DISSERTATION

FUNGI FROM SELENIFEROUS HABITATS AND THE RELATIONSHIP OF SELENIUM TO FUNGAL OXIDATIVE STRESS

Submitted by

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY AMI LEA WANGELINE ENTITLED FUNGI FROM SELENIFEROUS HABITATS AND THE RELATIONSHIP OF SELENIUM TO FUNGAL OXIDATIVE STRESS BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT OF DISSERTATION

FUNGI FROM SELENIFEROUS HABITATS AND THE RELATIONSHIP OF SELENIUM TO FUNGAL OXIDATIVE STRESS

Saprobic rhizosphere fungi from four seleniferous sites in Colorado and Wyoming were isolated from feeder roots of selenium (Se) hyperaccumulator and non-accumulator plants. The 259 fungal isolates were identified to genus and evaluated for Se tolerance. Among the 24 represented genera, eleven genera comprised 86% of the isolates. The majority of the isolates from the seleniferous sites were visually unaffected by 10 mg L^{-1} Se, irrespective of host plant (hyperaccumulator vs. non-accumulator) and original isolation agar (0 or 10 mg L^{-1} Se). Two previously undescribed species of *Alternaria* were isolated from hyperaccumulators; one isolate was highly selenophilic. Rhizosphere fungi also were isolated from plant roots from a control site where no Se accumulators were present; these fungi were highly sensitive to Se at 10 mg L^{-1} and, as a group, exhibited reduced ($p \leq 0.05$) tolerance when compared to the isolates from the seleniferous sites.

A selected group of isolates was evaluated for Se tolerance, accumulation and volatilization, as well as their trolox equivalent antioxidant capacity, total phenolics and recovery after exposure to UV light. Several *Alternaria* and related genera and one *Fusarium* isolate showed up to a five-fold increase in oxidative quenching ability when grown on media containing Se. Further, some of these isolates accumulated Se in their tissues, exhibited a significant increase in growth rate in the presence of Se, and exhibited decreased recovery time after exposure to UV light in the presence of Se. One *Aspergillus* isolate tolerated high levels of Se ($\leq$600 mg Se L^{-1}), volatilized Se, and failed to accumulate Se. This fungus may be a good candidate for Se mycoremediation. Five of the twelve isolates characterized utilize or to benefit from the Se as judged from a combination of tolerance and an increase in reactive oxygen species quenching and total phenolics without a negative impact on growth. The effects of these responses were seen in the reduction of recovery time from UV light. These fungi may provide a biological resource for oxidative stress treatment and model organisms to study oxidative stress and antioxidant capacity in relation to Se.

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Introduction

History of Selenium

The element Selenium (Se) is a metalloid that is chemically similar to sulfur (S) and tellurium (Te), is toxic in large amounts, but probably is essential for most organisms as an active center in certain enzymes (Wilber 1980, Sors *et al.* 2005a). Se is derived from the Greek word *selene-* meaning moon and was first identified by Jöns Jakob Berzelius in 1817.

In the intermountain Western US, Se is found mainly in the shale of the Pierre and Niobrara formations as well as some areas of the Greenhorn and Morrison formations, all of which were deposited during the late Cretaceous period, 77-68 MYA (Byers 1935, 1936; Beath 1982). Se in these sediments is believed to have originated from volcanic emanations deposited by rain into the Western Interior Seaway (Byers 1938; Rosenfeld and Beath 1964). The resultant mineral-rich shale is concentrated in the Central and Western US where the seaway was located and essentially non-existent in the South and on the East coast. These shales are not uniform in Se content; concentrations range from less than 1 mg kg^{-1} to over 100 mg kg^{-1} in certain areas. Outcrops of seleniferous shale and their occurrences underlying the surface soil often are recognized by the presence of "indicator" plants which hyperaccumulate Se and only are found where Se is available (Rosenfeld and Beath 1964). The Se present in the shale can be mobilized by uptake and translocation in plants, by leaching in agricultural runoff, by mining certain materials and from other natural and industrial sources (Hossner *et al.* 1992, Lemly 1999, Finkelman *et al.* 2002, Sors *et al.* 2005a, b). Changes in the presence and availability of Se can lead to chronic and acute Se poisoning in other inhabitants of the ecosystem through accidental substitution for sulfur (S) by Se in essential enzymes. Se often biomagnifies in ecosystems and endangers virtually all inhabitants (Rosenfeld and Beath 1964, Beath 1982, Bañuelos *et al.* 2002).

One of the most infamous examples of Se poisoning is from Kesterson National Wildlife Refuge in Las Baños, California. Agricultural runoff from fields overlaying Se-rich shale that collected in evaporation ponds led to extremely high concentrations of Se in the sediment. Bioaccumulation and biomagnification of the Se resulted in deformities and death in a variety of wildlife and resulted in a severe reduction in local bird population numbers (Ohlendorf *et al.* 1986). Other examples of poisoning, both acute and chronic, are found throughout the Front Range of the Rocky Mountains in animals feeding on hyperaccumulator plants (Rosenfeld and Beath 1964). Poisoning also occurs in aquatic ecosystems throughout the US and is considered an ongoing, potentially devastating threat to organisms in these habitats (Lemly 1999, Hamilton 2004). Historically, Se poisoning is thought to have affected the horses and pack mules involved in bringing reinforcements and supplies to Custer's army, thus contributing to the outcome of the Battle of Little Big Horn (Hintz and Thompson 2000).

After Se compounds have been mobilized into the ecosystem, regardless of method, they typically will remain in a bioavailable form due to the arid/semi-arid climates in the Western US that prevent the complete reduction of Se to its less toxic, non-soluble elemental form (Beath 1982).

Selenocompounds and Oxidative stress

Oxidative stress (OS) occurs in all organisms when the amount of reactive oxygen species (ROS) generated exceeds the organism's capacity for radical quenching. This can be caused by various factors including metabolic upset and environmental influences such as temperature fluctuation and UV exposure. OS can result in damage to lipids, enzymes, DNA and other cellular components. The response to OS can be manifested in plants and fungi as reduced growth, inability to complete their life cycle, or even death, depending on the severity of the stressor (Angelova *et al.* 2005). In addition, oxidative damage in humans is associated with a variety of diseases including cardiovascular disease and cancer (Chisolm 1992, Valko *et al.* 2006).

Each organism has its own set of machinery to combat these ROS though redox reactions facilitated by enzymes, including catalase, superoxide dismutase and glutathione peroxidase. A unique quality of the glutathione peroxidase (GPX) enzyme in animals, prokaryotes and some algae, is that it requires a seleno-amino acid (SeCys) which utilizes selenium in place of sulfur (Rotruck *et al.* 1973, Stadtman 1974, 1990, Fu *et al.* 2002) making Se an essential element for these organisms. GPX also is found in plants and fungi, but neither group has been found to use a seleno-amino acid as a component of GPX, nor have they been shown to require Se as an essential element, even though Se supplementation increases GPX levels in lettuce as well as growth rate in some plants (Xue *et al.* 2001).

Selenium has received a large amount of attention as an antioxidant in its own right as well as for its anti-tumor properties (Whanger 2004, Valko *et al.* 2006) and often is touted as a beneficial component in vitamins and mineral supplements. Se even has been found to suppress oxidative radical mediated sclerotia formation in the fungus *Sclerotium rolfsii* (Ellil 1999). However, Se has a small window between essential and toxic concentrations and often is reported for its negative impact on plants, fungi and animals at high concentrations. This toxicity occurs through DNA mutations, decreasing the fluidity of phospholipid membranes and through protein malfunction due to the replacement of sulfur containing amino acids with their seleno-homolouges (Rosenfeld and Beath 1964, Ohlendorf *et al.* 1986, Hefnawy 2002, Hartwig *et al.* 2003, Wycherly 2004).

Selenium hyperaccumulator plants

Taxa from several plant families have the unique ability to accumulate Se to around 1% of their dry weight. These plants typically are found only on seleniferous soil and may require Se for completion of their life cycle (Trelease and Trelease 1938; Beath *et al.* 1939; Rosenfeld and Beath 1964). There are at least 24 species of *Astragalus* (Fabaceae) that can hyperaccumulate Se, and several other members of the genus that can survive on seleniferous soil without accumulating Se (Rosenfeld and Beath, 1964). Other Se hyperaccumulators include several species of *Stanleya* (Brassicaceae), two species of *Xylorhiza*

(Asteraceae), and two species of *Oonopsis* (Asteraceae); all are found mainly in the US. In Australia there are two Se hyperaccumulating genera, *Neptunia* and *Morinda* (Peterson and Butler 1967, 1971). There are many examples of secondary Se accumulators, which accumulate Se when they are found on seleniferous soil, but whose distributions are not limited by the presence of Se (Rosenfeld and Beath 1964).

The mechanisms of tolerance as well as the potential for various applications, including phytoremediation, have been well studied in hyperaccumulator plants. The exceptional ability of these plants to hyperaccumulate Se involves the conversion of inorganic Se compounds into non-protein seleno-amino acids, thereby preventing toxicity to the plant by accidental insertion of a seleno-amino acid in place of its sulfur homologue (Brown and Shrift, 1981). Interestingly, the most commonly occurring non-protein seleno-amino acid found in Se hyperaccumulators, Se-methylselenocysteine (Shrift and Virupaksha, 1965), also is the most beneficial form for human consumption (Whanger 2004). Genetic and biochemical studies of the enzymes of the sulfur assimilation pathway have not been able to explain fully how Se hyperaccumulation occurs, nor do these plants have Se homologues of several sulfur molecules, including glutathione; this suggests that the presence of a separate Se metabolic pathway has yet to be elucidated (Shrift and Virupaksha 1965, Sors *et al.* 2005a, b).

Selenium and Fungi

Se interactions with fungi have been studied for the better part of the past century and were first documented in the early 1900's with a report by Stover and Hopkins (1927) on the fungicidal action of Se-containing compounds. The bulk of research concerning Se and fungi is applied, such as Se being used as a medicinal treatment for fungal dermatological disorders (Wilbur 1980, Kyle and Dahl 2004), as a fungicide or fungicide additive (Razak 1991, Hanson *et al.* 2003, Kloc *et al.* 2003), as a nutritional supplement or potential toxicant in edible fungi (van Elteren *et al.* 1998, Kalac and Svoboda 2000), and as a component of a bio- or phytoremediation system to deal with Se contamination (Thompson-Eagle *et al.* 1989, Brady *et al.* 1996).

There has been some research done on the metabolism of Se in filamentous fungi, including uptake and volatilization (Tweedie and Segel 1970, Barkes and Flemming 1974, 1976, Ramadan 1989, Gharieb *et al.* 1995), but little on the influence of Se on fungi in relation to their environment, competition or survival (States 1966, Ellil 1999, Hefnawy, 2002). Recent research demonstrates that mycorrhizal fungi enhance Se accumulation in garlic, resulting in a significant (10 fold) increase of Se in the plant tissue when compared to non-mycorrhizal controls (Larsen *et al.* 2006). Even though there has been a large amount of research done on the plants of seleniferous habitats, virtually nothing is known about the fungi associated with the Se hyperaccumulators in these ecosystems.

Study Goals

The first goal of this study was to examine the distribution, diversity and tolerance of culturable, saprobic (facultative or obligate) fungal genera associated with the rhizosphere and rhizoplane (will cumulatively be referred to as rhizosphere) of hyperaccumulator and non-hyperaccumulator plant species in seleniferous areas along the eastern Front Range of the Rocky Mountains and to describe unique (new and undescribed) fungi found in these habitats. These studies are presented in Part I (Chapters 1 and 2).

The second goal, presented in Part II, was to examine the Se tolerant fungi identified in Part I and to evaluate further how Se is metabolized based on examination of an increased range of tolerance, accumulation and volatilization, as well as the possible relationship of Se to oxidative radical quenching within the fungi. These Se-tolerant fungi have the potential to fill in gaps between the well studied, but different, ways that Se is metabolized in plants and animals. Additionally, they might be utilized in two other important Se applications, namely the removal of excess Se in cases of environmental toxicity and the supplementation of Se in situations that impact animal and human health and disease prevention.

PART I:

SURVEY OF FUNGI FROM SELENIFEROUS HABITATS

CHAPTER 1

Selenium tolerance in rhizosphere fungi from Se hyperaccumulator and non-hyperaccumulator plants from the Rocky Mountain Front Range

Introduction

Seleniferous Cretaceous shales are widespread throughout the High Plains region of the USA. These shales are found as outcrops and as the underlying parent material for soils that support plants with the ability to hyperaccumulate Se in their tissues to around 1% dry weight (Rosenfeld and Beath 1964). Virtually nothing is known about the fungi associated with the rhizosphere and rhizoplane of these plants. The objective of this study was to examine the distribution, diversity and selenium tolerance of culturable fungal genera associated with the rhizosphere of hyperaccumulator and non-accumulator plant species in seleniferous areas along the Eastern Front Range of the Rocky Mountains.

The three hypotheses tested were:

H_1: Se hyperaccumulator plants will harbor a less diverse group of fungi when compared to non-accumulators.

H_2: The fungi recovered from Se hyperaccumulator plants will exhibit greater Se tolerance than the fungi isolated from non-accumulator plants.

H_3: Agar containing Se will select for Se tolerant fungi as opposed to Se-free agar which will allow all fungi to grow.

These hypotheses are based on the assumption that Se is typically anti-fungal (Wilbur 1980, Razak 1991, Hanson *et al.* 2003, Kloc *et al.* 2003, Kyle and Dahl 2004) and thus can act as a selection agent in determining fungal diversity and distribution.

Materials and methods

Plant material— Plants were collected from five different sites in Colorado and Wyoming (USA) for isolation and culture of fungi from their rhizosphere (Table 1). The Lysite (WY) and Casper (WY) sites were chosen based on historical presence of Se accumulators (J.D. Love, University of Wyoming, USGS, pers. communication), while both the Laramie (WY) site and the Fort Collins site (CO) were chosen based on the current presence of Se accumulators (J.S. States, Northern Arizona State University, retired, pers. communication, E.A.H. Pilon-Smits, Colorado State University, pers. communication). The final site, Nunn (CO), was chosen as a potential control site due to the lack of obvious Se hyperaccumulators.

Four Se hyperaccumulator species, *Astragalus bisulcatus* (Hook.) A. Gray, *Astragalus racemosus* Pursh, *Xylorhiza glabriuscula* Nuttall and *Stanleya pinnata* (Pursh) Britton, were collected (whole plant) when they were present at the

Table 1. Host plants and sites from which fungal isolates originated

Site/ Host ID	Site Collected	GPS Coordinates	Elevation (m)	Scientific Name	# collected
1-AR	Lysite, WY	43°20.09N 107°48.50W	1663	*Astragalus racemosus*	2
1-SP				*Stanleya pinnata*	1
1-XG				*Xylorhiza glabriuscula*	1
1-NA				*Leucanthemum* sp.	1
2-NL	Casper, WY	42°50.77N 106°31.63W	1708	*Astragalus* sp.	1
2-AB				*Astragalus bisulcatus*	1
3-AB	Laramie, WY	42°51.17N 106°31.07W	1695	*Astragalus bisulcatus*	1
4-AB	Ft. Collins, CO	40°32.70N 105°07.87W	1578	*Astragalus bisulcatus*	2
4-AA				*Astragalus* sp.	1
4-SP				*Stanleya pinnata*	2
4-NL				*Medicago sativa*	1
4-NM				*Alyssum* sp.	1
5-NL1	Nunn, CO	40°44.98N 104°53.45W	1704	*Astragalus* sp.	1
5-NL2				*Psoralea* sp.	1
5-NM1				*Lepidium* sp.	1
5-NM2				*Sisymbrium* sp.	1
5-NA				*Gutierrezia sarothrae*	1

seleniferous sites. Nine non-hyperaccumulator plants from the same families (Fabaceae, Asteraceae, and Brassicaceae, respectively) were collected from these same four seleniferous sites, as well as from a fifth site (Table 1) where no hyperaccumulators were present. All plants were identified using keys to Colorado Flora (Weber 1987, 1990) and comparison with specimens in the Colorado State University Herbarium.

No attempt was made to evaluate either the plant or fungal diversity or composition for the seleniferous habitat beyond the rhizosphere interface of the host plants. Additionally, the host plants were not selected in a particular pattern or frequency to facilitate site comparisons at an ecological level. Discussion of ecosystem distinct soil fungal communities and their "principal forms," as described by Christiansen (1981) and States (1966), are used for generalized comparison since no specific studies of the rhizosphere of the host plants or seleniferous rhizosphere communities are available for comparison.

Selenium analysis— To quantify the levels of Se present in the plants and to verify the plant's status as a Se accumulator or non-accumulator, shoot tissue was dried for 48 h at 80°C. For each collection site and species, different plant parts (young leaves [taken from the top quarter of the plant], old leaves [taken from bottom half of plant] and flowers) each were analyzed in triplicate for Se after acid-digestion according to the method of Zarcinas *et al.* (1987). The root tissue was destroyed during fungal isolation and was not available for Se analysis. The total Se concentration in each digest was measured using inductively coupled plasma–atomic emission spectrometry (ICP–AES; Thermo

Elemental, Franklin Lakes, NJ) according to the method of Fassel (1978) using appropriate standards and quality controls.

Fungal isolation, identification and tolerance evaluation— All available feeder and non-woody tap roots of each collected plant were cut into 1cm pieces that were disinfested by immersion for 10 seconds in 10% (v/v) household bleach solution (6% sodium hypochlorite), rinsed in sterile water and plated on 0.5 strength Malt Extract Agar (0.5 MEA, Difco, Detroit MI, USA) either without Se or with 10 mg Se L^{-1} supplied as Na_2Se0_4 (Sigma Chemical, Chicago IL, USA). The Petri dishes were sealed and incubated at 22°C under continuous light.

When fungal growth occurred from the root cuttings, the cultures were either hyphal tipped or single spored and transferred to a new Petri dish of the same agar. The cultures were initially identified to genus or species using morphological characters (Ellis 1971, 1976, Domsch *et al.* 1980) and are listed for reference in Appendix A (note: some of the isolates identified to genus may be the same species).

Each isolate was transferred to two slants of 0.5 MEA, one without Se and the other with 10 mg Se L^{-1}, for tolerance evaluation by visual comparison of the two colonies. Each pair of slants was assigned a value describing the difference in growth between the Se and non-Se culture, thus quantifying the inhibition caused by the Se. The values assigned were as follows: 1 = little to no inhibition (<25% reduction in colony diameter or density), 2 = somewhat inhibited (25-75% reduction in colony diameter or density), 3 = inhibited (>75% reduction in colony diameter or density).

Statistical analysis—Species diversity indices were calculated for each individual plant collected using Simpson's diversity index, D (Simpson 1949):

$$D = \frac{\sum n_i(n_i - 1)}{N(N-1)}$$

where n_i = the total number of organisms of a single species (or species group) and N = the total number of organisms of all species. Values range between 1 and 0, the lower the value, the greater the diversity.

Statistical analyses for differences in Se concentrations in the host plant tissues ($p \leq 0.05$) were performed using the software program JMP-IN (SAS Institute, 1999) using ANOVA, and a post-hoc student's t test. JMP-IN also was used to evaluate differences in Se tolerance by using the non-parametric Kruskal-Wallis test to evaluate differences within the groups (inhibited, somewhat inhibited and not inhibited) and the Wilcoxon matched pairs test to determine the differences between pairs of data (sites, genera, host plants).

Results

Se analysis— With few exceptions, the Se hyperaccumulator plants contained high levels of Se, as expected. These results support previous studies that show the "indicator plants" are found on seleniferous soils and contain relatively high levels of Se (Rosenfeld and Beath 1964). The Se concentration in plant shoot tissues ranged from less than 1 mg Se kg^{-1} DW in several of the non-accumulator species to almost 19,000 mg Se kg^{-1} DW in flowers of the Se

Table 2. Se content in mg Kg^{-1} of various plant parts from original host tissue. Significant differences in Se accumulation in pooled Se hyperaccumulators by site are designated as asterisks. Fort Collins had the highest Se and Casper had the lowest Se.

Site	Plant	Flower (mean ± SD)	Young leaf tissue[c] (mean ± SD)	Old leaf tissue[d] (mean ± SD)
Lysite, WY**	*Astragalus racemosus*	277.64 ± 40.89	120.29 ± 17.04	15.80 ± 2.04
	Stanleya pinnata	2266.35 ± 275.48	695.60 ± 88.86	90.94 ± 24.65
	Xylorhiza glabriuscula	164.81 ± 59.43	93.73 ± 14.90	62.53 ± 6.08
	Leucanthemum sp.	1.83 ± 1.34	1.29 ± 1.83	0.96 ± 1.36
Casper, WY***	*Astragalus* sp.	0.48 ± 0.96	2.38 ± 0.54	0.71 ± 1.21
	Astragalus bisulcatus	8.91 ± 2.14	8.83 ± 2.00	3.94 ± 0.61
Laramie, WY**	*Astragalus bisulcatus*	817.45 ± 12.65	878.19 ± 83.96	417.06 ± 164.83
Ft. Collins, CO*	*Astragalus bisulcatus*	18897.76 ± 1024.37	17221.68 ± 1957.6	13641.31 ± 1276.17
	Astragalus sp.	ND[a]	[b]7482.02 ± 333.8	
	Stanleya pinnata	2621.61 ± 221.40	1170.69 ± 91.69	836.3 ± 128.64
	Medicago sativa	3.27 ± 1.02	[b]2.73 ± 0.81	
	Alyssum sp.	ND[a]	[b]0.37 ± 1.42	
Nunn, CO	*Astragalus* sp.	ND[a]	[b]1.46 ± 0.77	
	Psoralea sp.	0.05 ± 0.09	[b]0.48 ± 0.86	
	Lipidium sp.	ND[a]	[b]1.28 ± 1.17	
	Sisymbrium sp.	ND[a]	[b]1.44 ± 0.23	
	Gutierrezia sarothrae	0.20 ± 0.28	[b]1.51 ± 0.97	

[a] Plants were not flowering at time of collection

[b] Leaf tissue was combined for analysis due to small amounts

[c] Originated from the top 25% of the plant

[d] Originated from the lower 50% of the plant

accumulator *A. bisulcatus* (Table 2). The flowers typically had the highest levels of Se, whereas the older leaves contained lowest levels. After pooling all plant parts of Se accumulators by site, the Se accumulator plants from Ft. Collins had significantly more ($p<0.001$) Se than all other Se accumulators by site, while *Astragalus bisulcatus* from Casper had significantly ($p=0.007$) less Se than accumulator plants from the remaining seleniferous sites, with no differences between Se accumulators from Lysite and Laramie. The order of Se occurrence is as follows: [most Se → Ft. Collins > Lysite, Laramie > Casper ← least Se].

