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Isolation and Identification of Fungal Endophytes from Grasses along the Oregon Coast

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Abstract

Fungal endophytes have been shown to improve abiotic and biotic stress response in plants. Grasses growing along the Oregon coast are exposed to harsh conditions and may harbor endophytes that enable them to survive and grow under these conditions. Endophytic fungi were isolated from thirty-four grass plants representing eight different grass species at four different locations along the Oregon coast. The ITS-1, 5.8S, and ITS-2 regions of each isolate were amplified, sequenced, and used to perform a BLAST search against the nucleotide database collection at National Center for Biotechnology Information. One-hundred-eleven different fungal isolates were classified into thirty-nine genera with two isolates that did not show a match greater than 95%. These endophytes will be investigated to determine their potential for improving the adaptability of grasses and other crop plants to grow in diverse environments where they are subjected to multiple biotic and abiotic stresses.

Keywords

Fungi, Endophytes, Abiotic Stress, Grass, Biotic Stress, Salt Stress

1. Introduction

It is estimated that the human population will reach nine billion by 2050. This population increase combined with climate change will require an increase in food production under less than optimal conditions. In the mid-late 20th century, the "Green Revolution" was the result of breeding efforts aimed at improved crop cultivars, the introduction of hybrids, and increased agricultural inputs in terms of fertilizer, pesticides, water, herbicides

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and crop management practices. We are now facing a similar global food security challenge that will require continued improvements in crop yields and agricultural production practices. Climate change and the expansion of agricultural production to marginal lands will also require innovative ways to increase abiotic and biotic stress tolerance and improve nutrient uptake efficiency in crop plants to meet future global food demands.

Many plants contain endophytic organisms that Wilson (1995) defined as "fungi or bacteria which, for all or part of their life cycle, invade the tissues of living plants and cause unapparent and asymptomatic infections entirely within plant tissues but cause no symptoms of disease" [1]. Fungal endophytes are known to produce antibacterial substances [2] and have been shown to improve the tolerance of host plants to a variety of biotic and abiotic stresses [3]-[5]. The presence of fungal endophytes has been shown to increase the survival and persistence of their host plants in a diverse range of environments and may also protect them from insects, pathogens, and herbivores. *Neoptyphodium* sp., members of Clavicipitaceae group of fungi, infects grasses and has been extensively studied because of their significant impact on agriculture. This fungal species produces alkaloids which are toxic to livestock and can limit the utility of these grasses in forage and pasture applications [6]-[9]. However, the alkaloids and other metabolites produced by these endophytes [10]-[15] also provide benefits to their host by increasing resistance to insects [16] [17], grubs [18] and nematodes [19] [20], enhancing nutrient uptake [4] and increasing drought tolerance [21]. In addition to metabolites, the symbiotic relationship results in the increased production of reactive oxygen species (ROS), which may be critical to maintaining the mutualistic fungal/plant interaction [22]. This increase in ROS is thought to provide oxidative stress protection by stimulating the host's antioxidant pathways [22]-[24] that facilitate enhanced biotic and abiotic stress tolerance for the host plant. In addition to these pathways, there are likely many other unidentified mechanisms associated with the symbiosis that is beneficial to the plant's and endophyte's survival.

Although less well studied, another group of endophytes that also have been shown to provide enhanced abiotic and biotic stress tolerance to their hosts, is the nonclavicipitaceous fungal endophytes of the subkingdom *Dikarya* [25]. This group has a greater diversity of species that are able to colonize multiple plant tissues in a wider range of host plants than their *Neotyphodium* counterparts. In some cases, the benefit to both symbionts is obvious, as neither the host nor the endophyte is able to survive individually under stress conditions that they can survive when in symbiosis with each other [26]. The endophyte *Fusarium culmorum*, isolated from dunegrass growing in coastal habitats, has been shown to be necessary for salt tolerance of this plant [25]. Furthermore, when this endophyte was inoculated into tomato and rice, it imparted salt tolerance to these plants [25] [27]. The fungal endophyte *Curvularia protuberate* (and its associated virus), which were isolated from *Dichanthelium lanuginosum* (panic grass) growing near geothermal regions in Lassen Volcanic and Yellowstone National Parks [26], was able to confer heat tolerance when inoculated into panic grass and tomato [25]. Interestingly, endophytes from both locations were able to confer drought tolerance to infected plants. The increased tolerance of these plants correlated with lower levels of ROS in leaves of the symbionts when exposed to stress, possibly due to endophytes scavenging ROS or altering the plant's production or scavenging of ROS. The authors coined the term "habitat-adapted symbiosis" to describe this ability of fungi isolated from plants in a specific habitat to confer habitat-specific stress tolerance to those plants [25].

Another endophytic fungus currently being explored for agricultural potential, *Piriformospora indica*, was first isolated from xerophytes growing in a desert in India [28]. It was later shown to promote growth and increase biomass in a wide range of plants [29]-[31], induce resistance against pathogens in barley, wheat, *Arabidopsis* and tomato [30] [32] [33], increase abiotic stress tolerance of plants [34] [35], and reduce egg density of the parasitic soybean cyst nematode [36]. Interestingly, changes in plant hormone homeostasis were suggested to be important for this fungal endophyte to colonize different plant species [37] [38]. Furthermore, it was shown that enzymes involved in the antioxidant pathway were critical for increased biotic and abiotic stress resistance in the presence of *P. indica* [31] [32] [35] [39]-[42]. This endophyte shows great potential for increasing abiotic and biotic stress tolerance and increasing biomass and seed production in multiple plant species (for review see [43]).

