CULTIVATION OF ENDOPHYTIC FUNGI INHABITING *ABIES BALSAMEA* USING TWO TECHNIQUES: A COMPARATIVE STUDY

By

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ABSTRACT

Two cultivation techniques: leaf segment cultivation and dilution-series cultivation, were compared for recovery of endophytes from needles of *Abies balsamea* collected in Saint John, New Brunswick, Canada. Endophytes were cultured on 2% malt extract agar and morphology of the endophytic growth was used to aid identification of distinct isolates. Endophytes were isolated from *A. balsamea* needles using the leaf segment cultivation method at a frequency of 13%, all replicates of two distinct isolates. Endophytes were isolated form eight of 25 prepared isolation plates for the dilution-series method. All endophytic growth from the dilution-series method was single-celled yeast species, while the endophytic growth from the leaf segment method was exclusively filamentous fungal species; thus, dichotomy was seen between the two methods of isolation.

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1. Introduction

All plants tested to date are host to internal microorganisms (Nair & Padmavathy, 2004). The focus of my research was on the endophytic fungal microorganisms isolated from *Abies balsamea* needles. Usage of the term endophyte has dated back to 1866 (De Bary, 1866). One common definition of endophytic fungi was proposed by Petrini (1991): "all [fungal microorganisms] inhabiting plant organs that[,] at some point in their life, can colonize internal plant tissues without causing apparent harm to the host."(179). However, other definitions have been used (Hyde & Soytong, 2008) including "fungi that form unapparent infections within leaves and stems of healthy plants" (Carroll, 1988) and "fungi that colonize a plant without causing visible disease symptoms at any specific moment" (Schulz & Boyle, 2005). All the proposed definitions of endophytic fungi have focused on the same theme—fungi living inside plant tissues.

Endophytic fungi are ubiquitous to each plant studied thus far (Rodriguez et al., 2009). These microorganisms have been isolated from various parts of plants: the leaves, stems, bark, petioles and reproductive structures (Rodriguez et al., 2009). The numbers of endophytic species isolated in a given plant species varies (Huang et al., 2008), and it is probable that some or perhaps most endophytes are never isolated due to competitive interactions of endophytic species (Hyde & Soytong, 2008).

Isolating endophytic fungi has been a challenge and the diversity is limited (Hyde & Soytong, 2008; Huang et al., 2008). Despite this, few isolation techniques have been developed and reviewed in attempts to increase the number of endophytic fungal species being isolated (Schulz et al., 1993; Guo et al., 2000; Unterseher & Shnittler, 2009). Two techniques are leaf-segment cultivation and serial dilution of macerated leaf material (Schulz et al., 1993; Unterseher

& Shnittler, 2009). Leaf segment cultivation involves surface sterilizing the plant tissues (i.e., needles, leaf, bark, etc.) using effective sterilizing agents and placing cut segments of plant tissue directly on growth medium (Schulz et al., 1993). Dilution-series cultivation involves homogenizing the surface-sterilized plant tissue, serially diluting the macerated plant material, and then cultivating the homogenized plant tissue on growth medium (Unterseher & Shnittler, 2009). Comparing leaf segment cultivation and dilution-series cultivation will provide information about which isolation method yields a greater number of endophytic species and therefore greater diversity.

Unterseher and Schnittler (2009) studied the effects of isolating foliar endophytic species via leaf segment cultivation and dilution-series cultivation in order to determine species richness and endophyte diversity in *Fagus sylvatica*, more commonly known as beech (Unterseher & Schnittler, 2009). Their study demonstrated a difference in species richness for each of the two methods; leaf segment cultivation showed greater species richness, as a measure of diversity, than dilution-series cultivation. Comparing Unterseher and Schnittler's (2009) study to a reference study, which had determined the endophytic fungal species composition of *Fagus sylvatica*, allowed for the comparison of isolation frequencies of each method: leaf segment cultivation and dilution-series cultivation. The isolation frequency for leaf segment cultivation was 77% of total endophytic species, and dilution-series cultivation had a 54% isolation frequency. Eight distinct fungal taxa were isolated with the dilution-series method that were not seen in the leaf segment cultivation method, and 16 were recovered with leaf segment cultivation but not with dilution-series (Unterseher & Schnittler, 2009). Unterseher and Schnittler (2009) concluded that more studies were needed before a methodological shift from leaf segment cultivation to dilution-series could be advocated.