Fungal distribution—The plants collected from the four seleniferous sites (Lysite, Casper, Laramie and Fort Collins) yielded 259 rhizosphere fungal isolates (approximately 18 per plant) that belong to 24 different genera (some isolates may be the same species). The majority (229 of 259, 88%) belong to 11 main genera or generic groups (groups of related genera are combined for display purposes: *Alternaria* group = *Alternaria, Embellisia,* and *Ulocladium; Mucor* group = all mucoraceous or coenocytic fungi excluding *Absidia* and *Pythium*; and *Penicillium* group = *Penicillium, Pacilomyces,* and *Talaromyces*) (Figure 1). The control site (Nunn) yielded 28 isolates from eight genera; 16 of the isolates (54%) were from the same main genera isolated from the seleniferous sites. The distribution of the main genera was variable by site, with some groups not being represented at all sites (Figure 1). Members of both the *Alternaria* group and the *Mucor* group were isolated at all seleniferous sites, while *Fusarium* and members of the *Penicillium* group were isolated from all five collection sites.

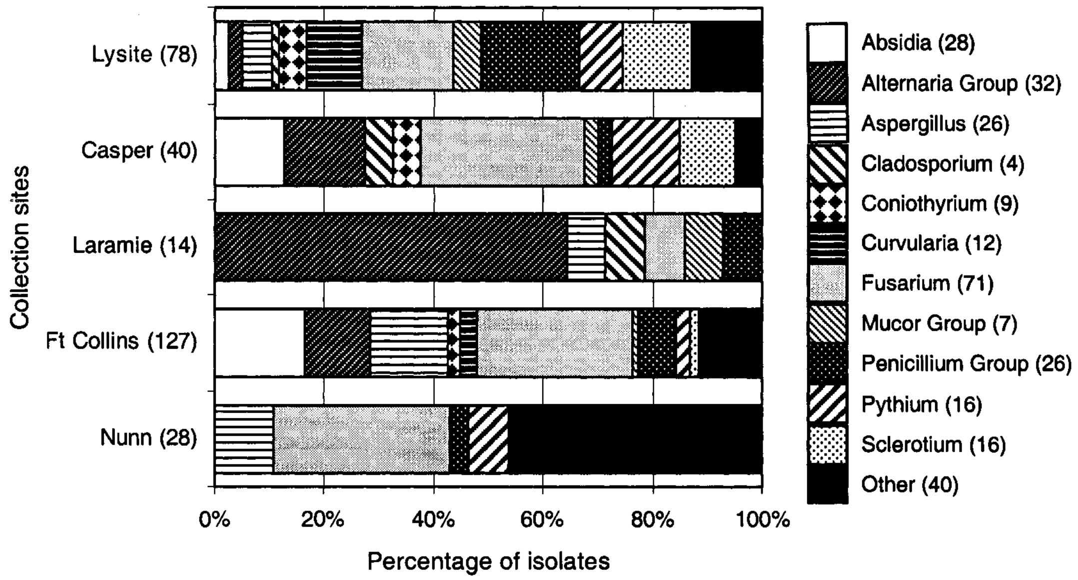

Figure 1. Distribution of main genera and generic groups by site. The four seleniferous sites are Lysite (WY), Casper (WY), Laramie (WY) and Ft. Collins (CO). The non-seleniferous site is Nunn (CO). Parenthetic numbers after site designate total number of isolates included in that bar, and parenthetic numbers after genus or generic group name designate the total number of isolates from that genus or generic group included in the chart.

Measures of species diversity (D) of the fungi from each collected plant showed that there is a large amount of variability, even in the case of individuals of the same species of host plant from the same site. For example, fungi isolated from *A. racemosus* from Lysite provided D=0.36 for one individual and D=0.15 for another. The species diversity by individual plant ranged from a high of D=0.09 in both *S. pinnata* from Lysite and *A. bisulcatus* from Casper to a low of D=0.40 from *A. bisulcatus* from Laramie. Interestingly, the average Se hyperaccumulator yielded a diversity index of D=0.25 while the non-accumulators yielded an average of D=0.20, suggesting a trend that greater diversity is supported by the non-accumulators, though the trend is not significant (p=0.44). When comparing the Se accumulators from each site and removing the outlier *A. racemoses* (D=0.36) from Lysite, Laramie and Fort Collins, both have significantly less (p=0.007) diversity than Lysite and Casper (Table 3).

Two of the *Alternaria* isolates were described as new species and one is highly selenophilic (Wangeline and Reeves 2007). These new species are further discussed in Chapter 2.

Two species of *Absidia* were isolated as frequently as other common groups such as *Alternaria* and *Aspergillus* at the Fort Collins (CO) site, and also were isolated several times from Lysite (WY) and Casper (WY), even though these *Absidia* species are not reported to be principal organisms in grassland ecosystems (Christensen 1981). While all sites yielded *Fusarium* isolates, three of the four seleniferous sites as well as the control site provided large

Table 3. Simpson's diversity index (DI) measurements by collection site and pooled host plant species and non-accumulator family groups from seleniferous sites (Lysite, Casper, Laramie and Fort Collins) and from a non-seleniferous site (Nunn). Parenthetic numbers represent multiple accessions of the same plant species within a single site. Se accumulators from Fort Collins and Laramie have significantly less diversity than Se accumulators from Casper and Lysite (p=0.007, after the removal of the outlier *A. racemosus*, D=0.36, from Lysite); no other significant differences were found.

Plant/Site groupings		Selenium Accumulator	Simpson's DI
Collection site			
Lysite, WY	Pooled plants (n=5)		0.11
	Astragalus racemosus (1)	Y	0.36
	Astragalus racemosus (2)	Y	0.15
	Stanleya pinnata	Y	0.09
	Xylorhiza glabriuscula	Y	0.14
	Leucanthemum sp.	N	0.16
Casper, WY	Pooled plants (n=2)		0.14
	Astragalus bisulcatus	Y	0.09
	Astragalus sp.	N	0.23
Laramie, WY	(single plant)		0.40
	Astragalus bisulcatus	Y	0.40
Ft. Collins, CO	Pooled plants (n=7)		0.17
	Astragalus bisulcatus (1)	Y	0.24
	Astragalus bisulcatus (2)	Y	0.39
	Astragalus sp.	Y	0.24
	Stanleya pinnata (1)	Y	0.29
	Stanleya pinnata (2)	Y	0.33
	Medicago sativa	N	0.28
	Allysum sp.	N	0.12
Nunn, CO	Pooled plants (n=5)	N	0.24
Se accumulators			
Pooled over Se sites			0.14
A. bisulcatus			0.16
A. racemosus			0.23
Astragalus sp.			0.24
S. pinnata			0.13
X. glabriuscula			0.14
Non-accumulators			
Pooled over Se sites			0.10
Asteraceae			0.16
Astragalus sp.			0.23
Other Fabaceae			0.28
Brassicaceae			0.12

numbers of *Fusarium* species, which is a defining genus for grasslands. In contrast, the Laramie (WY) site yielded a majority of *Alternaria* isolates; *Alternaria* is reported to be a principal genus for desert-grassland ecosystems (Christensen 1981).

Members of the main fungal genera were isolated from both Se hyperaccumulator and non-accumulator plants. However, nine of the eleven main genera were isolated more frequently (more than 50% of total isolates in that genus) from hyperaccumulators, while *Aspergillus* and *Curvularia* were isolated more often from non-accumulators (Appendix A).

Se tolerance— The ability of the fungi to tolerate Se was directly related to habitat (Figure 2). Among the rhizosphere fungi from the non-seleniferous control site (Nunn, CO), the majority (86%, 24 of 28 isolates) were sensitive to Se at 10 mg Se L^{-1}. The fungi from this site showed significantly lower ($p<0.001$) tolerance compared to each of the four seleniferous sites (Figure 2). Within the seleniferous sites, the tolerance of the fungal isolates to 10 mg Se L^{-1} (Figure 3) did not correlate with the original host plant (hyperaccumulator vs. non-accumulator, Figure 3A, 3C), nor with original isolation agar (+ or – Se, Figure 3B), nor between seleniferous sites as there were no significant differences found for any of the above in spite of the variable amounts of Se found in the host plants (Table 2).

Some members of the Zygomycota (*Absidia* and *Mucor* group) were more sensitive to Se than were most other genera and generic groups ($p\leq 0.05$). *Absidia* showed significantly lower tolerance than all groups except for

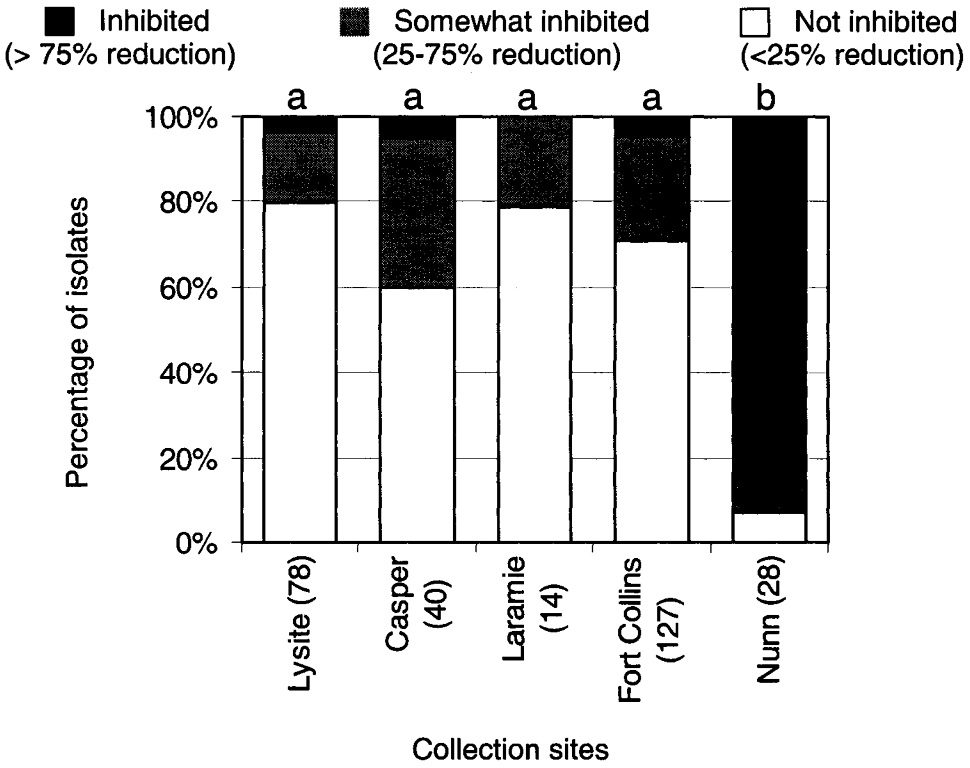

Figure 2. Inhibition of all isolates from all collection sites when cultured on malt extract agar containing 10 mg Se L^{-1}. Inhibition is measured as a reduction in colony diameter. Parenthetic numbers after labels designate the total number of isolates included in that bar. Fungi from the Nunn site (b) were statistically ($p<0.001$) less tolerant than the fungi from the other four sites (a) which had no significant differences among them.

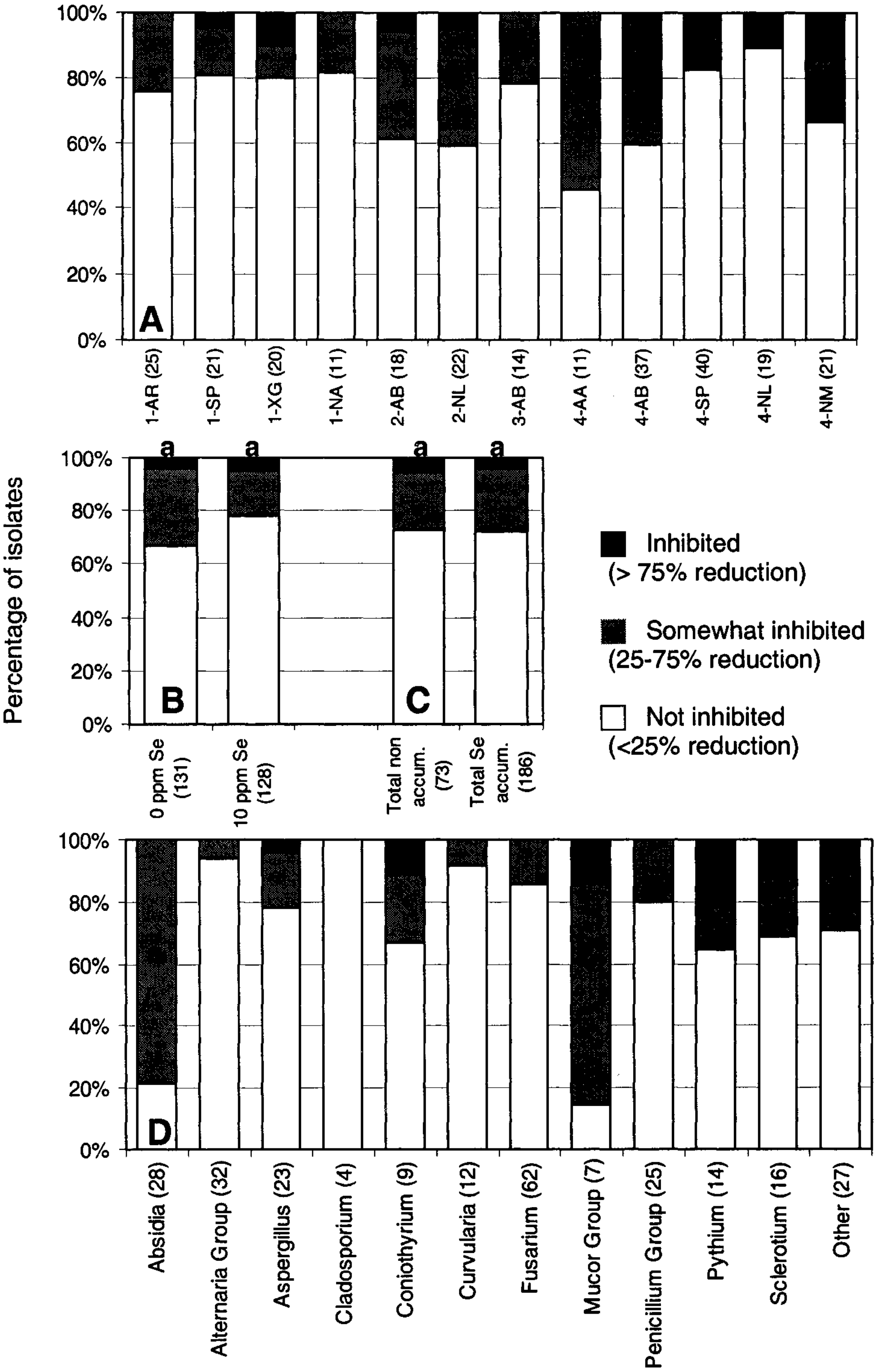

Figure 3. Inhibition of all isolates from seleniferous sites when grown on malt extract agar containing 10 mg L^{-1} Se measured as a reduction in colony diameter. (A) separated by original host plant (see Table 1 for site/host abbreviations); (B) separated by original isolation agar regardless of host plant, site, or genus; (C) pooled isolates from Se hyperaccumulators and from non-accumulators; and (D) separated by genus group regardless of host plant or site. Parenthetic numbers after labels designate the total number of isolates included in that bar. Significant differences ($p \leq 0.05$) are shown as different lowercase letters within a single chart in B and C.

Coniothyrium, the *Mucor* group and *Pythium*. The *Mucor* group showed significantly lower tolerance than all groups except *Absidia* and *Pythium* (Figure 3D).

Discussion

Se analysis—The amounts of Se found in the plants (Table 2) are representative of variability seen in Se accumulators (Cowgill 1979, Galeas *et al.* 2007) and also demonstrate the potential for fungal decomposers to come into contact with tissue containing extreme levels of selenium. High levels of Se found in these plants likely requires some form of tolerance in the fungi that decompose the detritus. These results also confirm the assignment of the title of "Se accumulator" and "non-accumulator" to the host plants. An exception is *A. bisulcatus* from Casper, which did not have significantly different levels of Se above those found in the non-accumulators. The Se analysis also showed that the Se accumulators at Fort Collins had significantly ($p<0.001$) more Se than accumulators from other sites, while Se concentration in the Se accumulators from Laramie and Lysite were not different from each other, but both had greater Se concentrations than did the Se accumulator from Casper.

Fungal distribution—The distribution of the genera and generic groups from each of the seleniferous sites differed slightly, but all sites contained typical genera (*Fusarium* and *Alternaria*) isolated from grassland soils. Additionally, all sites contained isolates of *Penicillium* (Figure 1), which are common in seleniferous soils, (States 1966).

Interestingly, two species of *Absidia* were frequently isolated, in spite of the fact that these species are not reported to be associated with either grasslands nor with any other particular ecosystem and typically are not recovered from soils (Christiansen 1981). In addition, *Absidia* is not as tolerant of Se as many of the other genera, suggesting that Se tolerance within this genus is not a contributing factor to its wide-spread occurrence in these habitats. This may be a result of the fast growth habit and long-lived zygospores in *Absidia* or lack of typical competitors due to the presence of Se. Conversely, *Trichoderma*, which is isolated regularly from all habitats (Christiansen 1981), was only isolated three times from one site. In fact, *Trichoderma* is inhibited completely by Se in the laboratory and likely is excluded from seleniferous habitats due to this sensitivity.

The species composition of fungal isolates from the non-seleniferous site (Nunn, Figure 1) was representative of grasslands, based on the large proportion of *Fusarium* isolates, but the composition differed from the seleniferous sites mainly in the lack of *Alternaria* and *Curvularia* isolates. Soil mycoflora within an ecosystem vary considerably with plant composition and cover, moisture, and nutrient availability (Christiansen 1981, States 1981), all of which likely have effects on the species available to colonize the rhizosphere of these plants.

The species diversity index accounts for both species richness (total number of species) and species evenness (numbers of each species present) and assumes that a habitat dominated by a few species is less diverse than one where species are found in equal quantities. However, using culturable fungi to assess diversity only reflects a portion of the actual diversity, particularly the species richness, because of large numbers of non-culturable fungi that likely were present in the rhizosphere microhabitat. Despite the limitations, examining culturable fungi in relation to diversity is widely used and accepted (Christiansen 1981, States 1981).

Based on the isolates recovered, *Astragalus bisulcatus* from Laramie (WY) provided the lowest fungal diversity. The fact that nine of the fourteen fungal isolates were *Alternaria* resulted in reduced species evenness. A likely contributing factor to the lack of diversity at this site is a prior history of attempts to kill or remove the Se hyperaccumulators on this site, causing disturbance to the rhizosphere ecosystem and allowing for survival of only the most resilient fungi. Conversely, Lysite and Casper had high diversity in both species richness and evenness, which is likely the result of two different contributing factors. Lysite (WY) was the only site on which all three families of Se hyperaccumulators grew and is also relatively undisturbed due to the seclusion and uninhabitability of the land. The lower amounts of Se (p=0.007) in the plants at the Casper site indicates a reduction in the severity of Se as a selector and allows for greater diversity when compared to the other study sites. The last seleniferous site, Fort Collins (CO), had less diversity than Lysite, which may be attributed to either disturbance or Se levels, both of which are greater at the Fort Collins site.

One interesting find in regard to the fungal species diversity is that when all of the plants from seleniferous sites were pooled into either Se hyperaccumulators or non-accumulators, the non-accumulators had slightly greater diversity. This difference was not significant; thus hypothesis 1 (H_1), that Se accumulators would have a less diverse rhizosphere than non-accumulators, was not supported. However, differences may be demonstrated in the future when a larger number of cultures are studied, especially cultures from non-accumulator host plants. If the extreme level of Se in the hyperaccumulator plants selects against some fungi in the rhizosphere, then the implication is that a more diverse group of fungi is able to live on nearby non-accumulating species. Such data would also support a hypothesis that the ability of fungi to live in close association with Se hyperaccumulators is not inherent to all fungal groups.

Se tolerance—Some Se compounds are anti-fungal in various applications (Wilber 1980, Razak *et al.* 1991, Hanson *et al.* 2003, Kyle and Dahl 2004) implying that fungi generally are sensitive to Se. Indeed, the majority of rhizosphere fungi isolated from the non-seleniferous site were sensitive to 10 mg Se L^{-1}. However, the majority of rhizosphere fungi isolated from the four seleniferous habitats were not inhibited by 10 mg Se L^{-1}. These data support hypothesis 2 (H_2), that fungi from Se accumulators would be more tolerant to Se than fungi from non-accumulators. However, this hypothesis was not supported within seleniferous sites (Figure 3C). *Aspergillus leporis*, which was isolated several times from multiple sites, was Se tolerant when isolated from seleniferous habitat (from both Se accumulators and non-accumulators), but not Se tolerant when isolated from the non-seleniferous site.