Many of the endophytes described above have been identified in plants that are exposed to abiotic and biotic stresses. Grasses growing along the Oregon coast are exposed to salinity stress as well as other abiotic and biotic stresses. In order to survive in this demanding environment, these grasses may contain a unique population of fungal endophytes. The long-term goal of this study is to isolate fungal endophytes associated with these grasses that have the potential to enhance plant growth/biomass and/or impart abiotic and biotic stress tolerance to forage, turf, and energy-related grasses and/or other plant species. This paper describes the initial isolation and

identification of fungal endophytes from various grasses growing in sandy soils and exposed to ocean spray and mist along the Oregon coast.

2. Materials and Methods

2.1. Sample Collection and Fungal Isolation

Samples (root crown, leaves, and stems) were collected from grasses growing in areas exposed to ocean spray, mists and tides along the Oregon coast. Grasses were collected from sites near Harbor Vista (Lat/Long 44.021629 - 124.133127), Coos Bay (Lat/Long 43.366501 - 124.217888), Bob Creek Wayside (Lat/Long 44.244493 - 124.111582), and Yachats (Lat/Long 44.3105 - 124.103976) (**Table 1**). Grass species that are present in the Willamette Valley were preferentially selected. At each site, attempts were made to collect different grass species, and within each species, 1-3 individuals were collected from different locations within each site.

Samples were stored in plastic bags in a cooler with ice during collection and refrigerated until processing for endophyte isolation. All samples were processed within 48 h of collection. Samples were rinsed with water to remove soil and debris, swirled in a beaker containing distilled water and two drops of Tween 20/100 ml, and rinsed again prior to cutting the tissue. Any dead plant tissue and most of the roots were removed from the plant, and the remaining plant was dissected into tissues corresponding to the root crown (1 - 1.5 cm), leaves (4 - 6 cm in length), and stems (4 - 6 cm in length) prior to surface sterilization. After plant tissues were visibly clean, the tissues were rinsed in tap deionized water and then placed between damp paper towels to prevent them from drying out until tissues were sterilized prior to plating for fungal isolation. Stems and root crowns were surface sterilized by placing the tissue in 90% ethanol for 1 min, 3% sodium hypochlorite (from bleach) with 2 drops of Tween-20/100 ml for 3 min, sterile double distilled water (DDW) for 1 min, 70% ethanol for 1 min, and a quick

Table 1. Plant ID # and identification of grasses collected at designated locations.

Location	Plant ID	Species	Location	Plant ID	Species
Coos Bay	CB2	*Bromus*	Bob Creek Wayside	BS1	*Lolium*
	CB3	*Ammophilia*		BS2	*Bromus*
	CB4	*Festuca*		BS3	*Bromus*
	CB5	*Hordeum*		BS4	*Phalaris*
Harbor Vista	HV1	*Phalaris*		BS5	*Festuca*
	HV2	*Phalaris*		BS6	*Bromus*
	HV3	*Festuca*		BS7	*Festuca*
	HV4	*Festuca*		BS8	*Festuca*
	HV5	*Bromus*		BS9	*Phalaris*
	HV6	*Festuca*			
	HV7	*Ammophila*	Yachats	YH1	*Phalaris*
	HV8	*Bromus*		YH2	*Bromus*
	HV9	*Festuca*		YH3	*Bromus*
	HV10	*Agrostis*		YH4	*Descampsia*
	HV11	*Bromus*		YH5	*Agrostis*
	HV12	*Bromus*		YH6	*Phalaris*
	HV13	*Festuca*		YH7	*Agrostis*
	HV14	*Festuca*			

rinse in sterile DDW. Leaf tissue was sterilized by placing leaves in 70% ethanol for 2 min, 2% sodium hypochlorite (from bleach) for 3 min, sterile DDW for 1 min, followed by a quick dip in 90% ethanol. After sterilization, the end (~2 - 3 mm) of stem, leaf or root crown was cut off and discarded. The remaining sample was cut into 2 - 3 mm sections and divided between two plates containing either Bacto™ Potato Dextrose Agar (PDA) or BBL™ Corn Meal Agar with added Malt (1 g/L) and Yeast Extract (2 g/L) (CMMY) (Becton, Dickinson & Co; Sparks, MD) containing 50 mg/L of carbenicillin and streptomycin. Plates were incubated at room temperature and examined for emerging fungi every 2 - 3 days. As fungi emerged, they were transferred to PDA plates to obtain pure cultures. Prior to initial plating, several samples were imprinted onto media and these imprinted plates were monitored for lack of fungal growth to ensure the effectiveness of the sterilization technique [44].

