endophytes in both situations could be the result of the interaction of the host plants with the endophytes residing in its host tissues, which have been known to alter their competitive interactions with other plants (Cheplick et al., 1989; Marks et al., 1991) and to enhance their production of secondary metabolites (Schutz, 2001). This offers a possible explanation on the higher recovery rate of antagonistic endophytes from weed plants, which coincidently were sampled from rural landscapes, compared to ornamentals which were mostly from urban landscapes.

Our results also indicated that the number of antagonistic endophytes recovered from medicinal plants were also relatively high. This observation is inclined to support the suggestion by Strobel & Daisy (2003), on the possibility that the natural products from medicinal plants may not necessarily originate from the plants per se, but may be attributed to the secondary metabolites produced by endophytes or as a result of the endophyte-host interaction. In addition, the higher number of antagonistic endophytes recovered from dicotyledonous plants observed in this study suggested that non-grass species can also be good sources of beneficial endophytes, especially for the control of FocR4.

Conclusions:

To conclude, we hope the results here offer a more creative strategy to narrow the search of endophytes displaying bioactivity towards FocR4. Endophytes as bioagents towards FocR4 can be source from weed or medicinal plants, isolated from rural areas. The selection of host plant is essential since they govern the novelty and biological activity of products associated with the endophytes isolated. This can be further expanded to include sourcing for endophytes with metabolites suitable for medicinal and industrial applications.

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References