Interestingly, groups of related fungi show similarity in their patterns of tolerance (Figure 3D). For example, the *Alternaria* group, *Curvularia* and *Cladosporium* are all dematiaceous hyphomycetes, and they have relatively high Se tolerance. These dematiaceous hyphomycetes comprise three of the five groups with no completely inhibited isolates, and the majority are unaffected by Se. In contrast, the coenocytic Zygomycetes, *Absidia* and the *Mucor* group, present a distinct pattern of nearly all of the isolates being partially but not completely inhibited by the Se. These patterns suggest that Se tolerance in these groups, or lack thereof, is likely either: 1) a physiological trait inherent to all members of the particular groups of fungi, e.g., using melanins to bind the Se in the dematiaceous hyphomycetes and thus conferring tolerance, or lacking compartmentalization and thus prohibiting Se tolerance, as in the coenocytic fungi, or 2) an inherited trait passed down from a Se tolerant ancestor, potentially unique to the fungi found in these habitats. The observed tolerance may well be a combination of both. No isolates of the mentioned groups (dematiaceous hyphomycetes or Zygomycetes) were isolated from the non-seleniferous site to compare Se tolerance ability.

The concentration used in this study (10 mg Se L^{-1}) is above the Se concentration found in the non-hyperaccumulator plants and also above that reported for typical seleniferous soils (Galeas *et al.* 2007). Thus the observed Se tolerance among fungi from non-accumulators from seleniferous sites is somewhat unexpected, and is contrary to hypothesis 2 (H_2) that anticipated two distinct groups of fungi, one Se tolerant and the other Se sensitive. Hypothesis 3 (H_3) also was not supported since Se tolerant fungi were recovered equally on both isolation agars. In their native habitats, soil fungi may experience temporal or local spikes in Se concentration when soil Se levels vary because of weather conditions, Se hyperaccumulator litter decomposition (Byers 1938, Cowgill 1979, Galeas *et al.* 2007), or non-homogeneous distribution of Se in the soil.

In view of the 100-fold higher hyperaccumulator root Se concentration (up to 1,000 mg kg^{-1} DW) compared to non-accumulators (Freeman *et al.,* 2006) one might expect that rhizosphere fungi from Se hyperaccumulators would be more Se tolerant than those from non-accumulators. However, rhizosphere fungi isolated from Se hyperaccumulator plants did not differ in Se tolerance from those on non-accumulators ($p=0.796$) at a test level of 10 mg Se L^{-1}. A distinction in tolerance of the rhizosphere fungi from different hosts may appear if higher levels of Se were used to screen the fungi. Higher levels of Se would likely increase cellular damage and thus require mechanisms of detoxification or exclusion to maintain tolerance.

Conclusions

The presence of Se hyperaccumulator plants in the Lysite and Laramie (WY) areas has been known since the 1930's, and the plants likely have occupied these seleniferous habitats for much longer due to their ability to avoid the toxicity of Se. The Lysite area was considered by Beath (1982) to be a "super-toxic area...[because] nearly every kind of indigenous vegetation rooted in these soils is poisonous [because] so much of the soil selenium is in an available form." The high Se concentration likely has favored adaptations in plants and fungi alike and has allowed survival of Se tolerant plants and fungi under such unique conditions.

Overall, these results suggest that the fungal communities in seleniferous soils have evolved collective Se tolerance; some are possible specialists (such as *Alternaria seleniiphila*, described in the next chapter) that associate with the Se hyperaccumulators, and some are generalists and are found throughout the habitat. This unusual ability to tolerate Se is likely in response to the long-term and continual presence of Se mobilized by many of the plants in this habitat. The isolation of these Se-tolerant fungi and a better understanding of their Se tolerance mechanisms may lead to new applications, from environmental cleanup of Se using myco- or phytoremediation to antioxidant treatments for cancer and disease prevention, as has been demonstrated for various other ROS quenching fungal products, including fungal lycopene (Shon *et al.* 2003, Feofilova *et al.* 2006).

Chapter 2

Two new *Alternaria* species from selenium-rich habitats in the Rocky Mountain Front Range[1]

Introduction

Alternaria is a large and diverse genus of important plant and animal pathogens, endophytes, and plurivorous saprobes. Based on morphology as well as sequence analysis, *Alternaria* has several close relatives including *Embellisia, Ulocladium,* and *Stemphylium* (Simmons 1967, 1983; Chou and Wu 2002). Our current concepts of these genera originated with E. G. Simmons' classic paper (Simmons 1967) that updated and recognized *Alternaria, Stemphylium,* and *Ulocladium. Alternaria* is separated into 14 species groups based on conidial morphology, catenation and the overall 3-D structure of the conidiophore (Simmons 1992). Distinct morphological species-groups (Simmons and Roberts 1993), e.g. the *alternata* group or the *arborescens* group, place similar species into recognizable subgeneric groups that can be separated morphologically, molecularly (Roberts *et al.* 2000) and chemically (Andersen *et al.* 2002).Some members of *Alternaria*, *Embellisia,* and *Ulocladium* have been found in "toxic"

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[1] The majority of this chapter is published as Wangeline and Reeves, 2007

habitats containing high levels of metal or metalloid elements such as zinc, lead and selenium (Thompson-Eagle *et al.* 1989, David *et al.* 2000) as well as other extreme environments including high salinity and low temperatures (Simmons 1983, Guiraud *et al.* 1995). The current chapter describes two new small-spored *Alternaria* species that were isolated from the roots of plants that hyperaccumulate Se. These plants are known as “indicators” for soils derived from seleniferous parent material and are found throughout the central and western USA (Beath *et al.* 1939).

Materials and Methods

Fungal Isolation—Stanleya pinnata (Pursh) Britton and *Astragalus bisulcatus* (Hook.) A. Gray were collected from Pine Ridge Natural Area in Fort Collins, CO (40°32.70 N, 105°07.87 W, 1578 m) and a private ranch in Laramie, WY (42°51.17 N, 106°31.07 W, 1695 m), respectively. One-cm cuttings of the feeder roots were surface disinfested by immersion for 10 seconds in 10% (v/v) household bleach solution (6% sodium hypochlorite), rinsed in sterile water and placed in plastic Petri dishes containing 0.5 strength Malt Extract Agar (0.5 MEA, Difco, Detroit, MI, USA) with either 10 mg Se L^{-1} (*S. pinnata*) or without Se (*A. bisulcatus*), supplied as sodium selenate, Na_2Se0_4 (Sigma Chemical, Chicago IL, USA). The Petri dishes were sealed and incubated at 22°C under continuous fluorescent light. The resulting cultures were subsequently single-spored and maintained on 0.5 MEA at 22°C under continuous light in sealed Petri dishes.

Morphological examination—A 2 mm cube from each maintenance culture was transferred to the surface of V-8 juice agar (800 mL H_2O, 200 mL V-8 juice, 3 g $CaCO_3$, 15 g agar) and incubated at 22°C in unsealed Petri dishes under fluorescent light with 8 hour day/16 hour night cycle for 4 days. Additionally, to observe the undisturbed conidiophores, 1 × 1.5 cm agar plugs were inoculated in the center, placed between a glass slide and cover slip on top of a wet filter paper disk within a sealed Petri dish, and incubated as above and observed with both a dissecting and compound microscope, and photographed with a digital camera through the ocular lens.

DNA extraction, PCR amplification, sequencing and sequence analysis—A 2 mm cube from each maintenance culture was transferred to 30 mL of Czapek-Dox broth (Difco, Detroit, MI, USA) and grown at 22°C for 5 days under continuous light; a 1 cm diameter colony was harvested for DNA isolation. DNA was extracted with a MasterPure Yeast DNA purification kit and amplified with a FailSafe PCR System (Epicentre Biotechnologies, Madison, WI, USA) using primers ITS1 and ITS4 (White *et al.* 1990). The PCR products (approx. 540 bp) were purified and the ITS region (ITS1+5.8S+ITS2) was sequenced by Macrogen (Rockville, MD, USA). Sequences were deposited in GenBank (accessions EF110522 and EF110523).

Taxonomic Description

Alternaria astragali Wangeline & EG Simmons sp. nov. (Figure 4, A-C)

Ex cultura in agaro V-8 descripta. Hyphae in superficie agari distincte radiales.

Conidiophora primaria fusca, erectiuscula, ad ca. 250-400 × 3-5 μm. Conidia ovoidea vel longiellipsoidea, 10-35 × 5-7 μm, 0-7 transverse septata, longiseptis nullis vel 1-longiseptata in 1 segmento transverso, dilute vel modice brunnea, laevia, 4-10-catenulata. Conidiophora secondaria nunc unicellularia, ca. 3 × 3 μm, nunc 30-100 × 3-4 μm, nunc conspicue ad 250-300+ μm longa.
Habitatio typi in radice Astragalus bisulcatus, *coll. A. Wangeline, 8 June 2002, Laramie, Wyoming, U.S.A. Holotypus: pars ex cultura E.G.S. 52.122 (ex isol. A. Wangeline A-3, 12 June 2002) desiccata et in BPI 872198 conservanda.*
Etym: L., Astragalus (as host source)

On V-8 agar: **colony** of radiating surface hyphae bearing abundant, crowded, semi-erect primary conidiophores; **primary conidiophores** of variable length, commonly 250-400 × 3-5 μm usually unbranched with an apical chain of 4-10 conidia; **conidium bodies** ovoid or predominantly long-ellipsoid, 10-35 × 5-7 μm, with 0-7 transverse septa and usually no longitudinal septa, or occasionally with a single longiseptum in one of the widest transverse segments, dilute to medium dull brown, septa slightly darker, outer wall appears smooth; **secondary conidiophores** are usually a single apical cell but in most chains at least one, sometimes two conidia produce longer secondary conidiophores 30-100 μm long, in addition, a very high percentage of small conidia produce an apical secondary conidiophore that is 250-300 μm or more in length. The strikingly long secondary conidiophores are diagnostic of the taxon; they are intermixed with the even longer primary conidiophores, yielding a tangled thicket of dark, erect or variously twisted elements that bear the inconspicuous chains of small spores.

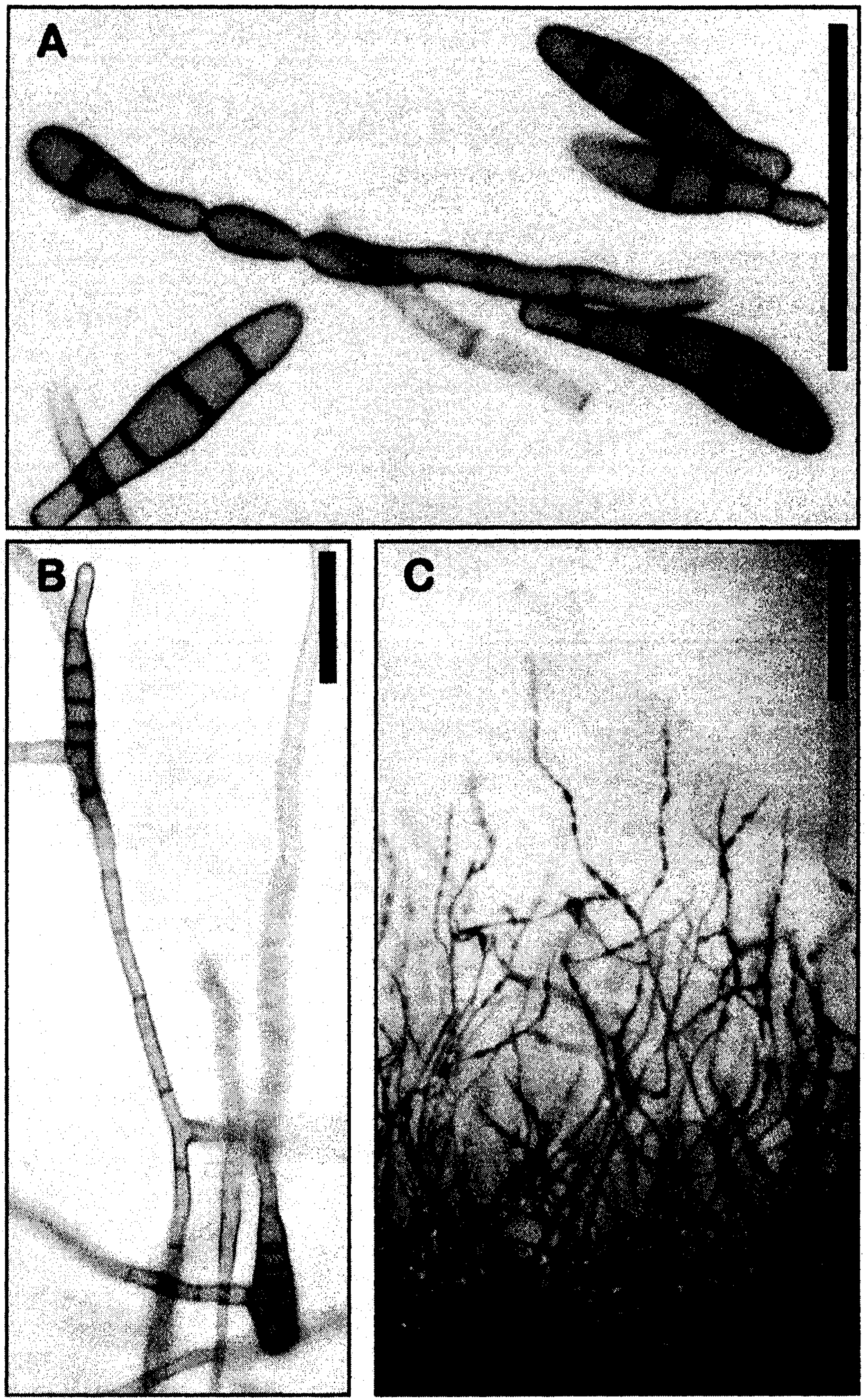


Figure 4. *Alternaria astragali*. Conidia (A), conidiophore showing secondary branching (B), and whole conidiophores (C) exhibiting characteristic sporulation pattern. Bars = 20μm for A and B, and 200μm for C.

Alternaria seleniiphila Wangeline & EG Simmons sp. nov. (Figure 5, A-C)

Ex cultura in agaro V-8 descripta. Conidiophora primaria fusca, erectiuscula vel diverse torta, plerumque sine ramis, 80-250 × 4-5 µm. Conidia ovoidea vel ellipsoidea, ad 20-40 × 8-12 µm, 1-7 transverse septata, 1-longi- vel oblique septata in 1-3 segmentis transversis, dilute vel modice brunnea, laevia vel punctata, 3-6-catenulata. Conidiophora secondaria vulgo 1-5-cellularia, 3-30 × 3 µm, etiam plerumque conspicue 50-110 µm longa.

Habitatio typi in radice Stanleya pinnata, *coll. A. Wangeline, 19 June 2002, Fort Collins, Colorado, U.S.A. Holotypus: pars ex cultura E.G.S. 52.121 (ex isol. A. Wangeline A-1, 20 June 2002) desiccata et in* ***BPI*** *872197 conservanda.*

*Etym.: NL, selenium + -phila (*having a strong affinity for selenium*)*

On V-8 agar: **colony** of abundant but not densely crowded long, erect or variously twisted primary conidiophores, 80-250 × 4-5 µm at maturity; **primary conidiophores** are simple or occasionally branched near the point of origin from surface vegetative hyphae usually unbranched within their aerial length, occasionally 1-2 geniculate conidiogenous extensions near apex, each simple conidiophore bears a short, usually unbranched apical chain of 3-6 conidia, occasionally a lateral secondary conidiophore and 1-2 conidia produced by an intercalary conidium in a chain; conidia produced in close succession with short 1-5-celled secondary conidiophore connectives ca. 3-30 × 3 µm, very long **secondary conidiophore** commonly produced from individual conidia in the chain terminating in 1-3 small conidia, these conspicuous secondary conidiophores are 50-110 µm long; **conidium bodies** are ovoid to ellipsoid without distinctive beak portion, maximum size range of ca. 20-40 × 8-12 µm at

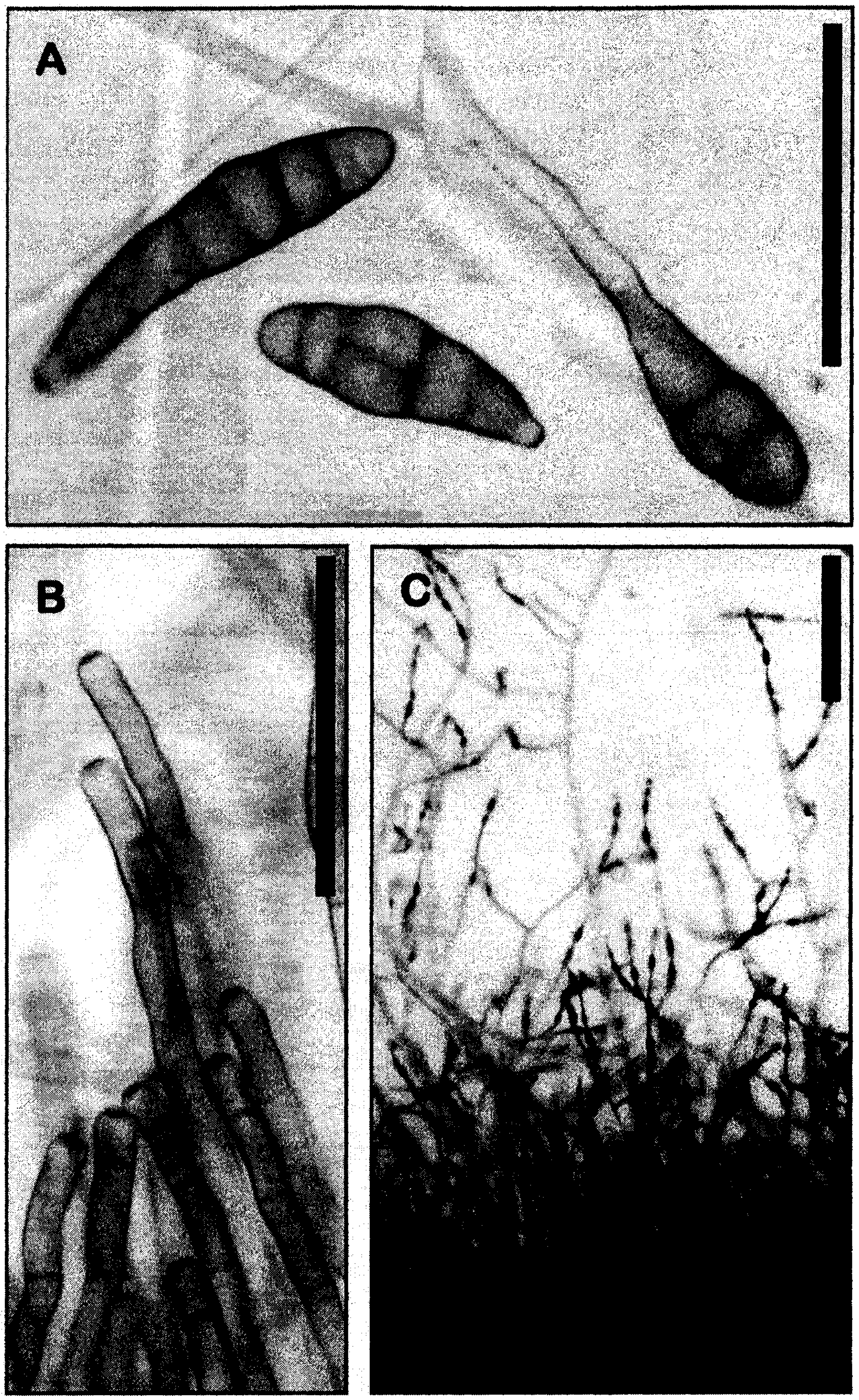


Figure 5. *Alternaria seleniiphila*. Conidia (A), conidiophore tips (B), and whole conidiophores (C) exhibiting characteristic sporulation pattern. Bars = 20µm for A and B, and 200µm for C.

maturity, with 1-7 transverse septa and one longitudinal or oblique septum in 1-3 of the transverse segments of some conidia, dilute to medium tan with slightly darker septa, outer wall smooth to evenly punctate.

Discussion

Various researchers have demonstrated that conidial size is an ineffective identification criterion when used alone to distinguish small-spored species of *Alternaria* (Simmons 1967, Andersen *et al.* 2002) and misuse of this feature has lead to rampant misidentification, especially in the case of *A. alternata.* There are standardized methods that should be used for morphological identification (Simmons 1992, Roberts *et al.* 2002) including observation of the appearance and branching habit of the distinct conidiophores of the small-spored *Alternaria* species. *A. astragali* and *A. seleniiphila* have unique conidiophore sporulation patterns specifically in relation to the length of their secondary conidiophores that are diagnostic for each taxon and which differ from that described for *A. alternata* (Simmons 1967, 1992).

A. astragali and *A. seleniiphila* exhibit Se tolerance, showing no visible effects when grown on agar containing 10 mg Se L^{-1} and grow to 0.95 and 0.61, respectively, of control cultures after 5 days on agar containing 600 mg Se L^{-1}. This ability is not unexpected due to their origin from seleniferous host material as well as the frequent presence of *Alternaria* and related genera in a variety of harsh environments. The ability of the described species to tolerate Se will likely contribute to further taxonomic distinction under metabolite examination.