2.2. DNA Extraction and ITS Sequencing

DNA was extracted from pure cultures following the simple miniprep method of Saitoh *et al.* [45]. A piece of mycelia about the size of a half dime was removed from the plate and placed into 500 µl of Lysis Buffer (200 mM Tris, 50 mM EDTA, 200 mM NaCl, 1% N-lauroylsarcosine-Na salt, pH 8.0). The tissue was lightly homogenized using disposable pestles. Samples were allowed to sit for approximately an hour at room temperature to allow for processing of multiple samples at a time. Samples were centrifuged for 5 min at max speed in an Eppendorf 5417C centrifuge. Approximately 300 µl of supernatant was removed to a new tube containing 750 µl of 100% ethanol. Samples were vortexed gently and stored at −20˚C overnight. Samples were centrifuged for 5 min at 13,000 ×g, the supernatant decanted, and the pellet was washed with 70% ethanol. The pellet was suspended in 100 µl of TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0).

The rDNA ITS region was amplified by PCR with primers ITS5 (GGAAGTAAAAGTCGTAACAAGG) and ITS4 (TCCTCCGCTTATTGATATGC) [46]. The amplicons include the ITS1, 5.8S and ITS2 region of rDNA and most amplicons were ~500-650 base pairs in length. The DNA was amplified using PrimeSTAR™ HS DNA Polymerase (Takara Clonetech, Madison, WI) following the manufacturer's instructions. The PCR reaction mix consisted of 1× PrimeSTAR buffer, 0.2 mM each dNTP's, 0.3 µM primers, ~20 ng of DNA, and 1.25 U PrimeSTAR HS DNA Polymerase in a final volume of 50 µl. Amplification was performed on an MJ Research PTC 200 or a Bio-Rad DNA Engine Peltier thermal cycler (BioRad; Hercules, CA) with the following program: 96˚C for 4 min; 35 cycles of 98˚C for 10 s, 57˚C for 5 s, 72˚C for 45 s; and a final 10 min extension at 72˚C; and then kept at 4˚C until removal. The PCR products (8 µl) were run on a 1.5% TAE agarose gel to analyze purity. Products with a single band were purified with the AccuPrep® PCR Purification kit (Bioneer, CA); and products with multiple bands were run on a 1.5% TAE agarose gel and purified using the Accuprep® Gel Purification Kit (Bioneer, CA). PCR products were sequenced on an ABI Prism® 3730 Genetic Analyzer at the Center for Genome Research and Biocomputing at Oregon State University or at Genscript USA Inc. (Piscataway, NJ). Sequences were submitted to a Targeted (Internal transcribed spacer region [ITS] from fungi type and reference material) Nucleotide Basic Local Alignment Search Tool (BLAST) Megablast (Optimize for highly similar sequences) and to a standard nucleotide nr/nt database BLAST against the nt collection at National Center for Biotechnology Information (NCBI, GenBank; www.ncbi.nih.gov) [47] [48].

2.3. Testing for Antibacterial Activity of Select Samples

A representative sample from each clade of the phylogenetic tree from the Megablast search results was tested for antibiotic production. Fungi were grown on plates without any antibiotics for 7 - 14 days. Bacteria (*Frigoribacterium* [*Frig*] and *Bacillus*, also collected from coastal grasses) were grown overnight in LB media, diluted 1:20 in LB, and then spread as a lawn on LB/PDA agar plates (500 mls; 2.5 g peptone, 1.25 g yeast extract, 2.5 g NaCl, 3.75 g agar, pH 6.5). A cork borer was used to remove 3 plugs (~7 mm in diameter) from each plate. A comparable sized plug of the fungus to be tested for antibiotic activity was placed into two of the bacterial plate holes and a negative media plug (no fungus) was placed in the third hole. Bacteria were allowed to grow for 24 - 48 h and then the plate was examined for the presence of zones of bacterial growth inhibition next to the fungal plug.

3. Results and Discussion

Thirty-four different plant samples were collected from various sites along the Oregon coast; four from Coos

Bay, 14 from Harbor Vista, nine from Bob Creek Wayside, and seven from Yachats (**Table 1**). Only plants that were exposed to ocean spray, mist and tidal water were collected for this experiment. Each plant was divided into root crown, stem and leaf; surface sterilized, and then cut into ~0.5 cm pieces and cultured on PDA and CMMY agar plates. Lack of fungal growth on tissue imprint plates indicated that the sterilization technique was sufficient to remove epiphytic fungi. It is possible that the sterilization was too harsh, and that more isolates could have been obtained with a less harsh method of sterilization. A total of 140 fungi were isolated from 34 different plants. After duplicate isolates (based on the ITS sequence, plant isolate number and culture morphology) from each individual plant segment (same fungi on the different media and from leaf, stem or root crown of the same plant) were eliminated, there were 111 different isolates remaining. Further elimination of identical isolates from within the same plant (same isolate found in either the root crown, stem and/or leaf) resulted in 107 distinct isolates from all plant samples at all locations. Based on the ITS sequence and culture morphology, the fungal isolates were classified into thirty-nine different genera, and two additional isolates designated as "unknown fungi" with sequences that did not have any BLAST hits with greater than 95% identity.