Prior to Unterseher and Schnittler's (2009) study, research on endophytic fungi relied mainly on isolation of organisms from surface sterilized leaf segments cultivated on agar (Hyde & Soytong, 2008). However, Paulus et al. (2003) and Collado et al. (2007) modified a high-throughput culturing method for use with fungi. The technique involved using a blender to macerate the plant tissue, and then centrifuging to separate the solid particles from the liquid suspension. Next, the liquid suspension was serially diluted before culturing into multi-welled plates (Paulus et al., 2003; Collado et al., 2007). Unterseher and Schnittler (2009) adapted the protocol above for their comparative study of isolating fungal endophytes.

The present research focused on the techniques leaf segment cultivation and dilution-series cultivation. The objective of the present research is to determine if serial dilution of macerated plant material or leaf segment cultivation, yields a greater endophytic fungal species richness.

2. Materials and methods

2.1 *Collection of plant material*

Branches from *Abies balsamea* (balsam fir) were collected from the University of New Brunswick campus, Saint John, New Brunswick (45°18'18" N, 66°4'49" W). The needles used for the research were ensured to be between the age of five years to ten years old.

2.2 *Surface sterilization*

Individual needles of *Abies balsamea* were surface sterilized per Johnson and Whitney (1989) protocol. The needle was placed in (1) 70% ethanol for 1 min, (2) 5.25% bleach for 10 mins, (3) dipped in 70% ethanol, and (4) sterile water for 15 seconds. After sterilization, the needles were blotted dry on autoclaved paper towel. The sterilized needles were streaked across

a plate of 2% malt extract agar using sterile forceps, making sure the surface of the needles made complete contact with the growth medium. A successful sterilization of the plant surface was indicated by a lack of growth on the 2% malt extract agar surface. Sterilized needles were cut in half lengthwise. One half was used in the leaf segment cultivation method, and the other half was used in the dilution-series method.

2.3 *Leaf segment cultivation method*

Sterilized needles were cut into four equal pieces, about 2.5mm × 2.5mm, and placed on 2% malt extract agar (MEA) in serial order: petiole end, two middle segments, and the tip (Figure 1). A total of 100 needle segments were cultivated, and were incubated at room temperature (approximately 25°C).

2.4 *Dilution-series method*

Sterilized needles were cut into smaller pieces to aid in the macerating protocol, and placed in vials containing 5 mL of 2% malt broth. Using a VD-12 Homogenizer (VWR International®), the mixture was blended at 3000 revolutions per minute for 60 seconds. The suspension served as a 1× dilution for further dilution-series. A 1:10000 serial dilution was pipetted on 2% malt extract agar plates using aliquots of 0.1 mL. Each aliquot was spread across 2% malt extract agar; to ensure equal distribution of the liquid suspension a glass rod was used to spread over the agar surface (no fewer than five concentric swipes and two parallel swipes) . A total of 25 isolation plates were created which were incubated at room temperature (approximately 25°C).

2.5 *Isolate Identification*

Plates with growth on the surface of the 2% malt extract agar were continually monitored. Unique isolates were determined based on morphology (color and shape of growing

endophyte edge) of the endophytic growth. Isolation frequency was calculated for the leaf segment cultivation method. The isolation frequency was calculated by dividing the number of needle pieces from which endophytes were cultured, by the number of total needle pieces prepared.