Furthermore, due to the frequent misidentification of *A. alternata* as well as its reported association with toxic Se environments, it is possible that previous reports of Se tolerant *Alternaria* could be isolates of one of these newly described species (Thompson-Eagle *et al.*1989).

The ITS region (ITS1+5.8S+ITS2) of both *A. astragali* and *A. seleniiphila* were sequenced and submitted to GenBank for reference, but cannot be used for identification purposes since this gene does not resolve closely related species in this group (Andersen *et al.* 2002).

PART II:

SELENIUM METABOLISM AND OXIDATIVE STRESS

CHAPTER 3

Oxidative radical quenching in relation to selenium, total phenolics, and induced oxidative stress in filamentous fungi

Introduction

Se plays a role in animals as a component of several antioxidant compounds, including the enzyme glutathione peroxidase. In addition, Se is often discussed as an antioxidant in its own right and has been found to stimulate DNA repair as well as to reduce tumor causing cells through apoptosis (Whanger 2004). Even though Se has not been demonstrated to be essential in plants or fungi, there have been selenocysteyl- tRNA's identified in both groups (Hatfield *et al.* 1992) suggesting that their genetic codes may support the production of selenoenzymes. Additionally, Se has been shown to suppress oxidative radical mediated sclerotia formation in the fungus *Sclerotium rolfsii* (Ellil 1999) which implies that Se, whether chemically or biologically, can directly affect reactive oxygen species (ROS) in fungi. The objectives of this study were: 1) to evaluate, via tolerance, accumulation and volatilization, how Se is metabolized within selected fungi from rhizospheres of Se hyperaccumulator plants, and 2) to examine the possible relationship of Se to the oxidative radical quenching ability of these fungi.

The hypotheses tested were:

H_1: There is a correlation between an isolate's phenolic content and its ability to quench the $ABTS^+$ radical, as is seen in some plants (Javanmardi *et al.* 2003).

H_2: Se tolerant fungi will benefit from being grown in the presence of Se as evidenced by their faster recovery from damage induced by UV light.

Materials and methods

Chemical reagents and supplies—The chemical reagent ABTS [2,2-Azino-bis (3-ethylbenzthiazoline-6-sulfonic acid)] was purchased from CALBIOCHEM (Darmstadt, Germany). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was purchased from Aldrich Chemical Co. (Milwaukee, WI). All other chemicals used were of analytical and HPLC grade and obtained from Sigma Co. (St. Louis, MO). Agar and broth media for fungal cultivation were purchased from Difco Laboratories (Detroit, MI).

Fungal isolation and identification—Fungal isolation is described in Chapter 1. Briefly, fungi were taken from feeder root cuttings of Se hyperaccumulator and non-accumulator species in the Fabaceae, Brassicaceae and Asteraceae families. All isolates from the final group (Table 4, BOLD) were identified to species with the exception of A74, *Embellisia* sp. Each isolate was identified using morphological characters with the addition of DNA

Table 4. Origin of fungal isolates used in metabolic studies. Bold font denotes isolates used in all discussed experimental procedures as opposed to only TEAC and total phenolics. ITS = ITS1-5.8-ITS2 ribosomal gene, TEF = partial translation elongation factor 1-alpha.

ID #	Scientific name	Host plant	Collection site	Selenium accumulator?	Accession # (GenBank)	Gene
A1	***Alternaria seleniphila***	***Stanleya pinnata***	**Ft. Collins, CO**	**Y**	EF110523	ITS
A3	***Alternaria astragali***	***Astragalus bisulcatus***	**Laramie, WY**	**Y**	EF110522	ITS
A4	***Ulocladium atrum***	***Astragalus bisulcatus***	**Casper, WY**	**Y**		
A40	*Ulocladium botrytis*	*Astragalus bisulcatus*	Casper, WY	Y		
A55	***Dreschlera biseptata***	***Alyssum sp.***	**Ft. Collins, CO**	**N**		
A67	*Alternaria sp.*	*Medicago sativa*	Ft. Collins, CO	N		
A74	***Embellisia sp.***	***Astragalus bisulcatus***	**Casper, WY**	**Y**	EF531694	ITS
A97	***Alternaria tenuissima***	***Astragalus bisulcatus***	**Ft. Collins, CO**	**Y**	EF531695	ITS
A142	*Alternaria sp.*	*Stanleya pinnata*	Ft. Collins, CO	Y		
AB62	***Absidia spinosa***	***Astragalus bisulcatus***	**Ft. Collins, CO**	**Y**		
AB73	*Absidia sp.*	*Astragalus sp.*	Casper, WY	N		
AB134	***Absidia spinosa***	***Astragalus bisulcatus***	**Ft. Collins, CO**	**Y**		
AS86	*Aspergillius sp.*	*Medicago sativa*	Ft. Collins, CO	N		
AS88	***Penicillium anatolicum***	***Stanleya pinnata***	**Lysite, WY**	**Y**	EF531696	ITS
AS117	***Aspergillus leporis***	***Stanleya pinnata***	**Ft. Collins, CO**	**Y**	EF531697	ITS
C43	*Curvularia sp.*	*Stanleya pinnata*	Lysite, WY	Y		
C54	*Curvularia sp.*	*Alyssum sp.*	Ft. Collins, CO	N		
CO6	*Coniothyrium sp.*	*Stanleya pinnata*	Lysite, WY	Y		
CO26	*Coniothrium amelides*	*Astragalus racemosus*	Lysite, WY	Y		
F29	*Fusarium sp.*	*Astragalus racemosus*	Lysite, WY	Y		
F30	***Fusarium acuminatum***	***Astragalus racemosus***	**Lysite, WY**	**Y**	EF531698	TEF
F49	*Fusarium sp.*	*Astragalus sp.*	Casper, WY	N		
F58	*Fusarium sp.*	*Astragalus sp.*	Ft. Collins, CO	Y		
F83	*Fusarium sp.*	*Astragalus sp.*	Ft. Collins, CO	Y		
F84	***Fusarium oxysporum***	***Stanleya pinnata***	**Ft. Collins, CO**	**Y**	EF531699	TEF
F90	*Fusarium sp.*	*Stanleya pinnata*	Lysite, WY	Y		

sequence analysis for eight of the twelve isolates. DNA sequence analysis is essential for the identification of some fungi because of the difficulty of separating out species of some genera, particularly *Fusarium*, using only morphological characters.

For DNA analysis, a 2 mm cube from an actively growing culture on 0.5 malt extract agar (MEA) was transferred to 30 mL of the defined medium Czapek-Dox broth (CDB) and grown at 22°C for five days under continuous light; after five days, a 1 cm diameter colony was harvested for DNA isolation. DNA was extracted with a MasterPure Yeast DNA purification kit and specific genes were amplified using a FailSafe PCR System (Epicentre Biotechnologies, Madison, WI, USA). Primers ITS1 and ITS4 (White *et al.* 1990) were used to amplify the ITS1-5.8-ITS2 ribosomal gene for isolates A1, A3, A74, A97, AS88 and AS117 and primers EF1 and EF2 (Geiser *et al.* 2004) were used to amplify a segment of translation elongation factor 1-alpha gene for isolates F30 and F84; the TEF partial sequence is preferred for identifying *Fusarium* species. Successful amplification of the PCR products (approx. 540 bp for ITS and 700 bp for TEF) was checked by electrophoresis in a 1% agarose gel, the products were purified, and the ITS region (ITS1+5.8S+ITS2) or the partial translation elongation factor 1-alpha (TEF) was sequenced by Macrogen (Rockville, MD, USA). Sequences were deposited in GenBank (Accession numbers in Table 4).

Fungal material for antioxidant capacity and total phenolic analysis—The selected isolates (Table 4, all) were grown for 14 days under continuous fluorescent light at 22°C in 30 mL CDB in flasks without Se or with 30 mg Se

L^{-1} (20 mg Se L^{-1} for *Absidia* cultures to allow for sufficient colony size due to greater sensitivity to Se in *Absidia*) supplied as Na_2SeO_4. The colonies were harvested using vacuum filtration and freeze dried in a Virtis Genesis, Model 25LL. The freeze sequence was 24 hours at each of the following temperatures: -40, 0, +18 and +28°C with the vacuum set at 200 millitorr until tissue was completely desiccated. Samples of freeze-dried fungal material of each isolate (+ and – Se) were ground and suspended as 1 mg tissue per 25 µL of 80% acetone. Samples were rotated for 1h in the dark, and centrifuged at 5400 g for 10 min, after which the supernatant was harvested and used for subsequent analysis.

Measurement of antioxidant activity—The antioxidant activity values were estimated by the Trolox equivalent antioxidant capacity (TEAC) assay (Miller and Rice-Evans, 1996). The relative capacity of fungal antioxidants to scavenge the ABTS+ radical was measured and compared to the antioxidant potency of Trolox (water-soluble Vit E, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) as a standard. The absorbance at 734 nm was measured using a SPECTRAmax-PLUS384, UV–vis spectrophotometer, and results are expressed as micromoles of Trolox-equivalent per gram dry weight (µmole TE/g DW).

Total phenolic compound analysis—The amount of total phenolics (TP) in the fungi were determined with the Folin-Ciocalteu reagent using the method of Singleton and Rossi (1965). The absorbance at 765 nm was measured using a SPECTRAmax-PLUS384 UV–vis spectrophotometer. Results were expressed as milligrams of gallic acid equivalent per gram of dry weight (mg GAE/g DW).

A select group of 12 isolates (Table 4, BOLD) was chosen to evaluate for Se tolerance, accumulation and volatilization based on the results of the TEAC and total phenolics assays.

Selenium Tolerance—Isolates were grown in sealed Petri dishes at 22°C under continuous fluorescent light on 0.5 strength malt extract agar (0.5 MEA) with the addition of 30, 300 or 600 mg Se L^{-1} as Na_2SeO_4 or without Se. Four replicates were used for each isolate for each treatment. Colony diameter measurements were taken at regular intervals (either daily or every other day) to establish a growth curve for each isolate. Additionally, obvious differences in aerial mycelium presence or density were recorded.

Volatilization—Volatile Se compounds give a distinct garlic-like odor. The 600 mg Se L^{-1} treatments from the Se tolerance experiment were evaluated qualitatively against the 0 mg Se L^{-1} treatment for the intensity of the Se odor given off from the colony for each isolate after 5 days of growth. The isolates were given a rating as follows: 0 for no detectable Se, + for low level Se odor, ++ for potent Se odor, +++ for extreme Se odor (which was able to be detected while the Petri dish was sealed).

Se analysis— Selected isolates (Table 4, BOLD) were grown at 22°C for 14 days under continuous fluorescent light in liquid culture of 30 mL Czapek-Dox broth (CDB) in flasks without Se or with 30 mg Se L^{-1} (20 mg Se L^{-1} for *Absidia* cultures to allow for sufficient colony size due to greater sensitivity to Se in *Absidia*) supplied as Na_2SeO_4. Before harvest, 30 mg Se L^{-1} (20 mg Se L^{-1} for *Absidia* cultures) was added to one of the Se free cultures for 10 min (dip treatment) to attempt to compensate for Se that may be attached to the cell wall but is not actually accumulated by the fungus. All cultures were rinsed with water and harvested with vacuum filtration. The cultures were subsequently freeze dried in a Virtis Genesis, Model 25LL. The freeze sequence was 24 hours at each of the following temperatures: -40, 0, +18 and +28°C. A sample from each isolate for each treatment was acid-digested for 6 h at 130°C in concentrated nitric acid according to the method of Zarcinas *et al.* (1987), after which the total Se concentration in each digest was measured using inductively coupled plasma–atomic emission spectrometry (ICP–AES; Thermo Elemental, Franklin Lakes, NJ) according to the method of Fassel (1978).

UV tolerance and recovery—Isolates were grown in sealed Petri dishes at 22°C under continuous fluorescent light on 0.5 strength malt extract agar (0.5 MEA) in two treatments, one without Se and one containing 30 mg Se L^{-1} as Na_2SeO_4 (20 mg Se L^{-1} for *Absidia*). After two days of growth, the lid of each culture dish was removed, the dish was inverted on the transilluminator, and the culture was exposed directly to UV light on a Foto/UV 300 Transilluminator with midrange 312 nm bulbs (Fotodyne, Hartland WI) for 0, 30, 60, 120, or 240 seconds. Five replicates of each isolate for each Se treatment and each UV exposure were used. After exposure to UV light, the Petri dishes were covered, resealed and incubated at 22°C under continuous fluorescent light. Colony diameter measurements were taken before UV exposure and at regular intervals (either daily or every other day) after exposure.

The wavelength and intensity of UV light from the transilluminator was found to be both broad range (UV-B and UV-C) and more powerful than listed by the manufacturer. The actual transmittance was measured using a Stellarnet EPP2000C - UV-NIR with a spectral resolution of about 1 nm. A quartz diffuser (cosine response) was used to accept a wide angle input and minimize reading hot spots. The diffuser was connected to the spectrometer with a 1000 micron fused silica fiber. The plot of the wavelength *vs.* intensity is shown in Figure 6.

Statistical Analysis—Statistics were conducted for each assay or experiment with the exception of the Se accumulation data on which no statistics were performed. Statistical analysis was conducted using JMPIN v.3.26 (SAS Institute, Cary, NC). Significant differences for TEAC and TP assays were determined using ANOVA with a post-hoc student's t-test for pair wise comparisons ($p \leq 0.05$). Correlation data were determined using the correlation of Y's function for both the large isolate group for TEAC and TP as well as the selected twelve isolates for various comparisons using a Pearson product-moment coefficient of correlation. Analyses for both the Se tolerance and the UV tolerance/recovery experiments were conducted using SAS (SAS institute, Cary, NC) with a repeated measures analysis in the PROC MIXED function including an autoregressive error structure. Significance ($p \leq 0.05$) is discussed both in the text and shown on figures and in tables where applicable.

Results

The TEAC assay revealed clear and generally significant differences in the effect that Se had on the quenching apparatus across genera and generic

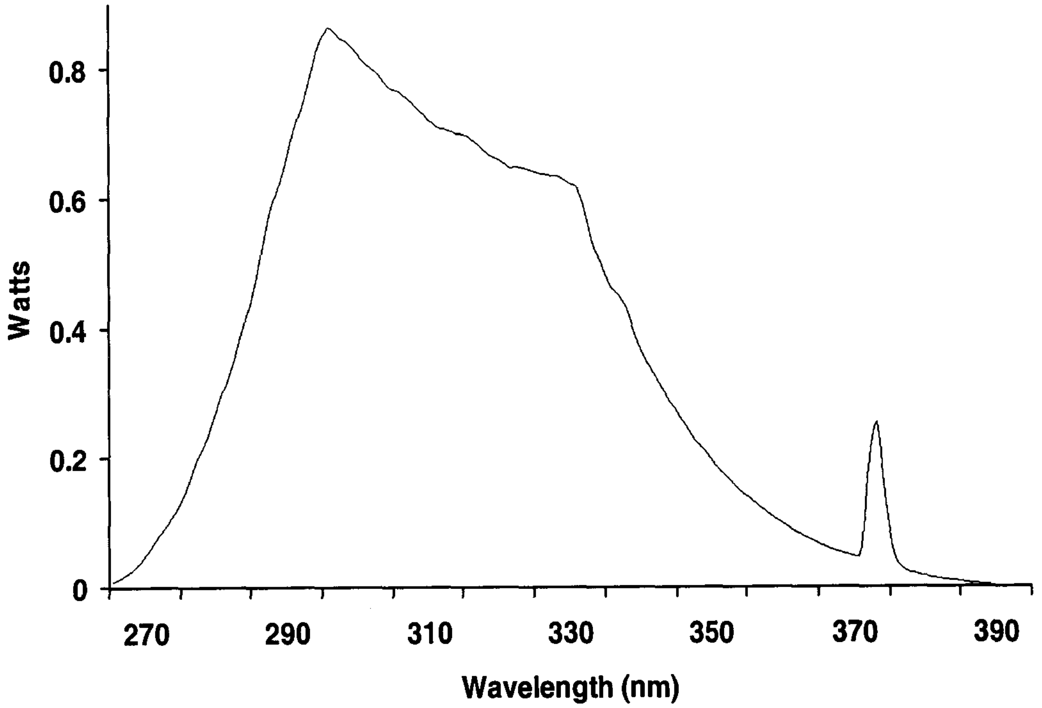


Figure 6. Spectrum of energy emitted by transilluminator used for UV exposure of fungal isolates

groups (Figure 7). Overall, the *Alternaria* (A) group (which includes *Alternaria* and related genera, including *Embellisia*, *Ulocladuim*, and *Dreshlera*) showed positive increases in radical quenching when grown with selenium for 8 out of 10 isolates (the quenching of A142 and A55 was negatively impacted). *Absidia* (AB) showed a negative relationship between quenching and Se for 2 of 3 isolates, while AB62 showed an increase in quenching. The *Aspergillus* (AS) group (which also includes *Penicillium*) showed a positive association for 2 of 3 (AS117 and AS86) and no change for the last isolate (AS88). *Curvularia* had one isolate that demonstrated increased quenching with Se and the other decreased, while *Coniothyrium* and *Fusarium* showed no significant differences between +/- Se treatments with the exception of F30, which showed an increase in quenching with Se.

The TP assay showed a trend similar to the TEAC assay, with some fungi showing an increase in phenolics with Se, while others had a decrease in phenolics in the presence of Se (Figure 8). An extremely high level of phenolics was seen in Se-treated *Embellisia* (A74) and in both treatments of *Penicillium anatolicum* (AS88).

The relationship between the ABTS quenching and TP was examined using the linear correlation function in JMP and determined to be fairly weak ($R=0.57$) but highly significant ($p<0.001$, Figure 9A). Plants with quenching that is equal to or exceeds 10 μmole TE g^{-1} DW are considered to be "high quenchers" (Cecil Stushnoff, Colorado State University, pers. communication). Based on this distinction in plants, and by removing the isolates that contain over 10 μmole

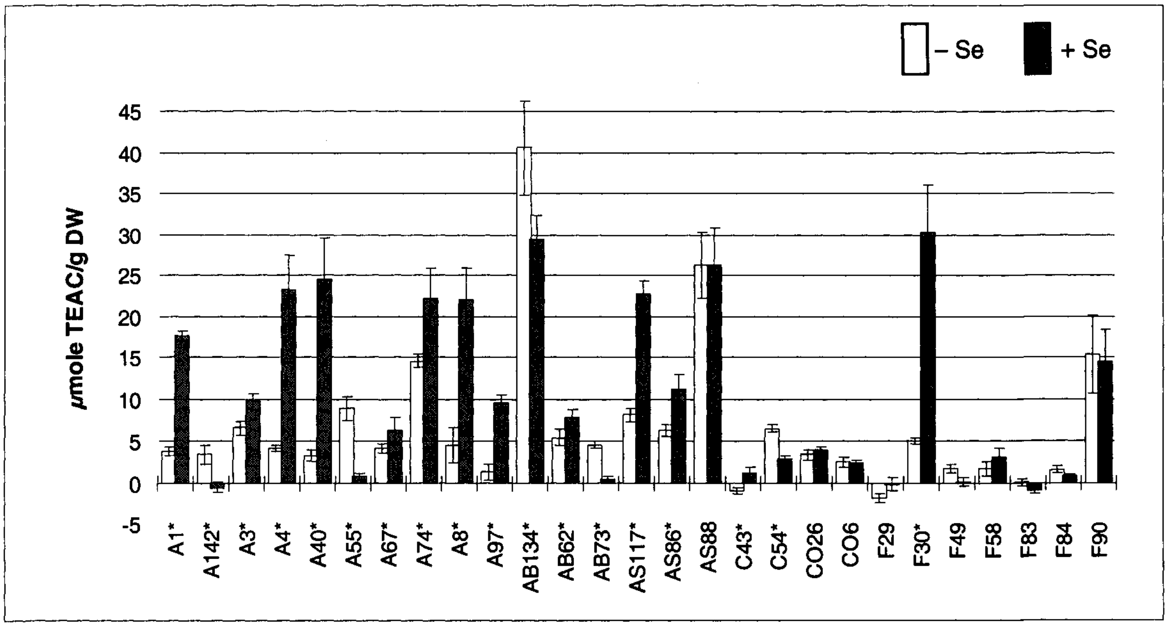


Figure 7. Quenching of the ABTS$^+$ radical by fungal isolates grown with and without Se expressed as Trolox (water soluable Vit. E) equivalents. Abbreviations for fungal isolates are in Table 1. Asterisks indicate differences that are significant ($p \leq 0.05$), error bars = SD.