The distribution of the fungal isolates at different locations is listed in **Table 2**. Some isolates are listed under a broader classification rather than a specific genera (*Ascomycete* sp., *Hypocreales* sp., *Mucorales* sp.) and in these cases the top BLAST hits were not associated with a genus, but were simply classified as belonging to the indicated group. Some species, such as *Fusarium* and *Ascomycota* sp., were found at all locations, while many genera were only isolated from plants at one of the locations (see **Table 2**, gray highlighted samples). Overall, two to three fungi were isolated from each plant at Bob Creek Wayside and Harbor Vista. Interestingly, while fewer plants were collected at Coos Bay, the plants that were collected averaged more fungal isolates per plant (~5.5) (**Table 2**) than plants from other areas (~2.5 - 4.1 isolates/plant). The Coos Bay site was more remote and there were no cultivated grasses growing in close proximity to the collection site. This was not the case at Bob Creek Wayside and Yachats, and in these areas some samples could have been escapes from cultivated grasses. This could potentially affect the types of endophytes and grasses isolated from each area.

The distribution of fungal isolates in the different grass species is presented in **Table 3**. While only one *Deschampsia* specimen was collected, this plant had the greatest number of fungal isolates associated with it. *Hordeum* and *Amophilia* also had slightly greater than average number of isolates, while only one fungal isolate was isolated from *Lolium*. Fungal isolates from both the Clavicipitaceous and the nonclavicipitaceous groups were isolated from these grass species (**Table 3**). Interestingly, clavicipitaceous fungi were isolated from *Festuca* sp. at Harbor Vista (Cf. *Acremonium* sp. and *Neotyphodium* sp.) and at Bob Creek Wayside (Epichloe sp.), but were not found in other grass species or at the other locations. There were 18 additional fungal isolates from the order *Hypocreales*, including multiple isolates most closely aligned to *Fusarium*, *Sarocladium*, and *Septoriella* from several grass species (**Table 3**) and individual isolates most closely related to *Beauveria* (from *Amophilia* sp.), *Chaunopycnis* (from *Hordeum* sp.), *Isaria* (from *Bromus*), and *Trichoderma* (from *Amophilia*). There were 32 different isolates from the order *Pleosporales* with eight isolates of *Phaeosphaeria* and seven of *Stemphylium*, five of *Alternaria*, three of *Phoma* and one or two of *Drechslera*, *Embellisia*, *Paraphaeosphaeria*, *Pleospora* and *Saccharicola*. Representatives of the *Dothideales* order were found at all locations and included two *Aureobasidium* sp. and five *Pseudoseptoria* isolates. A few fungi closely related to members of the order *Heliotales* (*Helgardia* and *Articulospora*) and *Mucorales* (*Umbelopsis* and *Mucorales* sp.) were also isolated from *Festuca*. Overall, fungal isolates collected were placed into five different orders based on their ITS sequences. A complete list of all unique isolates and the corresponding accession number for the top hit used to identify each isolate from the NCBI BLAST results can be found in **Table 4**.

3.1. Antibacterial Activity of Select Samples

Fungal endophytes are known to produce many secondary metabolites, some with antimicrobial activity (reviewed in [49]). The ITS sequence from isolates in this study were subjected to a targeted MOLE-BLAST search against "Internal transcribed spacer region [ITS] from fungi type and reference material" and the isolates were separated into 19 different loci (data not shown). At least one representative fungi from each loci was chosen to examine its potential for inhibiting bacterial growth of two bacteria, *Frig* and *Bacillus* sp., which were also isolated from grass species from this study. Preliminary results using fungal plug inhibition of bacterial growth revealed inhibition by representatives of *Penicillium* sp. (isolate from Coos Bay plant 2 and Harbor Vista plant 11), *Phlebia* sp. (from Bob Creek Wayside Plant 3), *Heterobasidium* sp. (from Harbor Vista plant 11),

Table 2. Fungal isolates (based on BLAST results) collected at each site. Isolates present at only one site are gray shaded. Only isolates that had BLAST sequence targets that were greater than 97% identical to test sequences are presented.

	Bob Creek Wayside	Coos Bay	Harbor Vista	Yachats
Alternaria sp.	2	1	2	
Articulospora	1			
Ascomycota sp.	2	5	3	3
Aureobasidium sp.		1		1
Beauveria sp.		1		
Cf. Acremonium sp.			1	
Chaetomium sp.				1
Chaunopycnis sp.		1		
Cladosporium sp.	1	1		6
Diaporthe sp.			2	
Drechslera		2		
Embellisia sp.		1		
Epichloe	1			
Exophiala sp.			1	
Fusarium sp.	1	1	2	1
Helgardia			2	
Helotiales				1
Heterobasidion sp.			1	
Homobasidiomycete	1			
Hypocreales				2
Isaria	1			
Microdochium sp.	1	2		1
Mucorales sp.			1	
Neotyphodium sp.			1	
Oidiodendron sp.	1	1		
Paraphaeosphaeria sp.			1	1
Penicillium sp.		1	1	2
Phaeosphaeria sp.		2	5	2
Phlebia sp.	1			
Phoma sp.			1	2
Pleospora sp.			1	
Pleosporales sp.				1
Pseudoseptoria sp.	1		3	1
Saccharicola sp.			1	
Sarocladium sp.	2	1		1
Septoriella sp.	1		2	
Stemphylium sp.	5			3
Trichoderma sp.		1		
Umbelopsis sp.			1	
Isolates with <95% ID			2	
# diff. fungi genera/site	22	22	34	29
# plants collected/site	9.0	4.0	14	7
# fungi/plant/site	2.4	5.5	2.4	4.1
Overall: Avg number of fungi/plant 3.14				

Table 3. Number of fungi of designated genera isolated and identified by ITS sequencing from each species at all locations. Only isolates that had BLAST sequence targets that were greater than 97% identical to test sequences are presented. The last column represents the order to which the fungi belongs.