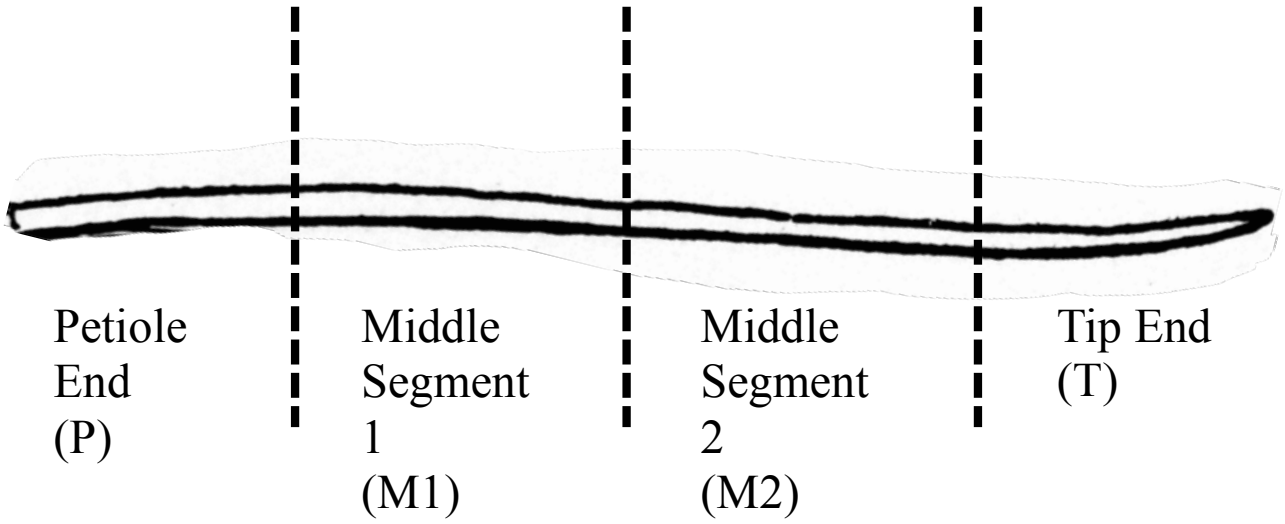

Figure 1. Schematic representation of *Abies balsamea* needle and the categorization of the individual cut segments

3. Results

Isolation frequency was determined for the leaf segment cultivation method of *A. balsamea* needles, and is defined as the number of needle pieces from which endophytes were cultured as a percentage of the total number of needle pieces prepared. Endophytes were isolated from *A. balsamea* using the leaf segment cultivation method at a frequency of 13% (13 out of the 100 needle segments) (Table 1), with two of the fungi being different based on morphology (Table 2). All endophytic growth on the 2% malt extract agar was filamentous fungi.

In the dilution-series method, endophytic growth was present on the 2% malt extract agar of eight out of the 25 prepared isolation plates (Table 3). All endophytic growth from the dilution-series method was single-celled yeast species.

Table 1. Presence of endophytic growth on 2% malt extract agar from segments of *Abies balsamea* sterilized needles.

	Segments			
Sample #	P [a]	M1 [a]	M 2 [a]	T [a]
1				+
2				
3			+	
4				
5				
6				
7				
8		+		
9				
10				
11				
12			+	
13				
14			+	
15		+	+	
16	+			
17				
18				
19				
20				+
21			+	
22		+	+	
23			+	
24				
25				

Note. Needle segments in the leaf segment cultivation method were designated as: P for the petiole end, M1 for the first middle segment, M2 for the second middle segment, and T for tip segments; + indicates the presence of endophytic growth on 2% malt extract agar.

[a] Refer to Figure 1 for explanation of categorization.

Table 2. Isolation frequency of endophytic growth from cut segments of *Abies balsamea* needle.

	Total number of segments	Number of segments from which endophytes were cultured	Isolation Frequency (%)	Number of different endophytes
Abies balsamea	100	13	13	2

Table 3. Presence of endophytic growth on 2% malt extract agar after homogenizing *Abies balsamea* needles and serially diluting.

Sample #	Presence of growth
1	+
2	
3	+
4	
5	
6	+
7	
8	+
9	
10	
11	
12	
13	
14	+
15	
16	
17	
18	
19	+
20	+
21	
22	
23	+
24	
25	

Note. + indicates the presence of endophytic growth on 2% malt extract agar.

4. Discussion

Unterseher and Schnittler (2009) adapted the modified high-throughput culturing method used by Paulus et al. (2003) and Collado et al. (2007) for their comparative study of isolating fungal endophytes. Their method included homogenizing the *F. sylvatica* leaves by placing them in a blender, with 200mL of sterile water, for 60 seconds. Subsequent use of three mesh sieves (630μm, 200μm, 100μm) were used to separate the larger fragments of solid leaf particles (Unterseher & Schnittler, 2009). Next, a centrifuge was used to separate the liquid from the solid supernatant and 10 mL of 0.1% (weight/volume) carboxymethyl cellulose (CMC) was added to the liquid suspension. The suspension served as a 1× dilution for further dilution-series. A 1:30 dilution with CMC was used; 50 mL aliquots of the serially diluted mixture were pipetted into the mutliwell plates for incubation (in daylight, at 20°C) (Unterseher & Schnittler, 2009). Unterseher and Schnittler (2009) attempted to enhance the growth of endophytic fungi by: (1) constantly inverting the multiwelled plates to guarantee an equal dispersion of particles on the nutrient agar, (2) allowing evaporation of excess water from the wells by exposing them to laminar flow for 30-60 minutes before sealing the lid, and (3) covering the plates with transparent plastic bags to ensure sufficient moisture and prevent drying from the peripheral walls.

When developing the dilution-series method for the present study, the methods proposed by Unterseher and Schnittler (2009), along with Paulus et al. (2003) and Collado et al. (2007), were used as a guide in determining how to homogenize the *A. balsamea* needles. In their study, *F. sylvatica*, a board leaf was homogenized, as opposed to a needle; thus, developing a method that effectively homogenized a needle was needed.