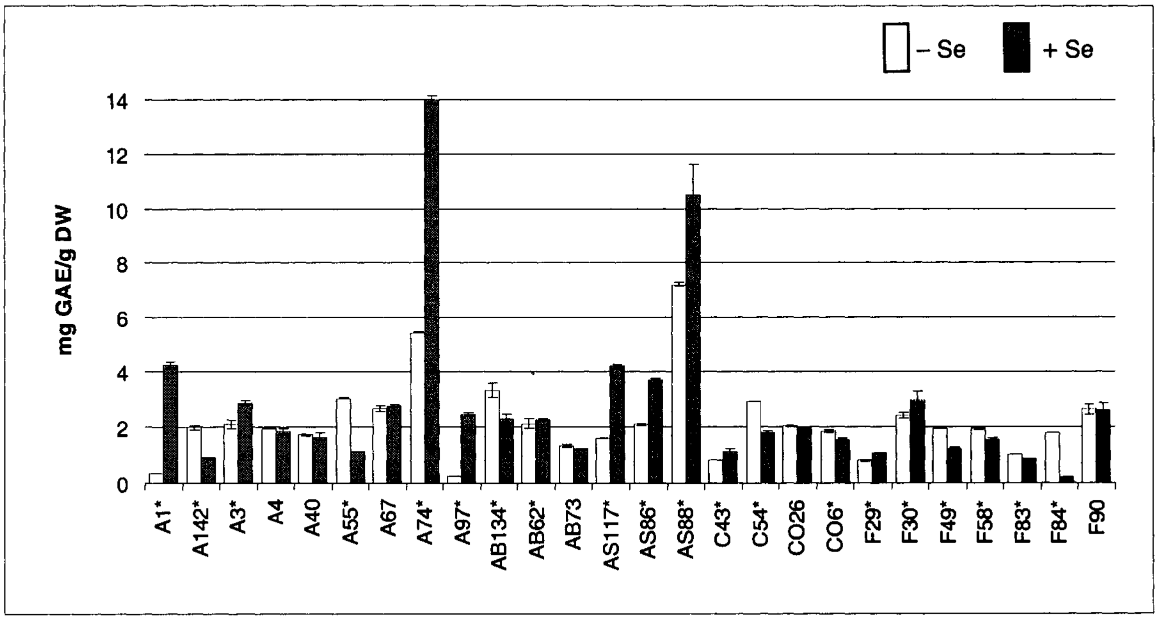


Figure 8. Total phenolic content of fungal isolates grown with and without Se expressed as Gallic Acid equivalents. Abbreviations for fungal isolates are in Table 1. Asterisks designate differences that are significant ($p \leq 0.05$), error bars = SD.

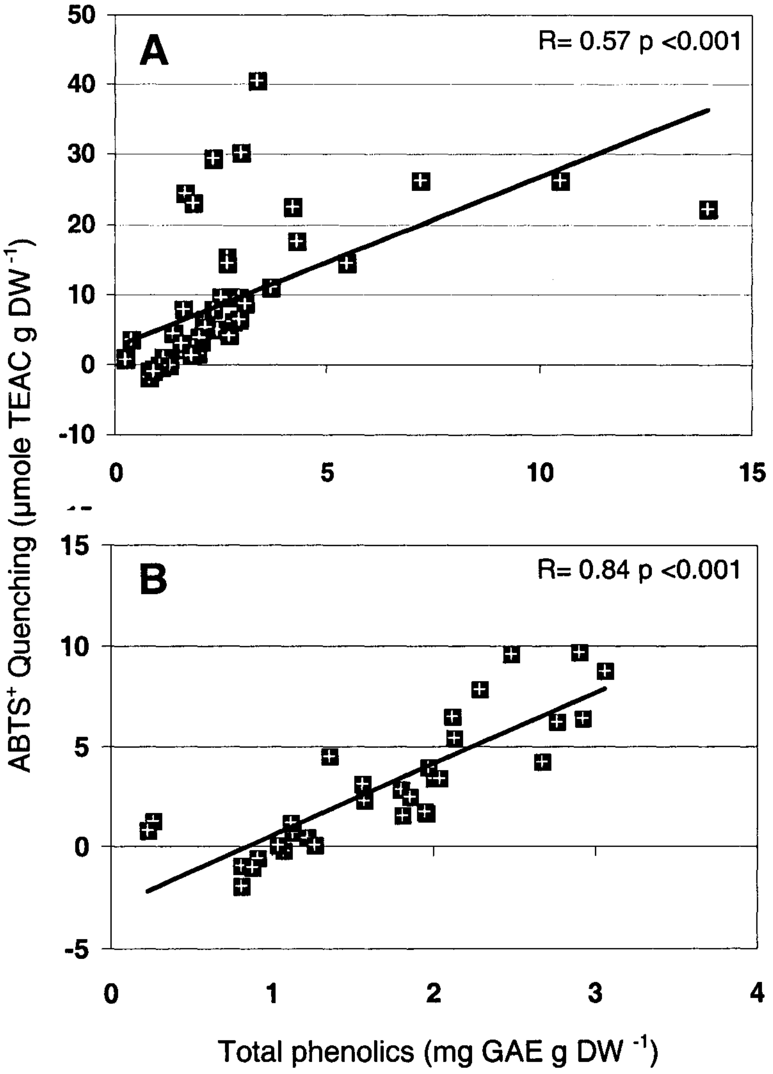


Figure 9. (A) Correlation of ABTS$^+$ quenching to phenolic content for all fungal isolates; (B) correlation of ABTS$^+$ quenching to phenolic content after all isolates with at least one treatment that had high quenching (> 10 µmole TEAC g DW^{-1}) are removed.

TE g^{-1} DW in quenching compounds for either treatment, the correlation coefficient R increases to 0.84 ($p<0.001$, Figure 9B).

A representative group of isolates with increased quenching in the presence of Se, along with a few isolates with a negative Se response were selected to continue the characterization experiments (Table 4, BOLD).

The isolates showed variable tolerance to Se, but most had obvious positive or negative growth differences between the control and Se treatments (Figure 10, Figure 11, Appendix B). There were statistically significant differences between +/- Se treatments for all fungi except for *Alternaria seleniiphila* (A1, Figure 10A) and *Alternaria tenuissima* (A97, Figure 10F) which had no difference between the –Se and 30 mg Se L^{-1} treatments. *Fusarium acuminatum* (F30, Figure 10K, Figure 11) was stimulated by Se and grew significantly better when grown on media containing Se. Conversely, three of the isolates, *Alternaria astragali* (A3), *Embellisia* (A74) and *Fusarium oxysporum* (F84), had significant reductions in colony size with increasing Se concentration (Figure 10B, 10E, 10L, Figure 11). *Alternaria tenuissima* (A97, Figure 10F) and *Penicillium anatolicum* (AS88, Figure 10I, Figure 11) had reductions that were not as obvious morphologically, but growth was significantly inhibited when compared to the control (only $\geq$ 300 mg Se L^{-1} for A97).

Additionally, for some of the isolates the Se supplementation resulted in obvious macroscopic morphological changes when compared to the control colony; the morphological differences were seen in both pigmentation and/or density of the mycelium (Figure 10). The impact of Se on the morphology was

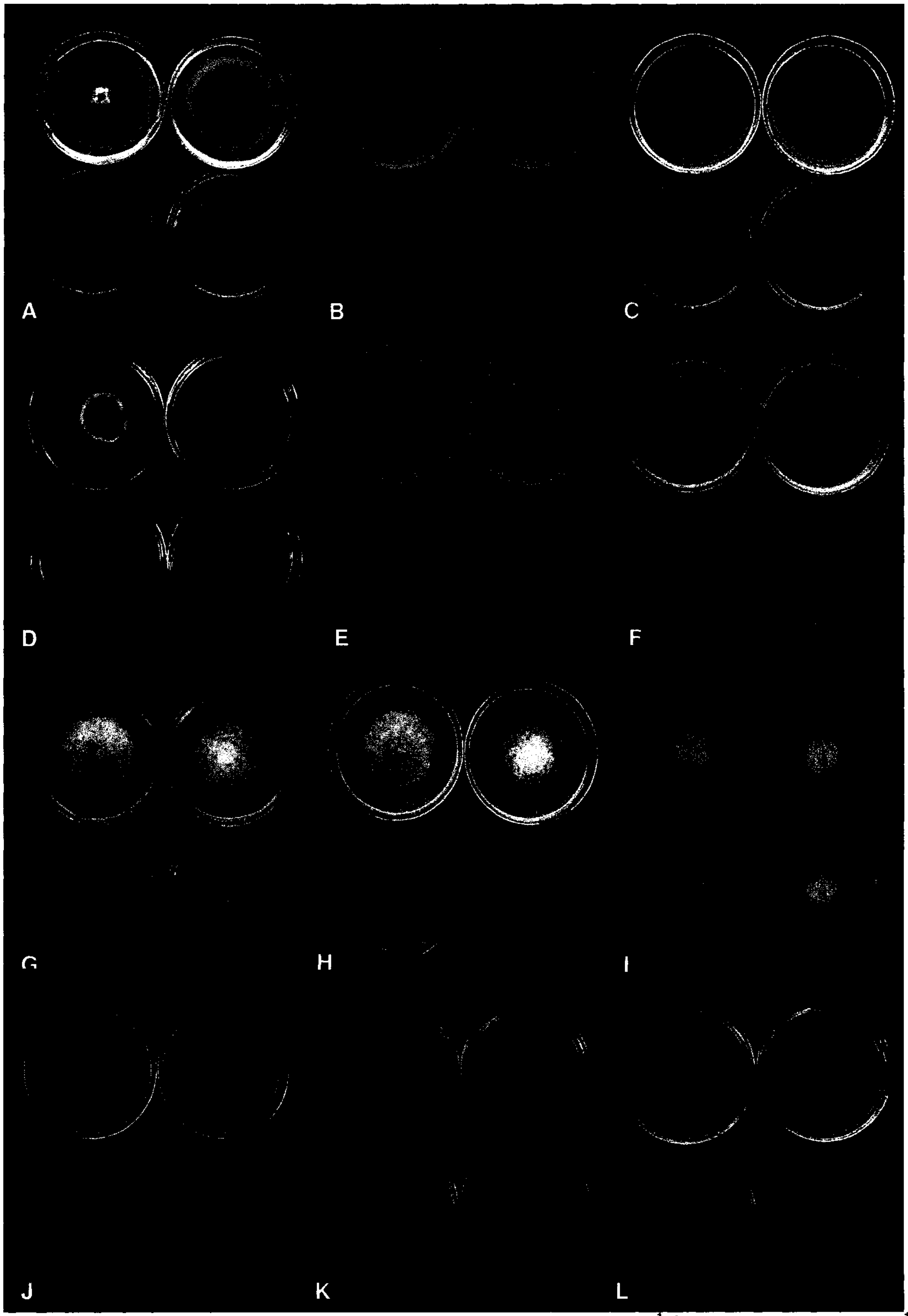


Figure 10. Fungal isolates grown without Se (top left), with 30 mg L^{-1} Se (top right), 300 mg L^{-1} Se (lower left) or 600 mg L^{-1} Se (lower right) after 7 days growth. (A) A1, *Alternaria seleniiphila*; (B) A3, *Alternaria astragali*; (C) A4, *Ulocladium atrum*; (D) A55, *Dreshlera biseptata*; (E) A74, *Embellisia* sp.; (F) A97, *Alternaria tenuissima*; (G) A62, *Absidia spinosa*; (H) A134, *Absidia spinosa*; (I) AS88, *Penicillium anatolicum*; (J) AS117, *Aspergillus leporis*; (K) F30, *Fusarium acuminatum*; (L) F84, *Fusarium oxysporum*.

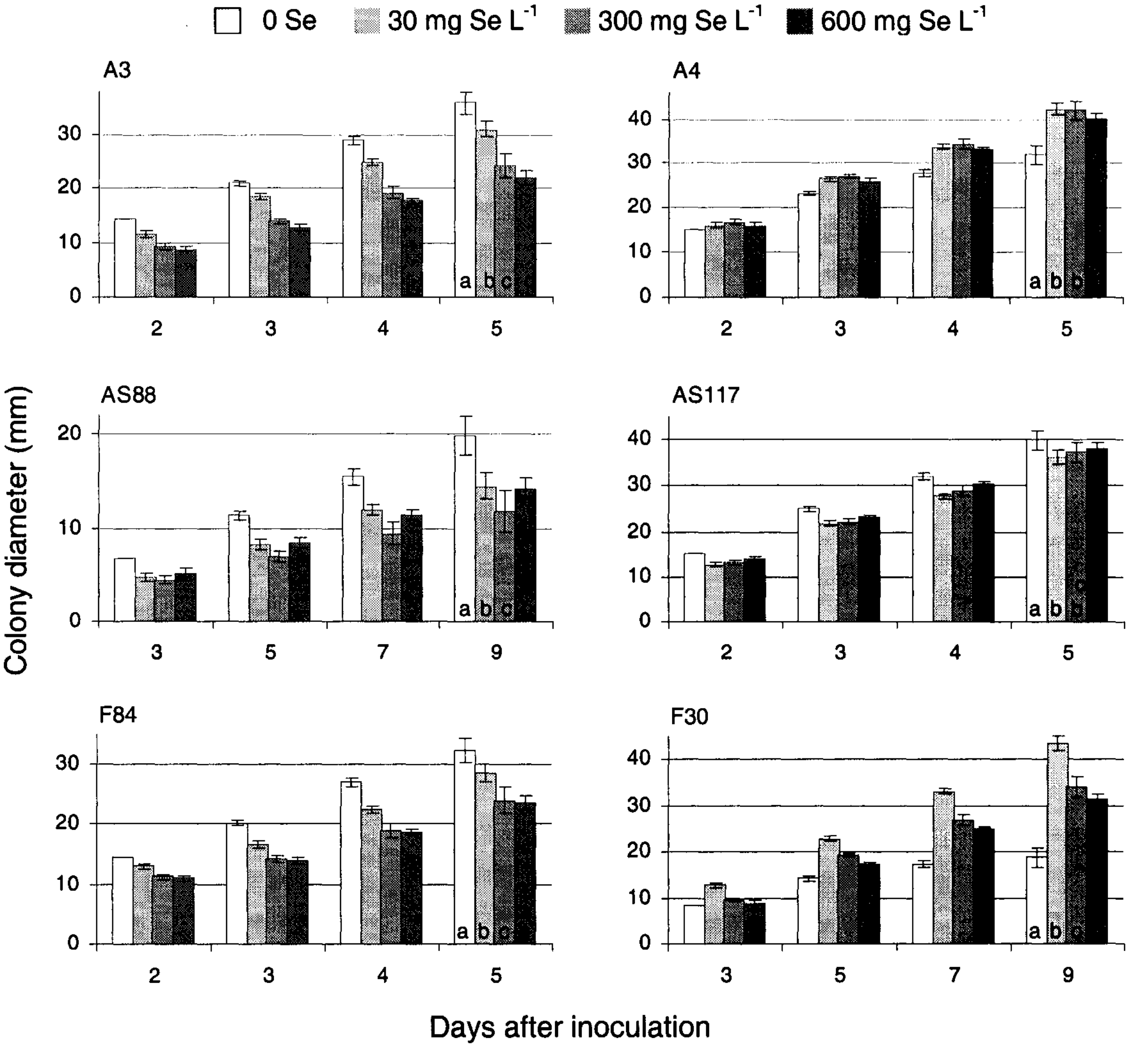


Figure 11. Selected Se tolerance growth charts for related isolate pairs. Isolates in left column (A3, AS88, F84) are adversely affected by Se, whereas isolates in the right column (A4, AS117, F30) are either unaffected or stimulated by the Se. Letters in the bars indicate significant differences for different letters ($p \leq 0.05$), error bars = SD. Remaining isolate growth charts shown in Appendix B.

the most pronounced in the *Absidia* cultures which produced no aerial hyphae when grown with Se (Figure 10G and 10H). The loss of aerial hyphae may imply that the observed increase in colony diameter in the presence of Se is not an indication of tolerance in *Absidia*. The *Dreshlera* colony grew significantly worse on the Se-containing medium and also showed a morphological effect, changing from a dense, pigmented colony to a sparse, translucent colony (Figure 10D). All growth charts for Se tolerance are shown in Appendix B.

The Se volatilization of these cultures was determined qualitatively by comparing the control culture (no Se) to the highest Se treatment (600 mg Se L^{-1}) and results are given in Table 5. *Fusarium* isolates had no noticeable Se odor, while *Aspergillus leporis* (AS117) released an extreme amount of Se. The remaining isolates all volatilized at an intermediate level (+) or at an intermediate/high level (++), in the case of *Penicillium* and *Alternaria tenuissima*.

All isolates, with the exception of *Aspergillus leporis* (AS117), accumulated Se when grown in a Se-containing medium (Table 5). Most of the isolates bioconcentrated Se to rather large amounts, up to 25 fold in *Ulocladium* relative to the growth medium. Apart from *Aspergillus*, the least Se accumulation was observed in the *Absidia* and *Penicillium* cultures.

The effects of UV exposure on the isolates grown with and without Se were studied to provide insight into the interaction of Se with UV-related oxidative stress. *Alternaria seleniiphila* (A1) and *Alternaria astragali* (A3) had significantly higher UV tolerance when grown with Se than without; the cultures recovered to or exceeded the growth of the control six days after UV exposure

Table 5. Volatilization and accumulation of Se in fungal isolates.

ID#	**Scientific Name**	**Se Volatilization**		**Se Accumulation** (μg Se g^{-1} DW)			
		+ Se (0 – +++)[a]	– Se	30ppm Se	Dip[b]	30ppm Se – Dip	0ppm Se
A1	*Alternaria astragalii*	+	0	831.92	100.86	731.06	0.71
A3	*Alternaria seleniiphila*	+	0	715.77	169.92	545.85	1.44
A4	*Ulocladium atrum*	+	0	794.15	2.85	791.30	0.41
A55	*Dreschlera biseptata*	+	0	623.74	164.74	459.00	0.67
A74	*Embellisia sp.*	+	0	301.67	30.56	271.11	6.80
A97	*Alternaria tenuissima*	++	0	729.90	120.27	609.63	1.25
AB134	*Absidia spinosa*	+	0	98.39	0.58	97.81	1.71
AB62	*Absidia spinosa*	+	0	46.37	1.87	44.50	0.27
AS117	*Aspergillus leporis*	+++	0	74.39	83.93	-9.54	2.05
AS88	*Penicillium anatolicum*	++	0	126.66	4.16	122.50	0.50
F30	*Fusarium acuminatum*	0	0	286.81	2.79	284.02	1.21
F84	*Fusarium oxysporum*	0	0	281.13	2.75	278.38	1.97

[a] The isolates were given a rating as follows: 0 for no detectable Se, + for low level Se odor, ++ for potent Se odor, +++ for extreme Se odor (which was able to be detected while the Petri dish was sealed).

[b] Dip treatment was isolate grown without Se then transferred to a 30ppm Se solution for 10 min, then rinsed with water before harvesting to account for Se attached to the cell walls but not actually accumulated

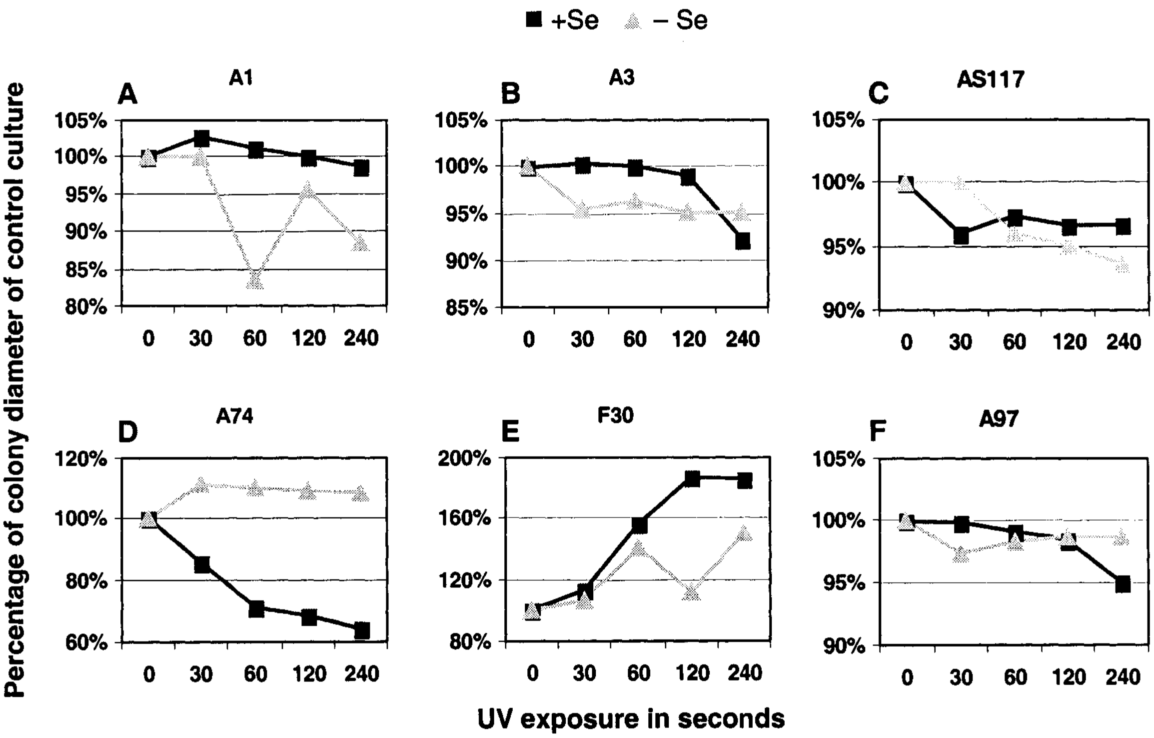


Figure 12. Recovery of selected fungal isolates grown with (30 mg L^{-1}) and without Se six days after exposure to UV light when compared with their control (no UV exposure). All comparisons between + and – Se for an isolate at a single UV exposure are significantly different for colony diameter (as displayed in Appendix D). Data are displayed as percentage of control to highlight the recovery trend, and are not analyzed statistically in this format.

(Figures 12A and 12B). In comparison, *Embellisia* (A74) had faster recovery in the cultures without Se (Figure 12D). *Fusarium acuminatum* (F30) was unique among the isolates in that both the + and – Se treatments were stimulated by the UV light well beyond the growth observed in the control, with +Se growing fastest (Figure 12E). In both *Aspergillus leporis* (AS117) and *Alternaria tenuissima* (A97) the effects appear to be UV dose dependent (Figures 12C and 12F). In A97, the culture grown with Se fared better with the two lower doses of UV, (30s and 60s) while the culture without Se had a better recovery for the two higher doses. In contrast, the – Se treatment for AS117 had better recovery at the lowest UV dose, while the + Se treatment recovered better for the remaining UV doses.