Fungi ↓ Grass*→	Br	Am	Fe	Ho	Ph	Ag	Lo	De	Total	Order
Alternaria sp.	3		1		1				5	*Pleosporales*
Articulospora			1						1	*Helotiales*
Ascomycota sp.	7	2	1					3	13	
Aureobasidium sp.	2								2	*Dothideales*
Beauveria sp.		1							1	*Hypocreales*
Cf. Acremonium sp.			1						1	*Hypocreales*
Chaetomium sp.					1				1	*Sordariales*
Chaunopycnis sp.				1					1	*Hypocreales*
Cladosporium sp.	1		1	1	1	2		2	8	*Capnodiales*
Diaporthe sp.	1		1						2	*Diaporthales*
Drechslera	1	1							2	*Pleosporales*
Embellisia sp.				1					1	*Pleosporales*
Epichloe			1						1	*Hypocreales*
Exophiala sp.			1						1	*Chaetothyriales*
Fusarium sp.	1	1	2					1	5	*Hypocreales*
Helgardia			2						2	*Helotiales*
Helotiales								1	1	*Helotiales*
Heterobasidion sp.	1								1	*Russulales*
Homobasidiomycete	1								1	
Hypocreales					2				2	*Hypocreales*
Isaria sp.	1								1	*Hypocreales*
Microdochium sp.	1			1	2				4	*Xylariales*
Mucorales sp.			1						1	*Mucorales*
Neotyphodium sp.			1						1	*Hypocreales*
Oidiodendron sp.	1	1							2	
Paraphaeosphaeria sp.			1		1				2	*Pleosporales*
Penicillium sp.	2				1	1			4	*Eurotiales*
Phaeosphaeria sp.	1	1	3		1	2		1	8	*Pleosporales*
Phlebia sp.	1								1	*Polyporales*
Phoma sp.	1				1			1	3	*Pleosporales*
Plectosphaerella sp.			1						1	
Pleospora sp.					1				1	*Pleosporales*
Pleosporales sp.	1								1	*Pleosporales*
Pseudoseptoria sp.	1		3					1	5	*Dothideales*
Saccharicola sp.	1								1	*Pleosporales*
Sarocladium sp.		1	1		2				4	*Hypocreales*
Septoriella sp.			3						3	*Hypocreales*
Stemphylium sp.	2		2		2	1	1		8	*Pleosporales*
Trichoderma sp.		1							1	*Hypocreales*
Umbelopsis sp.			1						1	*Mucorales*
Isolates with <95% ID			1			1			2	
SUMMARY	**Br**	**Am**	**Fe**	**Ho**	**Ph**	**Ag**	**Lo**	**De**	**Total**	
# of fungi/species	31	9	30	4	14	7	1	9	107	
# of plants	10	2	10	1	6	3	1	1	34	
AVG # of fungi/ Ind. sp.	3.1	4.5	3	4	2.3	2.3	1	9	3.15	

*Abbreviations: Br (*Bromus*), Am (*Amophilia*), Fe (*Festuca*), Ho (*Hordeum*), Ph (*Phalaris*), Ag (*Agrostis*), Lo (*Lolium*), De (*Descampsia*).

Table 4. List of unique isolates from each plant at each site, and identification of the most closely related species identified using the ITS sequence to perform an nr/nt BLAST search at the National Center for Biotechnology Information. Isolate designation refers to location (BS, CB, HV, YH); plant number (1-14); tissue type (L, SS, RC); and the final letter/number refers to different isolates from the specific sample. BS (Bob Creek Wayside), CB (Coos Bay), HV (Harbor Vista), YH (Yachats), L (leaf), RC (Root Crown), SS (stem).