Preliminary methods involved using a blender (similar to Unteseher and Schnittler), roughly 10-15 needles, and 2 % malt broth. Although successful in homogenizing the plant needles, use of the blender proved more efficient for large sample sizes (i.e., numerous leaves homogenized at once), as in Unterseher & Schnittler's (2009) study. In addition, use of the blender presented problems with regards to ensuring sterility of the equipment, and would not allow for the proper assessment of individual needle species richness; thus, creating a problem when comparing between the two techniques: leaf segment cultivation and dilution-series. Ultimately, a handheld homogenizer (VWR International® VD-12 Homogenizer) was used for the dilution-series method in the present study. Using a handheld homogenizer, allowed needles to be homogenized individually and ensured the ability to compare the effectiveness of the two methods, allowing the accurate assessment of endophytic species diversity.

Two differences from the Unteseher and Schnittler protocol included: (1) skipping use of the centrifuge and collecting sieves, and (2) cultivation on Petri dishes, as opposed to multiwelled plates. Use of the handheld homogenizer effectively homogenized the needle, leaving few solid particles in the suspension, thus omitting the use of a centrifuge and collecting sieves presented no issue with regards to methodology. Unteseher and Schnittler (2009) cultivated on multiwelled plates to reduce interspecific interactions between differing endophytic species, and to increase the possibility of detection of slow growing, weak competitors, while, at the same time, allowing the isolation of ubiquitous and dominant endophytic species. competitive interactions. In the present study, the goal was to compare species richness of the two methods. Cultivating in Petri dishes would allow the comparison to be completed.

As noted above, *A. balsamea* is a flat, needle-like plant tissue that is waxy and tough, and the present study assessed the species diversity isolated by both methods. Surface sterilization is

a technique commonly used before isolating endophytes (Hallmann et al., 2006). Theoretically, the sterilizing agent should kill any microbe on the plant surface without affecting the plant host. It is important to find the correct balance between over-sterilization and under-sterilization to ensure that the disinfectants were strong enough to completely disinfect the surface, yet not too strong so that the plant tissue was not penetrated resulting in the death of endophytes inhabiting the plant (Kjer et al., 2010). At times this can make sterilization difficult because conditions required to kill the microbes on the surface may be lethal for endophytic microorganisms (Hallmann et al., 2006), but without sterilization of the plant tissue surface, there is chance that some epiphytes remain on the surface of the needle and in turn grow on the 2% malt extract agar, skewing results (Ginn, 1998). Epiphytes that grow on the agar, may present difficulty in regards to interpreting results related to endophyte yield because these epiphytes may be mistakenly identified as endophytes and/or compete with endophytic growth (Ginn, 1998). Therefore, in order to be effective, the surface sterilization technique must be adapted to the respective tissues, and each disinfectant used should be sensitive enough to recover endophytic microorganisms, but at the same time be strong enough to eliminate epiphytes from the plant surface (Hallmann et al., 2006).

In the case of sterilization of *A. balsamea*, the surface sterilization process followed the Johnson and Whitney (1989) protocol. The disinfectants used were 6% sodium hypochlorite (bleach) and 70% ethanol, both of which are commonly used sterilizing agents (Schulz et al., 1993; Dong et al., 1994; Sieber, 2002). This surface sterilization protocol followed a three-step procedure in which ethanol is first used, followed by sodium hypochlorite, and then ethanol again (Schulz et al., 1993; Bills 1996; Sieber, 2002).

The isolation method is a critical step when working with endophytic fungi. The most commonly used isolation procedures are either maceration of the plant tissues and streaking on the nutrient agar (i.e., dilution-series method), or plating small sterilized segments of plant tissue onto nutrient agar (i.e., leaf segment cultivation) (Schulz et al., 1993; Guo et al., 2000; Paulus et al., 2003; Hallmann et al., 2006; Collado et al., 2007; Hyde & Soytong, 2008). Leaf segment cultivation and dilution-series method each pose strengthens and weakness (Unteseher & Schnittler, 2009; Schulz et al., 1993; Hallmann et al., 2006; Paulus et al., 2003; Collado et al., 2007), and the decision as to which isolation procedure is best for a given host depends on a multitude of factors that are plant-dependent (i.e., species, age, and tissue), as well as the available resources and total number of samples (Hallmann et al., 2006).