The effect of UV on the less Se tolerant *Absidia* cultures was cumulative for AB62 with the negative impact of the Se. The AB62 cultures grown with Se and exposed to 240 s UV exhibited severely reduced growth (Figure 13A). The AB134 culture also was negatively impacted by both Se and UV light, but the combined treatment showed little additive negative effect (Figure 13B).

Appendix C (percentage of control at day 6) and Appendix D (complete growth chart) contain charts for fungal responses to UV treatments. The remaining cultures all showed significant differences (+Se treatment resulted in better recovery for AS88, whereas +Se resulted in worse recovery for A4 and F84 at 60s and 240s), between the + and – Se for each UV treatment at day 6 with the exception of A55 for all UV exposure times and F84 for 30s and 120s (Appendix D).

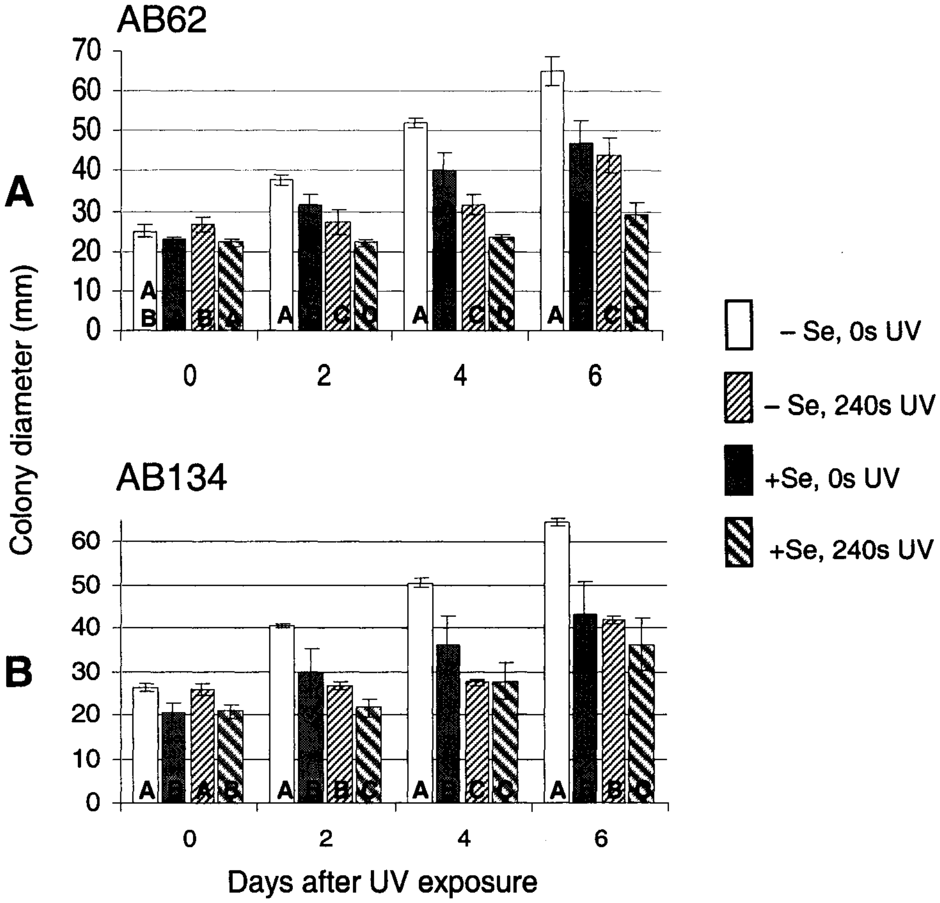


Figure 13. Growth charts for *Absidia* cultures after UV exposure. Significant differences are shown as different letters within bars ($p \leq 0.05$, error bars = SD). Growth charts for remaining isolates are shown in Appendix D.

Table 6. Correlations (R) between various groupings of the twelve selected isolates (A1, A3, A4, A55, A74, A97, AB62, AB134, AS88, AS117, F30, F84) for different assays. P value shown for correlations that were significant. TEAC = Trolox equivalent antioxidant capacity, Phenolics=Total phenolics, Se tolerance= colony diameter after 5 days growth without Se or with 30 mg Se L^{-1}, UV 120s = diameter after 6 days growth post exposure to UV light for 120 seconds, either without Se or with 30 mg Se L^{-1}. High quenchers = (>10µmole TEAC g DW^{-1}); Low quenchers = (<10µmole TEAC g DW^{-1}); Dark pigmented isolates = (A1, A3, A4, A55, A74, A97); Light or brightly colored pigmented isolates = (AB62, AB134, AS88, AS117, F30, F84).

	TEAC vs. Phenolics		TEAC vs. Se tolerance	TEAC vs. UV recovery		Phenolics vs. Se tolerance		Phenolics vs. UV recovery		UV recovery vs. Se tolerance	Se accumulation vs. UV recovery	
Group	**R**	**P**	**R**	**R**	**P**	**R**	**P**	**R**	**P**	**R**	**R**	**P**
All isolates	0.5075	0.0114	-0.1480	-0.6148	0.0014	-0.4847	0.0157	0.6542	0.0005	-0.2865	0.4167	0.0428
All + Se treatments	0.4437		0.0528	-0.6408	0.0247	-0.6225	0.0306	0.6128	0.0341	-0.4726	0.5947	0.0414
All – Se treatments	0.6334	0.0270	-0.4063	-0.7360	0.0064	-0.4167		0.7062	0.0103	-0.2155	0.2314	
High Quenchers	-0.2254		-0.0463	-0.7750	0.0085	-0.6236	0.0540	0.8285	0.0031	-0.2504	0.5201	
Low quenchers	0.7808	0.0010	0.1187	-0.3243		-0.1457		0.4417		0.0457	0.4573	
Dark pigmented isolates	0.6616	0.0191	-0.1887	-0.7293	0.0071	-0.4302		0.5363		0.1125	0.6825	0.0145
Light or brightly colored pigmented isolates	0.5104		0.0116	-0.6090	0.0356	-0.6194	0.0317	0.6755	0.0159	-0.3474	0.0738	

The twelve selected isolates were further evaluated to examine correlations between the various assays conducted. The results suggest several interesting interactions (Table 6). The isolates were examined as one group (all) and also separated into three different paired groupings: 1) + Se treatments or – Se treatments, 2) high (>10μmole TE g DW^{-1}) or low (<10μmole TE g DW^{-1}) ROS quenchers, and 3) dark (black or brown) or light (not black or brown) pigmentation. There were no significant correlations for any group between Se tolerance (after 5 days of growth) and TEAC or UV recovery (at 6 days post 120 s exposure). The correlations between TEAC and UV recovery, as well as between total phenolics and Se tolerance were both negatively correlated, i.e. the greater the amount of phenolics, the worse Se tolerance, while the relationship between total phenolics and UV recovery was positive, i.e. the more phenolics, the faster the recovery from the UV exposure. Interestingly, the dark pigmented isolates did not show a correlation between total phenolics and either Se tolerance or UV recovery as would have been anticipated due to the presence of melanins in the cell walls. Conversely, the light pigmented isolates showed significant correlations for both interactions. Se accumulation was positively correlated with all of the other measured parameters, but only significantly for UV recovery (p=0.0474). In relation to Se accumulation, positive significant correlations with UV recovery were seen for the entire group as well as the dark pigmented isolates. No other correlations with Se accumulation were found to be significant.

Discussion

About half of the fungal isolates (13 of 27) showed an increase in ROS quenching when grown in the presence of Se compared to the –Se treatment as evaluated by the TEAC assay. Some possible causes of this phenomenon are: 1) the fungi utilize Se as a component of a radical quenching mechanism, perhaps in seleno-enzymes such as in GPX that are analogous or homologous with those found in animals; 2) the fungi have quenching components (enzymes or otherwise) that are activated by the Se (as reported in potato and lettuce) where the result is a protective function (Xue *et al.* 2001, Seppänen *et al.* 2003); or 3) the fungi up-regulate their quenching response to combat the toxic/damaging effects of Se on hyphae and cytoplasm. Since interaction between ROS and Se has not been evaluated previously in these fungi, further experiments were conducted to provide additional information that might explain the causative relationship.

The second step in this evaluation was examination of phenolic compounds, since phenolic content in both plants and fungi often is cited as the primary contributor to their antioxidant capacity (Cheung *et al.* 2003, Javanmardi *et al.* 2003, Shon *et al.* 2003). The correlation between the quenching capacity and the total phenolics for the entire isolate group as a whole was highly significant but weak, at 0.57 ($p<0.001$, Figure 9A), yet when the exceptional quenchers, those that have at least one treatment with 10 μmole TE g DW^{-1} or higher, are removed, the correlation increases to 0.84 ($p<0.001$, Figure 9B). This may suggest that most of the quenching seen in the isolates with less than 10 µmole TE g DW^{-1} could be attributed to phenolic compounds. This supports hypothesis 1 (H_1) that quenching would be correlated with phenolic content. Conversely, the high level quenchers may be utilizing phenolics, but phenolics do not appear to account for the majority of the quenching machinery, as has been shown in some lichens (Odabasoglu *et al.* 2005). Other possible contributors could include enzymes (with or without seleno-amino acids), glutathione, Vitamins C or E, pigment-based quenchers such as carotenoids or other unknown mechanisms. The extraction methods performed on these samples allowed any of the above compounds to retain at least some activity. However, since the change in quenching ability seems to increase with Se in several cases, the enzymes that use Se, are activated by Se, or detoxify Se seem likely candidates to account for the high levels of quenching.

Regardless of whether the Se is being used in the fungi in a beneficial or detrimental way, there should be Se present in the fungus causing the reactions observed in the TEAC and TP assays. Indeed, this was the case for the select isolate group (Table 4, BOLD) with the exception of the *Aspergillus leporis* (AS117) isolate. This isolate had the highest level of Se volatilization, thereby explaining the lack of Se in the tissue. For Na_2SeO_4 to become a volatile form of Se in biological tissue, it must be converted in some way. This typically occurs in Se hyperaccumulator and non-accumulator plants via the sulfate assimilation pathway, leading to methylation of organic Se compounds or via other Se specific pathways yet to be elucidated in these species (Whanger 2004, Sors *et al.* 2005b). Fungi also are thought to utilize sulfate transporters to take up selenate, followed by the sulfate assimilation pathway (Tweedie and Segel, 1970). There also is evidence, based on the genetic code, that some filamentous fungi can specifically code for selenocysteine in proteins (Hatfield *et al.* 1992); this process involves a divergence from the sulfate assimilation pathway to a more Se specific pathway.

Overall, the results of this study begin to reveal how these fungal isolates deal with the occurrence of Se in their habitat. Based on this initial characterization, the isolates studied separate into three generalized groups.

The first group (A1, A4, A97 and F30) seems to benefit from, utilize or metabolize the Se in a non-harmful way. This group includes the isolates that grow faster in the presence of Se as well as those that have an increase in ROS quenching and TP without sacrificing growth. AS117 can be added to this group because, even though its growth was impacted slightly negatively by the Se, the growth reduction was relatively small compared to the concentrations that the fungus can tolerate (and likely volatilize).

In the second group (A3, A55, A74, AS88, F84) growth seems to be negatively impacted by Se, based on an increase in ROS quenching and/or TP at the cost of colony growth or a reduction in quenching and/or TP in addition to a reduction in growth.

The final group (AB134, AB62) contains isolates that have not revealed clear answers as to how they deal with the Se

To evaluate the legitimacy of the isolates as grouped above, the isolates were grown in the presence of Se and then exposed to UV light to induce

ROS, which potentially cause oxidative stress and cellular damage. Indeed, three of the four fungi that seemed to utilize Se to their benefit confirmed that Se protected them from the damaging effect of the UV light, as demonstrated by faster recovery compared to the same isolate grown without Se. These data support hypothesis 2 (H_2), that the fungi can use/benefit from Se as evidenced by recovery from UV light induced damage.

Are there characteristics that are shared by the selenophilic isolates that are lacking in the selenophobic isolates? The twelve experimental isolates were grouped based on various characteristics and then analyzed for correlations between the various assays and responses. Interestingly, the dark pigmented isolates, those that presumably contain melanins which have been shown to enhance stress tolerance, metal tolerance, and quenching of ROS (Fogarty and Tobin 1996, de Cássia *et al.* 2005), do not show a correlation between phenolic content and Se tolerance or UV recovery; however, they do show a positive correlation with Se accumulation. While the lack of correlation with phenolics may be unexpected in one sense, on the other hand many of the dark pigmented fungi are in the group that seems to benefit from the Se. Thus, if they are truly using the Se to combat the stress of the UV exposure, then no increase in phenolics would be anticipated. Both the light pigmented isolates and the group as a whole show a significant correlation between phenolic content and UV recovery suggesting that for many of the isolates studied, phenolic compounds may play a role in the ability of the fungi to recover after UV exposure, though many other compounds also are likely involved.

Se accumulation is significantly positively correlated with UV recovery for the fungal group as a whole, the + Se treatements and the dark pigmented isolates. This correlation is likely the strongest evidence presented that many of these fungi take up the Se and then utilize it in some way to combat oxidative stress, leading to a faster recovery from damage induced by UV light.

Another interesting comparison is seen in the correlation between TEAC and total phenolics for the groups separated by their quenching ability. Similar to what was discussed previously for the large group of isolates, within the twelve experimental isolates, the high quenchers show no correlation while in the low quenchers, TEAC is highly positively correlated with phenolics (R=0.78, p=0.001). These data suggest that the ability to have a high quenching capacity requires utilization of compounds other than phenolics.

Lastly, even though the correlations were not significant, it is worth noting that the low quenchers show a positive correlation between Se tolerance and both TEAC and UV recovery, whereas the high quenchers showed an inverse correlation for both relationships. This suggests that organisms with high levels of antioxidant capacity (TEAC) deal with Se and UV damage in a different way from those with low TEAC, as was demonstrated by pairs of related organisms with different quenching abilities (A1 and A55, F30 and F84).

Based on the tolerance, accumulation and apparent metabolization and utilization of Se by some of the isolates presented in this study, further research is warranted to investigate the presence of selenoenzymes in filamentous fungi. Additionally, research is needed to further elucidate how the Se is actually being utilized by these fungi to combat oxidative stress. Better insight into the mechanisms involved may eventually lead to applications of protection for various organisms from oxidative stress related cellular damage that can lead to disease and death.

Summary of discussions and conclusions

Selenium has become increasingly prominent in research over the last thirty years after the discovery of its essentiality in animals and the repeated Se contamination incidents involving blatant deformity and death. The three major groups of organisms typically studied in relation to Se are: humans for anticarcinogenesis, livestock for Se supplementation in areas of deficiency, and plants, mainly for phytoremediation. A group that is missing from this list is fungi. In spite of the fact that fungi are readily available, relatively fast growing and easy to manipulate, there is still a lack of information on the fungal domain as a whole, with some exceptions of crop or human pathogens.

The results from the survey of the fungi isolated from the Se hyperaccumulators were both interesting and unexpected. Since the concentration of Se typically found in the roots of Se hyperaccumulators is generally so much higher than the concentration found in the soil (Galeas *et al.* 2007), I anticipated much greater selection pressure resulting in diversity differences as well as tolerance differences between fungi isolated from Se hyperaccumulators and non-accumulators. The results show that neither hypothesis was supported. While there is more diversity in the non-accumulators, there is still quite a bit of fungal diversity in the Se hyperaccumulator rhizospheres, and many of the genera, if not species, overlap. In addition, I anticipated finding unique fungi due to the high toxicity of Se. While I did find two new species, the majority of the isolates are common species with uncommon abilities to tolerate and possibly use the Se. *Aspergillus leporis* is a great example; this species is Se tolerant when isolated from seleniferous habitat and Se sensitive when isolated from a non-seleniferous environment.

As for the metabolic studies, the fungi that are Se tolerant and that possibly utilize Se have great potential, both for further research and for promising applications of antioxidant use, including anti-cancer treatments as well as expansion of growth conditions for economically important fungi, by increasing their stress tolerance. In addition, the fungi may play a role in the hyperaccumulation of Se by plants. Evidence for this includes the fact that Se hyperaccumulators grown in the greenhouse typically do not accumulate Se to the levels observed in the field and that mycorrhizal fungi have been shown to increase the movement of selenate into a non-accumulating plant by 10 fold. Furthermore, the use of fungi in remediation applications could have a significant impact on the ability and time frame required for phytoremediation, translating to less money spent for a cleaned area.

Overall, this research has laid the groundwork for various paths of study involving the relationship of filamentous fungi and Se, while demonstrating the possibilities and the essential, if often invisible, role that these amazing organisms play in the circle of life.

Ami L. Wangeline, March 2007

My-cology is better than your -cology

Appendix A. All fungal isolates from seleniferous sites.

Study ID (used in dissertation and publications)	Scientific name (note: some isolates identified to genus may be the same species)	Collection site	Host plant	Host plant accumulates Se?	Isolation agar (+ or – Se)	Orignial sample ID	Fungal inhibition on 10 mg L-1
AB73	Absidia	Casper	Astragalus sp	N	+	02-D	0
	Absidia	Casper	Astragalus sp	N	+	30-A-2	1
	Absidia	Casper	Astragalus sp	N	+	32-A	1
AB60	Absidia	Fort Collins	S. pinnata	Y	–	65-D	0
AB61	Absidia	Fort Collins	S. pinnata	Y	–	65-F	0
	Absidia	Fort Collins	Astragalus sp	Y	–	60-A-2	1
	Absidia	Fort Collins	Astragalus sp	Y	+	61-D	1
	Absidia	Fort Collins	S. pinnata	Y	+	62-E	1
	Absidia	Fort Collins	A. bisulcatus	Y	–	67-B	0
	Absidia	Fort Collins	A. bisulcatus	Y	–	73-A	1
	Absidia	Fort Collins	A. bisulcatus	Y	–	74-A	1
	Absidia	Fort Collins	A. bisulcatus	Y	+	75-C	1
	Absidia	Fort Collins	A. bisulcatus	Y	–	76-A	1
	Absidia	Fort Collins	A. bisulcatus	Y	+	78-A	1
	Absidia	Fort Collins	A. bisulcatus	Y	–	79-B	1
	Absidia	Fort Collins	S. pinnata	Y	–	83-A	1
	Absidia	Fort Collins	S. pinnata	Y	–	84-A	1
	Absidia	Fort Collins	S. pinnata	Y	–	85-A	1
	Absidia	Fort Collins	A. bisulcatus	Y	+	86-C	1
	Absidia	Fort Collins	A. bisulcatus	Y	+	87-C	1
	Absidia	Fort Collins	A. bisulcatus	Y	+	88-B	1
	Absidia	Fort Collins	A. bisulcatus	Y	+	88-C	1
	Absidia cylindrospora	Lysite	A. racemosus	Y	–	41-C-2	1
	Absidia spinosa	Casper	Astragalus sp	N	–	20-A	1
	Absidia spinosa	Casper	Astragalus sp	N	–	40-D	1
AB134	Absidia spinosa	Fort Collins	A. bisulcatus	Y	+	75-E	0
AB62	Absidia spinosa	Fort Collins	A. bisulcatus	Y	–	67-A	0
	Absidia spinosa	Lysite	A. racemosus	Y	–	23-A	1
	Alternaria	Casper	Astragalus sp	N	–	25-B	0
	Alternaria	Casper	A. bisulcatus	Y	+	31-B	0
	Alternaria	Casper	A. bisulcatus	Y	+	31-F	0
A103	Alternaria	Fort Collins	S. pinnata	Y	–	84-B	0
A142	Alternaria	Fort Collins	S. pinnata	Y	+	89-C	0
A67	Alternaria	Fort Collins	M. sativa	N	–	71-C	0
A97	Alternaria	Fort Collins	A. bisulcatus	Y	–	76-B	0
	Alternaria	Fort Collins	M. sativa	N	–	71-A	0
	Alternaria	Fort Collins	M. sativa	N	–	71-E-1	0
	Alternaria	Fort Collins	M. sativa	N	+	82-B	0
	Alternaria	Fort Collins	S. pinnata	Y	–	83-B	0
	Alternaria	Fort Collins	S. pinnata	Y	–	84-C-2	0
	Alternaria	Fort Collins	S. pinnata	Y	–	85-B	0
	Alternaria	Fort Collins	S. pinnata	Y	+	89-D	0