Isolate	Species	Accesion #	% Coverage	% ID
CB4RCSSD	*Alternaria infectoria*	HG324079.1	100	100
HV8SSA	*Alternaria infectoria* str CNRMA10.1102	KP131537.1	99	100
HV8SSB	*Alternaria infectoria* str CNRMA10.143	KP131538.1	99	100
BS9SSA	*Alternaria infectoria* str CNRMA10.143	KP131538.1	99	100
BS2SSA	*Alternaria* sp. GYI-051221	FJ627005.1	99	100
BS7RCB	*Articulospora proliferata* str CCM F-11200	KP234351.1	100	97
CB3AL2	*Ascomycete* sp. DGC-2	AY230245.1	100	98
BS2RCB	*Ascomycota* sp. UNEX FECRGA 2012E081	KP698333.1	100	100
BS3LA	*Ascomycota* sp. UNEX FECRGA 2012E081	KP698333.1	100	100
HV8SSC	*Ascomycota* sp. UNEX FECRGA 2012E081	KP698333.1	100	99
CB4RCSSB	*Ascomycota* sp. UNEX FECRGA 2012E081	KP698333.1	100	100
YH4SSD	*Ascomycota* sp. UNEX FECRGA 2012E143	KP899390.1	100	99
HV11LA,RCF	*Ascomycota* sp. UNEX FECRGA 2012E217	KP899440.1	100	100
HV12RCA,SS	*Ascomycota* sp. UNEX FECRGA 2012E217	KP899440.1	100	100
CB3BRCB	*Ascomycota* sp. UNEX FECRGA 2012E497	KP899421.1	100	99
YH4RCC1	*Ascomycota* sp. *UNEX FECRGA 2012E547*	KP899402.1	100	99
CB2A-LD	*Ascomycota* sp. *UNEX FECRGA 2012E651*	KP698369.1	100	99
YH4RCB	*Ascomycota* sp. *UNEX FECRGA 2012E651*	KP698369.1	100	99
CB2ALC	*Ascomycota* sp. UNEX FECRGA 2012E497	KP899421.1	100	99
CB2ARCB	*Aureobasidium pullulans* isolate 24-3	KP783506.1	100	100
YH2RCD	*Aureobasidium* sp. 3 BRO-2013	KF367567.1	100	99
CB3ARCC	*Beauveria bassiana* str WM 09.202	KP131647.1	99	99
HV14RCE	Cf. *Acremonium* sp. SS-1583	AM262388.1	79	97
YH6SSC	*Chaetomium* sp. CGMCC 3.9441	JN209925.1	100	99
CB5-SSB	*Chaunopycnis* sp. ANT 03-065	DQ402530.2	100	100
YH4RCA	*Cladosporium cladosporioides*	AB975285.1	100	100
YH6RCB	*Cladosporium ramotenellum*	LN834387.1	100	100
YH7LA	*Cladosporium ramotenellum*	LN834387.1	100	99
YH2LA	*Cladosporium* sp. 4 SDM-2014	LN834427.1	100	100
YH4SSA	*Cladosporium* sp. 4 SDM-2014	LN834427.1	100	100
CB5SSA	*Cladosporium* sp. 5 SDM-2014	LN834419.1	100	100
YH7LC	*Cladosporium* sp. 5 SDM-2014	LN834419.1	100	100
BS7LA	*Cladosporium* sp. 5 SDM-2014	LN834419.1	100	100

Continued

HV5LA	*Diaporthe* cf. nobilis RG-2013	KC343153.1	100	99
HV3LA	*Diaporthe eres* strain UCCE1004B	KF017914.1	100	100
CB2BLC	*Drechslera dematioidea* str CBS 108962	JN712465.1	100	99
CB3BRCC	*Drechslera dematioidea* str CBS 108962	JN712465.1	100	99
CB5RCB	*Embellisia* sp. 9151S6	JQ796753.1	100	99
BS5LB,RCD	*Epichloe festucae*	X62987.1	100	99
HV9SSB	*Exophiala pisciphila* isolate AFTOL-ID 669	DQ826739.1	100	100
HV4RCA	*Fusarium acuminatum* str RJFAWY137YT2E	KR051403.1	100	100
YH4SSC	*Fusarium avenaceum*	AB975293.1	100	100
BS8LE	*Fusarium avenaceum*	AB975293.1	100	100
CB2ARCC	*Fusarium culmorum* isolate MF18	KP292806.1	100	100
HV7RCA	*Fusarium pseudograminearum* str NRRL28062	DQ459871.1	100	99
HV14SSD	*Helgardia aestiva* isolate RAE22	AY266145.1	97	98
HV13LB	*Helgardia aestiva* isolate RAE22	AY266145.1	100	99
YH4RC3	*Helotiales* sp. CWG-F1-E3	JF690986.1	97	99
HV11RC	*Heterobasidion occidentale* isolate PFC 5364	KC492948.1	100	100
BS2L1c	*Homobasidiomycete* sp. WRCF-B9	AY618675.1	96	99
YH6SSE	*Hypocreales* D_D31	KC311472.1	98	98
YH6SSFR	*Hypocreales* sp. IBL 03161	DQ682584.1	98	99
BS2LA	*Isaria* sp. 07MA19	JX270419.1	100	100
CB2BLA, RCB	*Microdochium bolleyi*	AM502264.1	100	100
CB5SSFung	*Microdochium bolleyi*	AM502264.1	100	100
YH1RCD	*Microdochium nivale*	AM502260.1	100	100
BS9RCA	*Microdochium phragmitis*	AM502263.1	100	99
HV9RCB	*Mucorales* sp. DU13	KM113751.1	100	99
HV13RC	*Neotyphodium coenophialum* str CBS 494.82	DQ119115.1	100	100
CB3ARCA	*Oidiodendron* sp. 06VT08	JX270395.1	100	98
BS3SS3	*Oidiodendron* sp. 06VT08	JX270395.1	100	99
YH1RCB	*Paraphaeosphaeria neglecta* str CBS 627.94	JX496101.1	100	100
YH5RCA	*Penicillium janthinellum* str GYJ1(1)	KM268660.1	100	100
CB2ALA, RCA	*Penicillium murcianum* str CBS 161.81	KP016844.1	100	100
YH6RCE	*Penicillium* sp. *IFB-E022*	EF211128.1	97	97
HV11RCD	*Penicillium nothofagi* CBS 130383	NR_121518.1	100	100
HV14SSA	*Phaeosphaeria avenaria* str QLF50	FJ623271.1	100	97
CB3ARCA	*Phaeosphaeria pontiformis*	AJ496632.1	100	99
HV10SSA	*Phaeosphaeria* sp. I147	GU062238.1	69	97
YH4RCC2	*Phaeosphaeria* sp. I147	GU062238.1	67	97