Leaf segment cultivation is most commonly used for isolating endophytic fungi (Schulz et al. 1993; Hallmann & Sikora 1994; Bills 1996). One major limitation of this method is that it is biased towards fast-growing, ubiquitous endophyte species (Unterseher & Schnittler, 2009). The competitive interactions of endophytic fungi subsequently limit the number of isolates growing on the nutrient agar, and may affect the amount of slow growing endophytic species represented (Unterseher & Schnittler, 2009). Thus, the main disadvantage of the leaf segment cultvaiton method is an overall decrease in endophytic diversity that is able to be isolated (Hyde and Soytong, 2008; Huang et al., 2008). However, the advantages of the leaf segment cultivation method have proven effective, as this method continues to be used for the isolation of endophytic fungi (Schulz et al., 1993). Leaf segment cultivation is fast, reliable, inexpensive, and works for a variety of plant tissues (i.e., broad leafed plants, needles, roots, etc.) (Schulz et al., 1993; Guo et al., 2000).

Dilution-series cultivation is the most commonly used method for the isolation of

endophytic bacteria (Paulus et al., 2003). More recently, the method has been modified for use with endophytic fungi (Paulus et al., 2003; Collado et al., 2007). The modified protocol is said to improve the isolation of slow-growing, less-competitive endophytic species because the fungal hyphae of the endophytes are directly cultivated on the growth medium. In the past, the maceration of the surface-sterilized tissue has been performed with: (1) a mortar and pestle (Mahaffee & Kloepper, 1997), (2) a homogenizer (Blankenship et al., 2001; Asis & Adachi, 2004), or (3) a blender (Paulus et al., 2003; Collado et al., 2007). The choice of which method to use was dependent on numerous factors: cell physiology, cell doubling time, and the hardness of the plant material. Few limitations and disadvantages of the dilution-series method have been proposed by Hallmann et al (2006). Sterility must be guaranteed during all steps of the maceration process to ensure there is no contamination of the macerated suspension (Hallmann et al., 2006). Another disadvantage that may reduce the number of isolates being recovered is the plant enzymes and toxins that can be released during the homogenizing step; these toxins may then interfere with the endophyte growth by inactivating or killing the microorganism (Hallmann et al., 2006). Release of the harmful plant enzymes and toxins can be mitigated by ensuring a cool temperature (i.e., homogenizing on ice), and quick dilution and streaking on nutrient agar (Hallmann et al., 2006). Overall, the dilution-series method is more labor intensive and time-consuming than the leaf segment cultivation method (Paulus et al., 2003; Collado et al., 2007).

Varying factors such as pH, temperature, aeration, and percent of water availability (moisture) are important considerations when selecting media (Hallmann et al., 2006). It is essential that moisture (available water) is present for growth, and in regards to the dilution-series method, no filamentous endophytic growth was present; only yeast was seen. The lack of moisture may have been due to media absorbing the available water, thus, hindering the fungal

hyphae in diluted suspension of macerated plant tissue from growing on the nutrient agar.

Of the 21 isolates obtained from both methods (13 from the leaf segment cultivation method and 8 from the dilution-series method), endophytic fungal and yeast growth was seen. In terms of dichotomy, the methods were successful in that, none of the endophytic species isolated by the leaf segment cultivation were yeast; instead, the isolating via that method was exclusively fungal. The isolation of endophytes via the dilution-series method was exclusively yeast; no endophytic fungi were isolated with that method. The dichotomy between the two methods of isolation is useful, especially considering the growth associated with each method was unique when compared to the other method. Overall, the big difference between the two methods: leaf segment cultivation and dilution-series cultivation, was the exclusive isolation of only fungi and yeast, respectively.

The leaf segment method showed a predominance (10 out of the 13 isolates) of endophytic growth from the middle segments of the sterilized *A. balsamea* needles, as opposed to the petiole or tip end of the needle (Table 1). All the endophytic growth was filamentous fungi, which is typically what has been isolated from use of the protocol of leaf segment cultivation (Hyde & Soytong, 2008). The dilution-series method isolated single-celled yeast. Before adapting the dilution-series protocol for use isolating endophytic fungi, the technique was used for extinction culturing of bacteria, another single celled microorganism (Paulus et al., 2003). Given that no yeast is typically isolated from the leaf segment cultivation, the difference between these two methods could be the diversity of microorganisms that the leaf segment cultivation and dilution-series cultivation isolate.

5. Conclusion

More research on the effectiveness of both methods is needed before a shift in methodology can be advocated. Intensive testing into the efficiency of the dilution-series method should be completed to conclusively determine which method has more benefits when it comes to isolating endophytes, and to determine under what conditions are best when using the leaf segment cultivation and/or dilution-series method. Future research related to this study may include enhancing the methodology of the dilution-series method to influence the initial isolation of endophytes from their host. Also, investigation into the effectiveness of the two isolation methods on different plant tissue types is warranted.

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