Study ID (used in dissertation and publications)	Scientific name (note: some isolates identified to genus may be the same species)	Collection site	Host plant	Host plant accumulates Se?	Isolation agar (+ or – Se)	Orignial sample ID	Fungal inhibition on 10 mg L-1
	Alternaria	Fort Collins	S. pinnata	Y	+	89-E	0
	Alternaria	Fort Collins	S. pinnata	Y	+	90-D	0
	Alternaria	Fort Collins	S. pinnata	Y	+	91-A	1
A27	Alternaria	Laramie	A. bisulcatus	Y	+	19-E	0
	Alternaria	Laramie	A. bisulcatus	Y	+	05-A-G	1
	Alternaria	Laramie	A. bisulcatus	Y	+	07-B	0
	Alternaria	Laramie	A. bisulcatus	Y	–	08-A	0
	Alternaria	Laramie	A. bisulcatus	Y	+	19-B	0
	Alternaria	Laramie	A. bisulcatus	Y	–	22-A	0
	Alternaria	Laramie	A. bisulcatus	Y	–	22-C	0
	Alternaria	Laramie	A. bisulcatus	Y	–	22-D	0
A113	Alternaria	Lysite	S. pinnata	Y	+	37-B	0
A3	Alternaria astragali	Laramie	A. bisulcatus	Y	–	06-C-B	0
A1	Alternaria seleniiphila	Fort Collins	S. pinnata	Y	+	91-B	0
	Aspergillius	Fort Collins	Alyssum sp	N	+	55-C	2
	Aspergillius	Fort Collins	A. bisulcatus	Y	+	75-G	0
	Aspergillius	Fort Collins	M. sativa	N	+	80-A	0
	Aspergillius	Fort Collins	M. sativa	N	+	80-B	0
	Aspergillius	Fort Collins	M. sativa	N	+	80-C	0
	Aspergillius	Fort Collins	M. sativa	N	+	80-E	0
	Aspergillius	Fort Collins	M. sativa	N	+	81-B	0
	Aspergillius	Laramie	A. bisulcatus	Y	–	06-B-2	1
	Aspergillius	Lysite	S. pinnata	Y	–	11-D	1
	Aspergillius	Lysite	S. pinnata	Y	–	16-F	1
	Aspergillius	Lysite	S. pinnata	Y	+	35-E	0
AS116	Aspergillus leporis	Fort Collins	S. pinnata	Y	+	62-D	0
AS117	Aspergillus leporis	Fort Collins	S. pinnata	Y	+	64-A	0
AS119	Aspergillus leporis	Fort Collins	S. pinnata	Y	+	66-E	0
AS128	Aspergillus leporis	Fort Collins	Alyssum sp	N	+	55-A	0
AS64	Aspergillus leporis	Fort Collins	S. pinnata	Y	–	68-G	0
AS82	Aspergillus leporis	Fort Collins	Alyssum sp	N	+	53-C	0
AS86	Aspergillus leporis	Fort Collins	M. sativa	N	+	80-D	0
	Aspergillus leporis	Fort Collins	M. sativa	N	–	70-A	1
	Aspergillus leporis	Fort Collins	A. bisulcatus	Y	–	73-C	0
	Aspergillus leporis	Fort Collins	M. sativa	N	+	81-A	0
	Aspergillus leporis	Fort Collins	M. sativa	N	+	82-A	0
	Bipolaris	Lysite	X. glabrusicla	Y	–	13-A	0
	budding yeast	Lysite	S. pinnata	Y	–	11-E	0
	budding yeast	Lysite	S. pinnata	Y	+	37-E	0
CL125	Cladosporium	Casper	Astragalus sp	N	+	30-B	0
	Cladosporium	Laramie	A. bisulcatus	Y	–	22-B	0
	Cladosporium	Lysite	X. glabrusicla	Y	–	39-E	0
CL5	Cladosporium herbarum	Casper	A. bisulcatus	Y	–	10-D-G	0
	Coniciobolis	Fort Collins	A. bisulcatus	Y	+	88-A	0

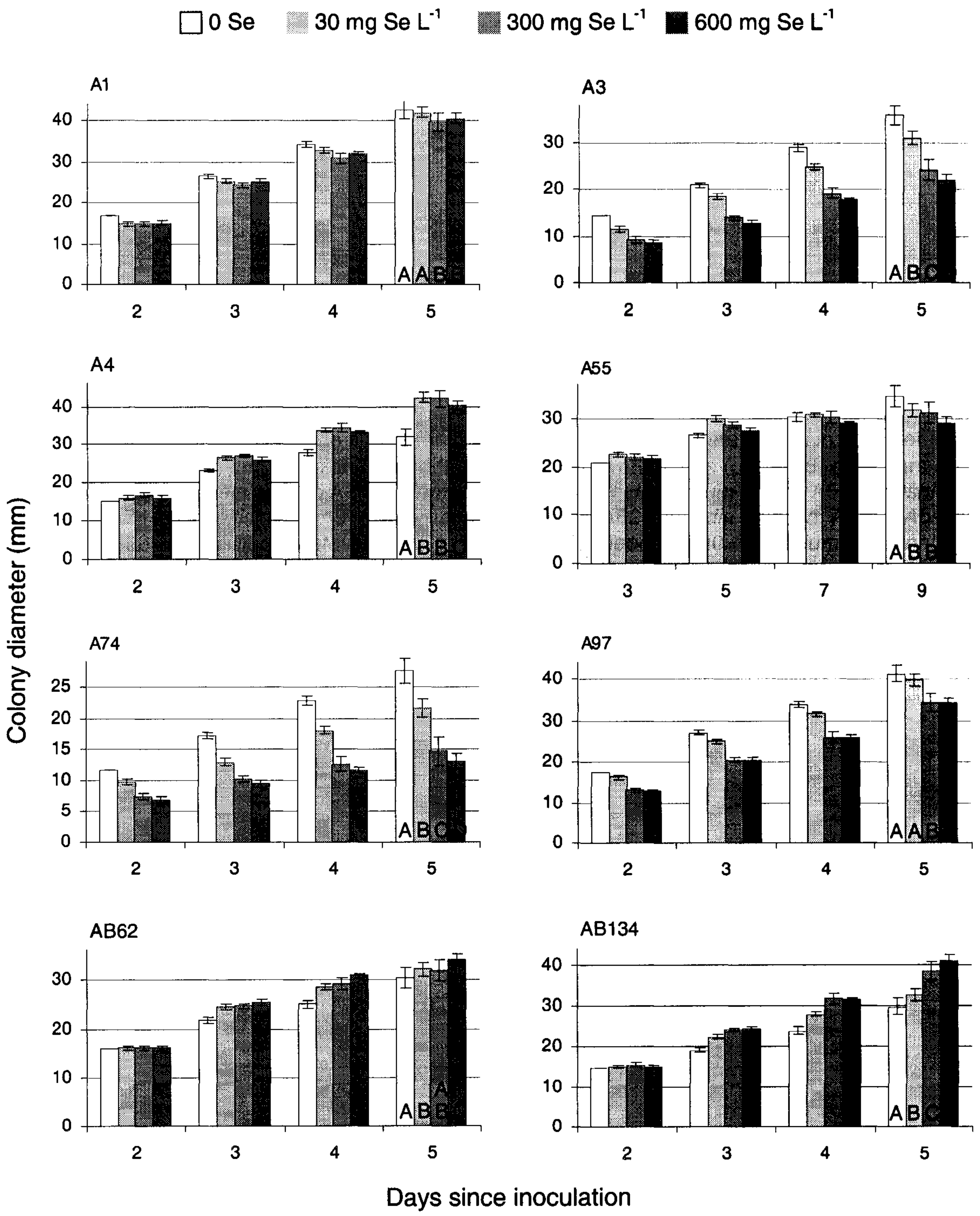
Study ID (used in dissertation and publications)	Scientific name (note: some isolates identified to genus may be the same species)	Collection site	Host plant	Host plant accumulates Se?	Isolation agar (+ or – Se)	Orignial sample ID	Fungal inhibition on 10 mg L-1
CO109	Coniothyrium	Casper	Astragalus sp	N	+	32-E	0
	Coniothyrium	Casper	A. bisulcatus	Y	+	18-E	0
	Coniothyrium	Fort Collins	Alyssum sp	N	–	54-B	2
	Coniothyrium	Fort Collins	Astragalus sp	Y	–	56-D	1
	Coniothyrium	Fort Collins	Astragalus sp	Y	–	59-E	1
CO26	Coniothyrium	Lysite	A. racemosus	Y	+	15-A	0
CO6	Coniothyrium	Lysite	S. pinnata	Y	–	11-A	0
	Coniothyrium	Lysite	S. pinnata	Y	–	16-A	0
	Coniothyrium	Lysite	Leucanthemum	N	+	26-B	0
	Cunninghamella elegans	Lysite	A. racemosus	Y	–	41-C-1	1
	Cunninghamella elegans	Lysite	A. racemosus	Y	–	41-E	1
C54	Curvularia	Fort Collins	Alyssum sp	N	–	50-A	0
	Curvularia	Fort Collins	Alyssum sp	N	+	53-D-1	1
	Curvularia	Fort Collins	Alyssum sp	N	–	54-C	0
	Curvularia	Fort Collins	Alyssum sp	N	+	55-B	0
C110	Curvularia	Lysite	Leucanthemum	N	+	34-B-2	0
C13	Curvularia	Lysite	Leucanthemum	N	–	24-D	0
C43	Curvularia	Lysite	S. pinnata	Y	–	16-C	0
	Curvularia	Lysite	Leucanthemum	N	+	34-C	0
C22	Curvularia clavata	Lysite	X. glabrusicla	Y	+	36-C	0
	Curvularia clavata	Lysite	X. glabrusicla	Y	+	03-D	0
	Curvularia clavata	Lysite	X. glabrusicla	Y	+	14-A	0
	Curvularia clavata	Lysite	Leucanthemum	N	+	34-F	0
A55	Dreschlera beseptata	Fort Collins	Alyssum sp	N	–	52-F	0
A74	Embellisia	Casper	A. bisulcatus	Y	+	09-A-Y	0
F49	Fusarium	Casper	Astragalus sp	N	–	25-C	0
	Fusarium	Casper	A. bisulcatus	Y	–	01-A-Y	1
	Fusarium	Casper	A. bisulcatus	Y	+	18-G	1
	Fusarium	Casper	Astragalus sp	N	–	20-B	0
	Fusarium	Casper	Astragalus sp	N	–	20-D	0
	Fusarium	Casper	Astragalus sp	N	–	20-F	1
	Fusarium	Casper	Astragalus sp	N	+	30-E	1
	Fusarium	Casper	Astragalus sp	N	+	32-C	0
	Fusarium	Casper	Astragalus sp	N	–	40-B	1
	Fusarium	Casper	Astragalus sp	N	–	40-C	0
	Fusarium	Casper	A. bisulcatus	Y	–	42-B	0
	Fusarium	Casper	A. bisulcatus	Y	–	42-G	0
F83	Fusarium	Fort Collins	Astragalus sp	Y	+	57-C	0
F93	Fusarium	Fort Collins	Alyssum sp	N	–	54-F	0
F98	Fusarium	Fort Collins	A. bisulcatus	Y	–	77-B	0
	Fusarium	Fort Collins	Alyssum sp	N	–	54-A	0
	Fusarium	Fort Collins	Alyssum sp	N	+	55-F	0
	Fusarium	Fort Collins	Astragalus sp	Y	+	58-C	0
	Fusarium	Fort Collins	Astragalus sp	Y	+	58-E	0

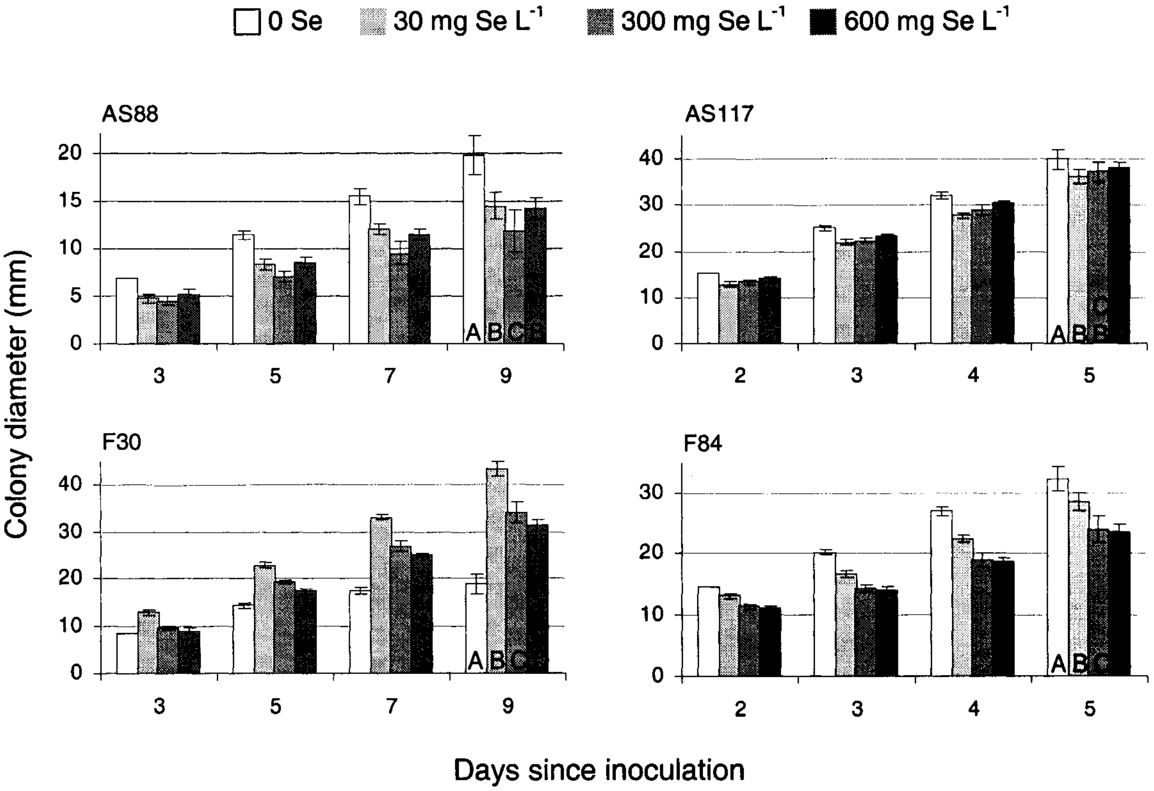
Study ID (used in dissertation and publications)	Scientific name (note: some isolates identified to genus may be the same species)	Collection site	Host plant	Host plant accumulates Se?	Isolation agar (+ or – Se)	Orignial sample ID	Fungal inhibition on 10 mg L-1
	Fusarium	Fort Collins	Astragalus sp	Y	–	60-A-1	0
	Fusarium	Fort Collins	S. pinnata	Y	–	63-A	0
	Fusarium	Fort Collins	S. pinnata	Y	–	63-B	0
	Fusarium	Fort Collins	S. pinnata	Y	–	63-D	0
	Fusarium	Fort Collins	S. pinnata	Y	–	65-B	0
	Fusarium	Fort Collins	S. pinnata	Y	+	66-H	0
	Fusarium	Fort Collins	A. bisulcatus	Y	–	67-D	0
	Fusarium	Fort Collins	S. pinnata	Y	–	68-A	0
	Fusarium	Fort Collins	S. pinnata	Y	–	68-B	0
	Fusarium	Fort Collins	S. pinnata	Y	–	68-D	0
	Fusarium	Fort Collins	A. bisulcatus	Y	+	69-C	0
	Fusarium	Fort Collins	A. bisulcatus	Y	+	75-B	0
	Fusarium	Fort Collins	A. bisulcatus	Y	–	76-D	1
	Fusarium	Fort Collins	A. bisulcatus	Y	–	77-C	1
	Fusarium	Fort Collins	A. bisulcatus	Y	+	78-C	0
	Fusarium	Fort Collins	A. bisulcatus	Y	+	78-D	0
	Fusarium	Fort Collins	A. bisulcatus	Y	–	79-A	1
	Fusarium	Fort Collins	M. sativa	N	+	82-C	0
	Fusarium	Fort Collins	S. pinnata	Y	–	84-C-1	0
	Fusarium	Fort Collins	A. bisulcatus	Y	+	86-A	0
	Fusarium	Fort Collins	A. bisulcatus	Y	+	86-B	0
	Fusarium	Fort Collins	A. bisulcatus	Y	+	86-D	0
	Fusarium	Fort Collins	A. bisulcatus	Y	+	87-A	0
	Fusarium	Fort Collins	A. bisulcatus	Y	+	87-B-1	0
	Fusarium	Fort Collins	A. bisulcatus	Y	+	87-B-2	0
	Fusarium	Fort Collins	S. pinnata	Y	+	91-D	0
	Fusarium	Laramie	A. bisulcatus	Y	–	06-D	1
F1	Fusarium	Lysite	X. glabrusicla	Y	–	04-B	0
F29	Fusarium	Lysite	A. racemosus	Y	+	28-B	0
F30	Fusarium	Lysite	A. racemosus	Y	+	28-E	0
F47	Fusarium	Lysite	A. racemosus	Y	–	21-A	0
F90	Fusarium	Lysite	S. pinnata	Y	–	16-E	0
	Fusarium	Lysite	X. glabrusicla	Y	–	17-B	0
	Fusarium	Lysite	X. glabrusicla	Y	+	27-E	0
	Fusarium	Lysite	X. glabrusicla	Y	+	27-F	0
	Fusarium	Lysite	A. racemosus	Y	+	33-A	0
	Fusarium	Lysite	A. racemosus	Y	+	33-B	0
	Fusarium	Lysite	S. pinnata	Y	+	35-A	0
	Fusarium	Lysite	X. glabrusicla	Y	–	39-D	0
	Fusarium	Lysite	A. racemosus	Y	–	41-B	0
F84	Fusarium oxysporum	Fort Collins	S. pinnata	Y	+	62-A	0
F58	Fusarium solani/ oxysporum	Fort Collins	Astragalus sp	Y	–	59-A	0

Study ID (used in dissertation and publications)	Scientific name (note: some isolates identified to genus may be the same species)	Collection site	Host plant	Host plant accumulates Se?	Isolation agar (+ or – Se)	Orignial sample ID	Fungal inhibition on 10 mg L-1
	Fusarium solani/ oxysporum	Fort Collins	Alyssum sp	N	–	50-F	0
	Geotricum	Fort Collins	Alyssum sp	N	–	50-C-2	1
	Mucor plumbeus	Lysite	Leucanthemum	N	–	12-D	1
	Mucoracious	Casper	Astragalus sp	N	+	02-B	1
	Mucoracious	Fort Collins	S. pinnata	Y	–	65-E	2
	Mucoracious	Laramie	A. bisulcatus	Y	+	05-B	0
	Mucoracious	Lysite	A. racemosus	Y	+	29-A	1
	Pacilomyces	Lysite	A. racemosus	Y	–	38-B	0
	Penicillium	Casper	A. bisulcatus	Y	–	01-C	1
	Penicillium	Fort Collins	Alyssum sp	N	–	50-D	1
	Penicillium	Fort Collins	Alyssum sp	N	–	54-D	0
	Penicillium	Fort Collins	S. pinnata	Y	+	62-B	0
	Penicillium	Fort Collins	M. sativa	N	–	70-E	0
	Penicillium	Fort Collins	M. sativa	N	–	71-D	0
	Penicillium	Fort Collins	S. pinnata	Y	–	83-C	0
	Penicillium	Laramie	A. bisulcatus	Y	–	06-B-1	0
	Penicillium	Lysite	A. racemosus	Y	+	15-E	0
	Penicillium	Lysite	A. racemosus	Y	–	21-D	0
	Penicillium	Lysite	A. racemosus	Y	–	23-E	0
	Penicillium	Lysite	Leucanthemum	N	+	26-A	1
	Penicillium	Lysite	A. racemosus	Y	+	28-D	0
	Penicillium	Lysite	A. racemosus	Y	+	29-B	0
	Penicillium	Lysite	A. racemosus	Y	+	29-B-B	0
	Penicillium	Lysite	A. racemosus	Y	+	29-D	1
	Penicillium	Lysite	S. pinnata	Y	+	35-F	0
	Penicillium	Lysite	X. glabrusicla	Y	+	36-D	0
	Penicillium	Lysite	S. pinnata	Y	+	37-A	0
	Penicillium	Lysite	A. racemosus	Y	–	38-E	0
AS88	Penicillium anatolicum	Lysite	S. pinnata	Y	–	11-B-2	0
	Penicillium/Pacilomyces	Fort Collins	Alyssum sp	N	+	51-D	0
	Penicillium/Pacilomyces	Fort Collins	S. pinnata	Y	+	90-A	0
	Penicillium/Pacilomyces	Lysite	A. racemosus	Y	+	29-E	0
	Penicillium/Talaromyces	Fort Collins	A. bisulcatus	Y	–	67-C	1
	Pyronema	Fort Collins	Alyssum sp	N	–	50-C-1	1
	Pythium	Casper	Astragalus sp	N	–	20-E	0
	Pythium	Casper	Astragalus sp	N	+	30-A-1	0
	Pythium	Casper	Astragalus sp	N	+	30-D	0
	Pythium	Casper	Astragalus sp	N	–	40-A	2
	Pythium	Casper	A. bisulcatus	Y	–	42-C	1
	Pythium	Fort Collins	A. bisulcatus	Y	+	69-D	0
	Pythium	Fort Collins	M. sativa	N	–	72-A	0
	Pythium	Fort Collins	S. pinnata	Y	+	90-B	2
	Pythium	Lysite	S. pinnata	Y	–	11-B-1	0