Continued

HV1RCA	*Phaeosphaeria* sp. I147	GU062238.1	67	97
HV11RCC	*Phaeosphaeria* sp. JP-2013 str WA0000019138	JX981472.1	100	99
CB4RCSSA	*Phaeosphaeria* sp. S-93-48	EF452730.1	99	99
HV6RCB	*Phaeosphaeria* sp. S-93-48	EF452730.1	99	99
YH5RCB	*Phaeosphaeria vagans* str CBS 604.86	KF251193.1	99	99
BS3L1	*Phlebia uda* strain FP-101544-Sp	KP135361.1	100	99
YH3RCB	*Phoma* sp.	KF646102.1	100	99
YH4RC4	*Phoma* sp.	KF646102.1	100	99
HV2RCB	*Phoma* sp.	KF646102.1	100	99
HV9RCA	*Plectosphaerella cucumerina* str WM 07.196	KP068972.1	99	100
HV2LA	*Pleospora* sp. 286A	GQ120976.1	100	99
YH2RCA	*Pleosporales* sp. ICMP 17119	HM116749.1	100	100
BS8SSB	*Pseudoseptoria obscura* str CBS 135103	KF251219.1	97	99
HV11SSB	*Pseudoseptoria obscura* str CBS 135103	KF251219.1	89	99
HV9SSA	*Pseudoseptoria obscura* str CBS 135103	KF251219.1	97	99
YH4SSB	*Pseudoseptoria obscura* str CBS 135103	KF251219.1	97	99
HV14LA	*Pseudoseptoria obscura* str CBS 135103	KF251219.1	97	99
HV5LB	*Saccharicola bicolor* isolate wb557	AF455415.1	99	99
YH6SSB	*Sarocladium strictum*	AB975290.1	100	99
BS4SSA	*Sarocladium strictum*	AB975290.1	100	99
BS7SSB	*Sarocladium strictum*	AB975290.1	100	100
CB3BLA	*Sarocladium strictum*	AB975290.1	100	100
BS8LA	*Septoriella phragmitis* str CPC 24118	KR873251.1	99	99
HV13LA	*Septoriella phragmitis* str CPC 24118	KR873251.1	99	99
HV14LB	*Septoriella phragmitis* str CPC 24118	KR873251.1	99	99
YH1LD	*Stemphylium solani* str SS21	AF203448.1	100	99
BS1LA	*Stemphylium solani* str SS21	AF203448.1	100	99
BS3LB	*Stemphylium solani* str SS21	AF203448.1	100	99
BS5LA	*Stemphylium solani* str SS21	AF203448.1	100	99
BS7LB	*Stemphylium solani* str SS21	AF203448.1	100	99
YH7LB	*Stemphylium solani* str SS21	AF203448.1	100	99
BS2LB	*Stemphylium vesicarium* isolate CCTU237	JX424812.1	100	100
YH1LA	*Stemphylium vesicarium* isolate CCTU237	JX424812.1	100	99
CB3BRCD	*Trichoderma viridescens* str TRS35	KP009338.1	100	100
HV9RC1	*Umbelopsis ramanniana* str NRRL 5844	KM017730.1	99	99
HV10SSB	Unknown			
HV6RCA	Unknown			

Phaeosphaeria sp. (from Harbor Vista plant 14) and *Aureobasidium* sp. (from Yachats plant 2). *Penicillium* species were initially included in the study as a potential positive control for the assay, because it is the source of the common antibiotic penicillin, and was expected to be positive.

Another isolate obtained from *Festuca* at Harbor Vista was also able to inhibit growth of *Frig* and *Bacillus* and based on the ITS sequence, this isolate was most closely related to a species of *Phaeosphaeria*. Interestingly, antibacterial activity of phaeosphenone, a compound isolated from *Phaeosphaeria*, has been previously reported [50]. *Phlebia* sp., which was isolated from *Bromus* growing at Bob Creek Wayside, was also able to inhibit the growth of these bacteria. *Phlebia* species produce several merulinic acids which show antimicrobial activity to multiple bacterial species [51]. *Heterobasidion* sp. are probably most recognized as the causal agent of rot in forest trees, however *Heterobasidion* has also been isolated as an endophyte in chili pepper [52]. An isolate from *Bromus* sp. at Harbor Vista was identified as *Heterobasidion* sp. based on the IT sequence homology and was shown to inhibit bacterial growth of *Bacillus* and *Frig*. Antibiosis has previously been associated with secondary metabolites of *Heterobasidion annosum* [53]. Another fungus, *Exophiala pisciphila* (which was later identified as *Aureobasidium pullulans*) produces exophilin A, which has antimicrobial activity against gram positive bacteria [54]. More recent studies have focused on novel types of antibacterial liamocins from various strains of *Aureobasidium pullulans* grown on different types of growth media and their antibacterial activity [55]. A fungal species isolated from *Bromus* sp. in Yachats, which based on ITS sequence was most closely related to *Aureobasidium* sp., inhibited growth of *Bacillus* and *Frig* in our studies. Several other isolates were tested, but they showed little or no inhibition of bacterial growth. The antibiosis observed in selected isolates indicates that these endophytes may be a source for other valuable secondary metabolites.