Study ID (used in dissertation and publications)	Scientific name (note: some isolates identified to genus may be the same species)	Collection site	Host plant	Host plant accumulates Se?	Isolation agar (+ or – Se)	Orignial sample ID	Fungal inhibition on 10 mg L-1
	Pythium	Lysite	X. glabrusicla	Y	+	14-B	0
	Pythium	Lysite	X. glabrusicla	Y	+	14-D	0
	Pythium	Lysite	S. pinnata	Y	+	35-D	1
	Pythium	Lysite	S. pinnata	Y	+	37-C	0
	Pythium	Lysite	X. glabrusicla	Y	–	39-B	2
	Rhizoctonia	Fort Collins	Alyssum sp	N	+	53-E	2
	Sclerotium	Casper	A. bisulcatus	Y	–	01-B	1
	Sclerotium	Casper	A. bisulcatus	Y	+	31-A	0
	Sclerotium	Casper	A. bisulcatus	Y	+	31-C	2
	Sclerotium	Casper	A. bisulcatus	Y	–	42-H	1
	Sclerotium	Fort Collins	S. pinnata	Y	–	68-H	0
	Sclerotium	Fort Collins	A. bisulcatus	Y	+	75-D	0
UG	Sclerotium	Lysite	S. pinnata	Y	–	11-C	0
	Sclerotium	Lysite	X. glabrusicla	Y	+	03-A	0
	Sclerotium	Lysite	X. glabrusicla	Y	–	13-D	1
	Sclerotium	Lysite	A. racemosus	Y	–	23-G	0
	Sclerotium	Lysite	Leucanthemum	N	–	24-A	0
	Sclerotium	Lysite	Leucanthemum	N	–	24-C	0
	Sclerotium	Lysite	X. glabrusicla	Y	+	27-C	2
	Sclerotium	Lysite	Leucanthemum	N	+	34-B-1	0
	Sclerotium	Lysite	S. pinnata	Y	+	35-B	0
	Sclerotium	Lysite	X. glabrusicla	Y	+	36-B	0
	Torula	Casper	A. bisulcatus	Y	–	01-D-W	0
	Tricohderma harzianum	Fort Collins	Astragalus sp	Y	–	60-C	1
	Tricohderma harzianum	Fort Collins	M. sativa	N	–	70-C	1
	Tricohderma harzianum	Fort Collins	Astragalus sp	Y	+	61-A	1
A8	Ulocladium atrum	Lysite	Leucanthemum	N	–	12-B	0
A4	Ulocladium atrum	Casper	A. bisulcatus	Y	–	10-A	0
A40	Ulocladium botrytis	Casper	A. bisulcatus	Y	–	10-B-B	0
	Unknown	Casper	Astragalus sp	N	–	20-C	0
	Unknown	Fort Collins	Alyssum sp	N	+	51-A	0
	Unknown	Fort Collins	Alyssum sp	N	–	54-E	0
	Unknown	Fort Collins	S. pinnata	Y	+	64-C	0
	unknown	Fort Collins	A. bisulcatus	Y	+	69-A	0
	Unknown	Fort Collins	M. sativa	N	–	71-E-2	0
	Unknown	Fort Collins	S. pinnata	Y	–	85-C	0
	Unknown	Fort Collins	A. bisulcatus	Y	+	87-D	2
	Unknown	Lysite	Leucanthemum	N	–	12-A	0
	Unknown	Lysite	A. racemosus	Y	+	15-D	0
	Unknown	Lysite	S. pinnata	Y	–	16-D-1	0
	Unknown	Lysite	S. pinnata	Y	–	16-D-2	2
	Unknown	Lysite	X. glabrusicla	Y	+	27-D-1	0
	Unknown	Lysite	X. glabrusicla	Y	+	27-D-2	1
	Unknown	Lysite	A. racemosus	Y	+	28-C	0

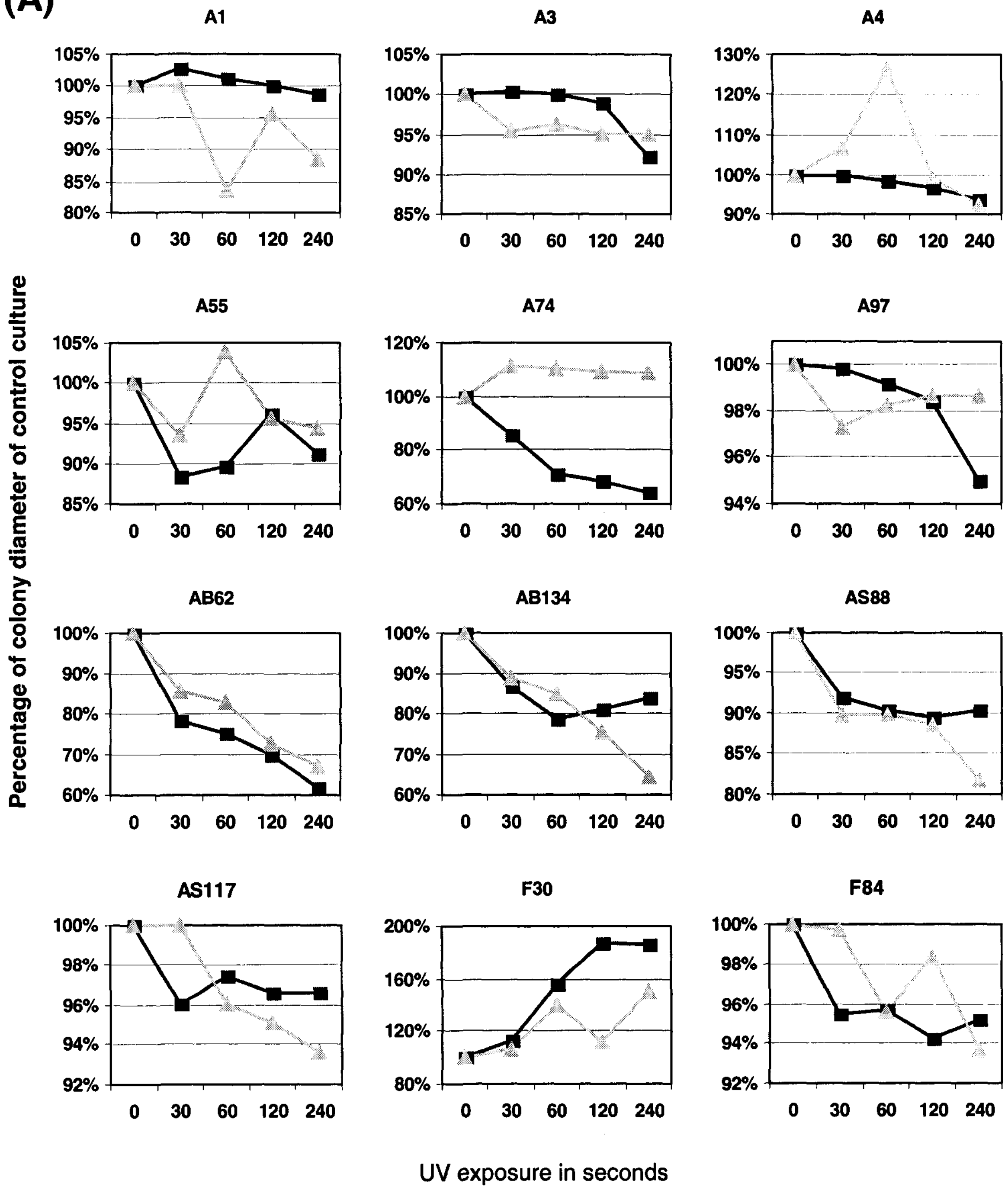
Appendix B. Se tolerance growth charts for select fungal isolates. Letters shown in bars (for final growth measurement) indicate significant differences for different letters ($p \leq 0.05$, error bars = SD). A1= *Alternaria seleniiphila*; A3= *Alternaria astragali*; A4= *Ulocladium atrum*; A55= *Dreshlera biseptata*; A74= *Embellisia* sp.; A97= *Alternaria tenuissima*; A62= *Absidia spinosa*; A134= *Absidia spinosa*; AS88= *Penicillium anatolicum*; AS117= *Aspergillus leporis*; F30= *Fusarium acuminatum*; F84= *Fusarium oxysporum*.



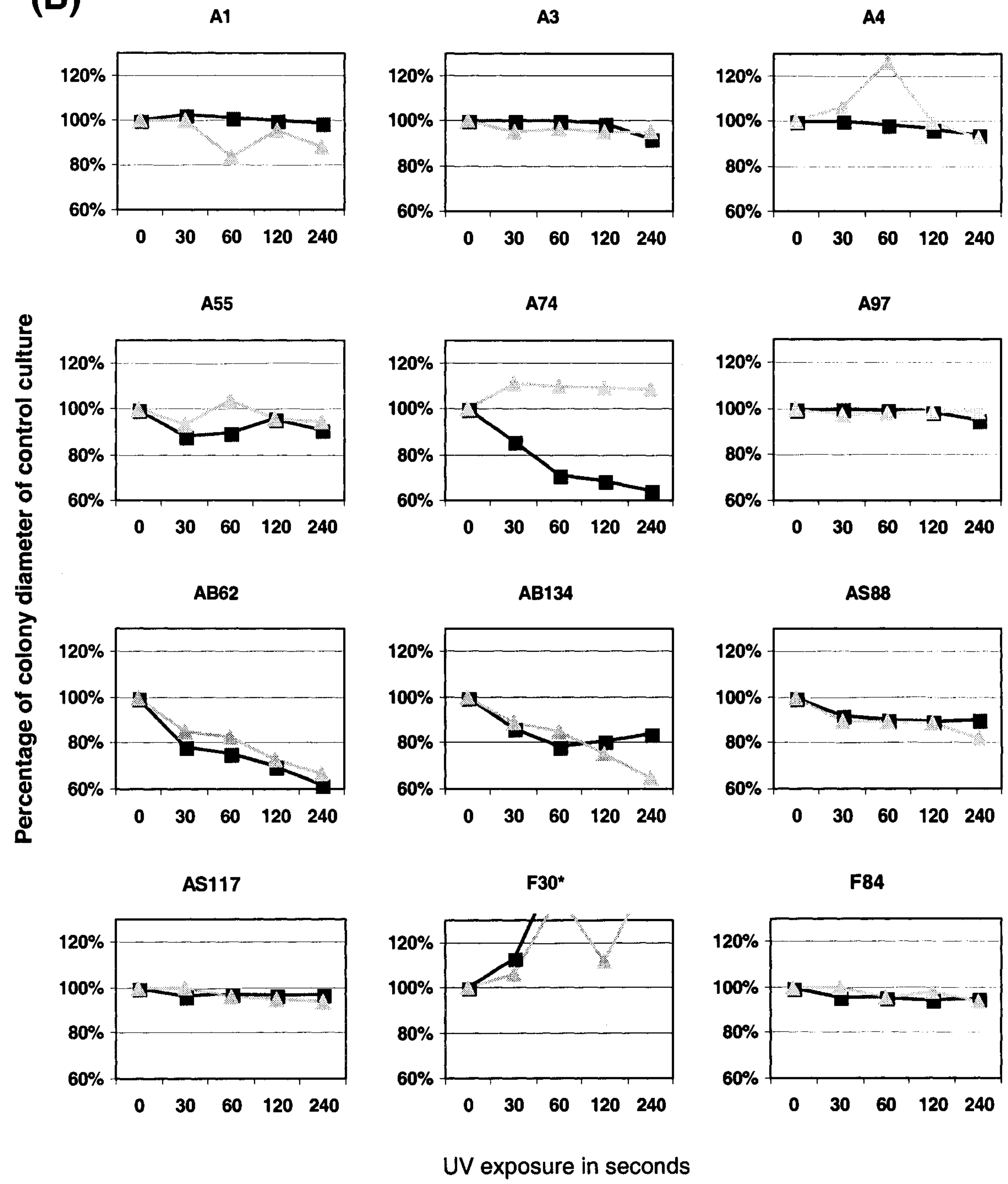

☐ 0 Se
▨ 30 mg Se L-1
▨ 300 mg Se L-1
■ 600 mg Se L-1
AS88
AS117
F30
F84
Colony diameter (mm)
Days since inoculation
0
5
10
15
20
10
20
30
40
3
5
7
9
2
3
4
5
A
B
C

Appendix C. Recovery of selected fungal isolates grown with (■) Se (30 mg Se L^{-1}, 20 mg Se L^{-1} for AB cultures) and without (▲) Se six days after exposure to UV light when compared with their control (no UV exposure).All comparisons between + and – Se for an isolate at a single UV exposure are significantly different. Shown in (A) at varying scales for + and – Se comparison with individual cultures. Shown in (B) at the same scales for comparison between cultures. A1= *Alternaria seleniiphila*; A3= *Alternaria astragali*; A4= *Ulocladium atrum*; A55= *Dreshlera biseptata*; A74= *Embellisia* sp.; A97= *Alternaria tenuissima*; A62= *Absidia spinosa*; A134= *Absidia spinosa*; AS88= *Penicillium anatolicum*; AS117= *Aspergillus leporis*; F30= *Fusarium acuminatum*; F84= *Fusarium oxysporum*. See note below regarding significance.

(A)



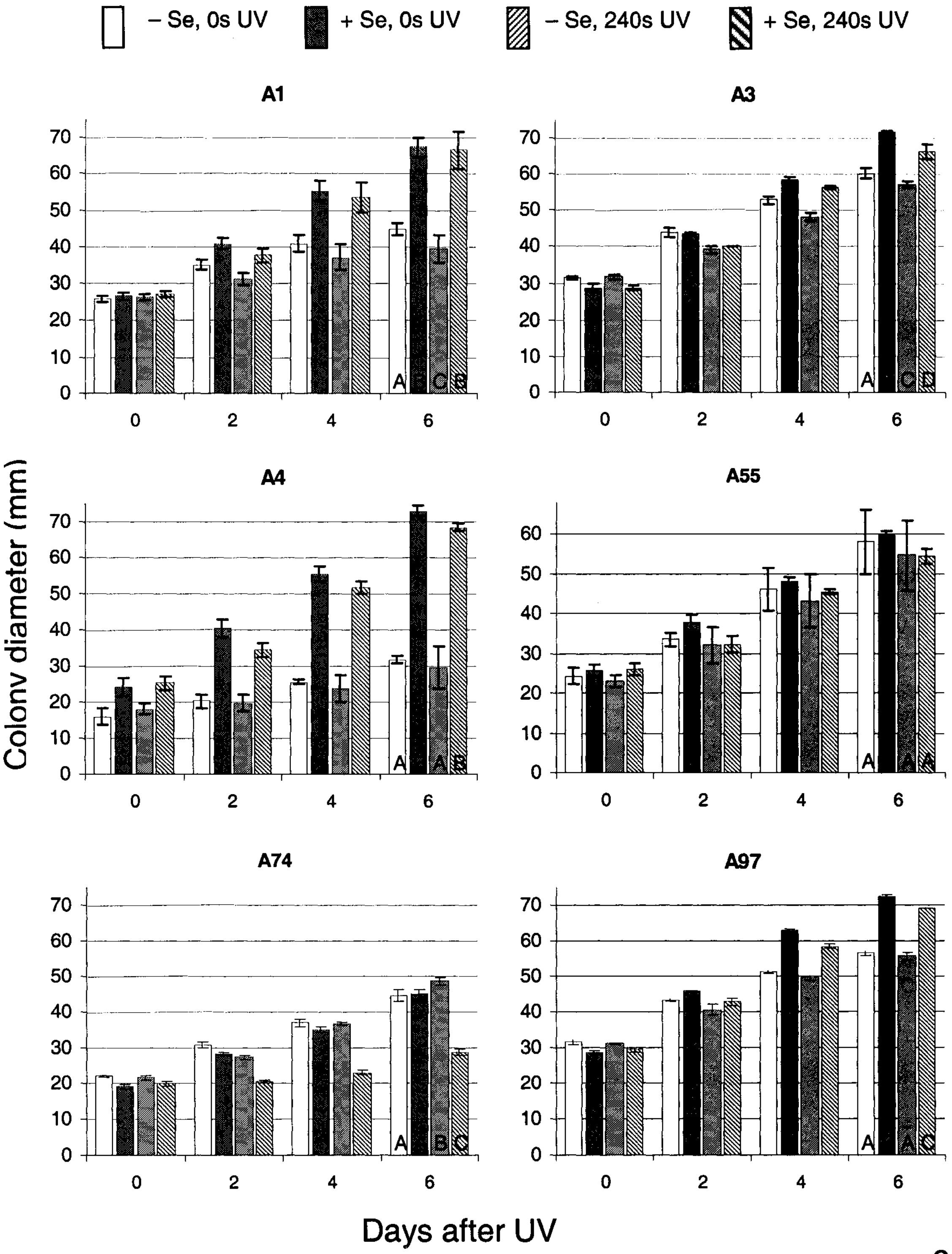
(B)

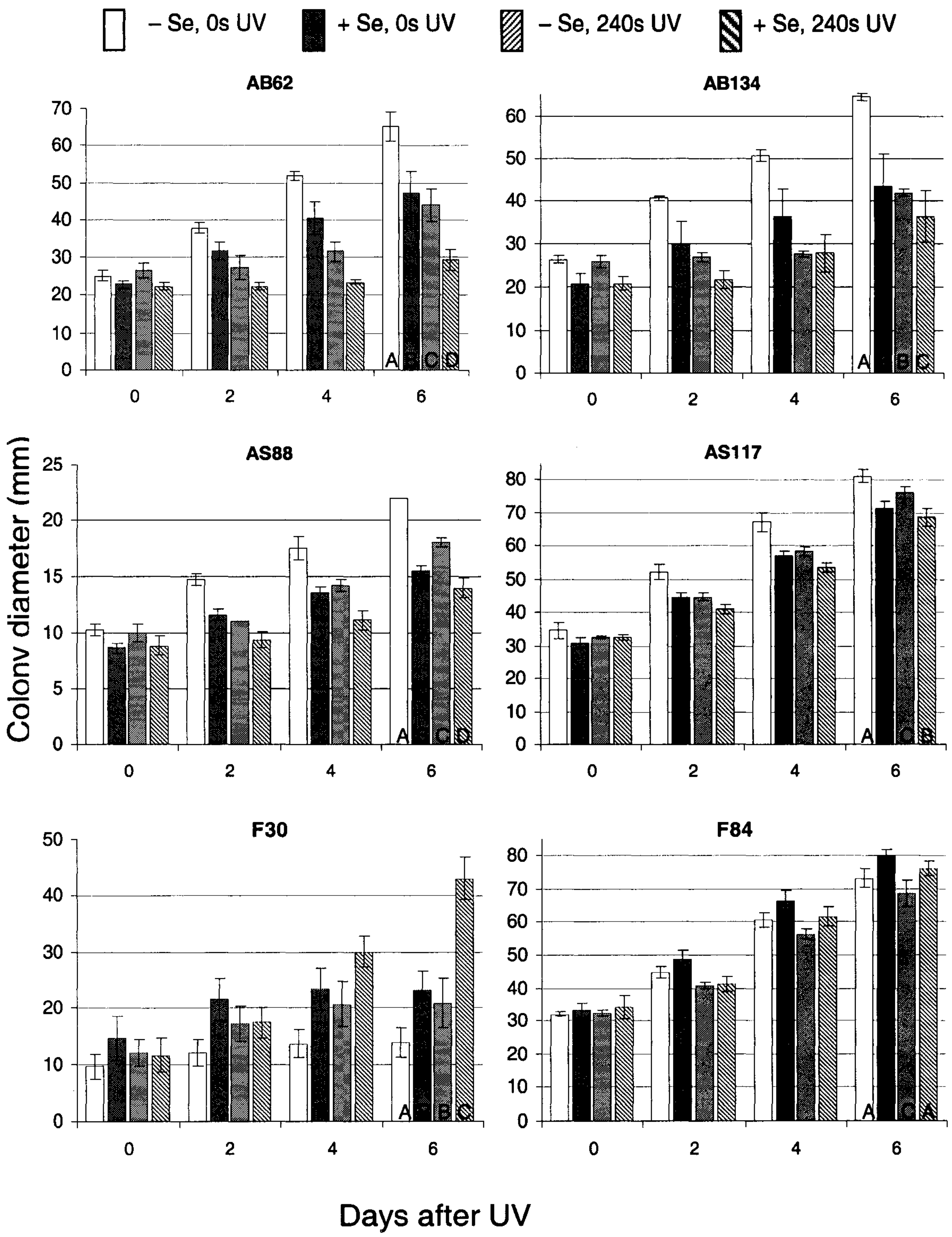


*F30 isolate is outside of the selected scale, see A for actual values

Note: all comparisons shown are significantly different (p ≤ 0.05) for each UV exposure time (e.g. at 30s, the + and – Se treatments are significantly different based on colony size) with the exception of: A55 which had no differences for any exposure time; A74 was not different at 0 UV exposure; F84 was not different at 30s and 120s UV, but was significantly different for remaining exposures. Statistics were conducted on colony diameter data, not on percentage of control as data are displayed here to highlight the differences in recovery from UV exposure for + and – Se treatments.

Appendix D. Growth pattern of select fungal isolates. Bars represent +/- Se treatments with no UV exposure, and after 240 seconds UV exposure. Letters within bars designate significant differences for different letters ($p \leq 0.05$, error bars = SD). A1= *Alternaria seleniiphila*; A3= *Alternaria astragali*; A4= *Ulocladium atrum*; A55= *Dreshlera biseptata*; A74= *Embellisia* sp.; A97= *Alternaria tenuissima*; A62= *Absidia spinosa*; A134= *Absidia spinosa*; AS88= *Penicillium anatolicum*; AS117= *Aspergillus leporis*; F30= *Fusarium acuminatum*; F84= *Fusarium oxysporum.*





▯ – Se, 0s UV
■ + Se, 0s UV
▨ – Se, 240s UV
▧ + Se, 240s UV
AB62
AB134
AS88
AS117
F30
F84
Colony diameter (mm)
Days after UV
0
2
4
6
A
B
C
D

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Publications from this dissertation work and related topics:

Hanson B, Lindblom SD, Garifullina GF, Wangeline A, Ackley A, Pilon-Smits EAH (2003) Selenium Accumulation Affects *Brassica juncea* Susceptibility to Invertebrate Herbivory and Fungal Infection. New Phytologist, 159: 461-469

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Wangeline AL, Ammon JA, Wangeline CR, Stushnoff C, Pilon-Smits EAH, Reeves FB (2007) Oxidative radical quenching in relation to selenium, total phenolics, and induced oxidative stress in filamentous fungi. In preparation.

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