3.2. Potential of Fungal Endophytes Collected in This Study

Fungi can form different types of associations with plants, some beneficial and some harmful. Parasitic, saprophytic and pathogenic fungi can be very deleterious to a plant, while endophytes and mycorrhizal fungi are considered beneficial symbionts which promote plant growth, confer enhanced resistance to various pathogens and pests, and improve survival under unfavorable environmental conditions [4] [5] [25] (reviewed in [56]). Several endophytes identified in this study have previously been shown to promote plant growth under normal and/or stressed conditions. In endophyte growth promoting studies in switchgrass, six different endophytes, including two genera, *Phaeosphaeria pontiformis* and *Alternaria* sp., isolated in our study, increased total biomass of switchgrass seedlings following inoculation. Several other endophytes, including some known crop pathogens isolated from asymptomatic switchgrass plants, decreased biomass when tested in greenhouse conditions [57]. Interestingly, in these switchgrass studies, some species of *Alternaria* promoted growth while others inhibited growth. Other endophytes isolated in this study belong to genera previously shown to promote plant growth including *Fusarium*, *Cladosporium*, *Phoma*, *Aspergillus* sp., *Chaetomium*, *Aureobasidium*, *Exophilia* and *Trichoderma* (reviewed in [58]).

Several other studies have described the isolation of endophytes from plants growing in salt stressed or harsh environments. As mentioned earlier, *Fusarium culmorum*, isolated from dunegrass growing in coastal habitats, has been shown to be necessary for salt tolerance of this plant [25] [27]. Interestingly, filtrates from *Penicillium* endophytes isolated from a dune plant (*Ixeris repenes*) and from a salt marsh plant (*Suaeda japonica* Makino), both determined to be gibberellin producing fungi, promoted growth when applied to test plants [59] [60]. Fungal endophytes (*Cladosporium* sp., *Penicillium* sp., and *Pyrenochaeta* sp.) isolated from roots of wild barley species growing under shallow, alkaline, salty and dry soil conditions, increased grain yield and shoot biomass of cultivated barley, and interestingly these benefits were more evident when plants were grown under low nutrient conditions [61]. In earlier studies, endophytes from wild barley were shown to be antagonistic to seedborne infections when inoculated onto seeds [62]. Species of *Sarocladium* have been reported as endophytes in other grass species [63] [64] and as a beneficial endophyte in maize which produces antibiotics inhibitory to *Aspergillus flavus* and *Fusarium verticillioides* [65] [66]. However, *Sarocladium* sp. are also known to cause rice sheath rot disease [67] and wilts in other species.

Schultz *et al.* [68] described the host/endophyte association as “A balanced antagonism”. It is interesting to note that some endophytes may be latent pathogens that could become pathogenic later when the host plant/fungal association is no longer beneficial to the endophyte, as is the case with an oak endophyte, *Discula quercina* [69]. Some of the putative fungal endophytes isolated in this study belong to species that are known patho-

gens and would not be considered for future studies without eliminating that possibility through further molecular analysis. Future studies will focus on testing these endophytes for their ability to increase abiotic/biotic stress tolerance in grass species. Additional isolation of endophytes from grasses at different times during their growth cycle and from areas more remote to human activity could also be beneficial for expanding this endophyte collection. This endophyte collection will allow us to begin evaluating their potential for increasing agricultural production in a sustainable, environmentally friendly manner.

4. Conclusion

Grasses provide forage and ecological benefits that contribute significantly to our agricultural, environmental, economic, and social well-being. Grasses are a valuable forage species, but are also becoming more important as buffers for watersheds, habitat for biologically diverse plants and animals, and as sinks for carbon sequestration. Adaptable, high-yield, low-input grass varieties and management strategies are needed to enhance the utility of these grasslands and to meet the goals of improved food and energy security. The Willamette Valley of Oregon produces over 50% of the world's cool season grass seed. The presence of fungal endophytes in grasses has been shown to improve the persistence and productivity of grasses when challenged with abiotic and biotic stresses, but information about the potential for isolating and adapting new endophytes from other grasses to improve rangeland, pasture, turf and bioenergy grasses is limited. The purpose of this study is to identify novel fungal endophytes that are native to Oregon that will allow us to improve grass stress tolerance without using direct genetic modification and without introducing foreign or exotic species into this diverse agricultural production area. The discovery of novel endophytes has the potential to improve yield and persistence, as well as increase the adaptability of these grasses to multiple stresses encountered in end-use environments.

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