Structural responses of *Daucus carota* root-organ cultures and the arbuscular mycorrhizal fungus, *Glomus intraradices*, to 12 pharmaceuticals

Derek G. Hillis^{a,*}, Pedro Antunes^b, Paul K. Sibley^a, John N. Klironomos^b, Keith R. Solomon^a

^a Department of Environmental Biology, Ontario Agricultural College, University of Guelph, Guelph, ON, Canada N1G 2W1

^b Department of Integrative Biology, University of Guelph, Guelph, ON, Canada

ARTICLE INFO

Article history:

Received 25 September 2007

Received in revised form 1 May 2008

Accepted 27 May 2008

Available online 25 July 2008

Keywords:

Root-organ cultures

Arbuscular mycorrhizal fungi

Pharmaceuticals

Personal care products

Phytotoxicity

Mycotoxicity

ABSTRACT

Pharmaceuticals and personal care products may enter the terrestrial environment through the amendment of agricultural soils with manure or biosolids with potential impacts on beneficial soil microbe populations. The beneficial symbiotic relationship between most plant species and arbuscular mycorrhizal fungi is a primary determinant of plant health and soil fertility. As such, there is increasing recognition of the need to study the impacts of anthropogenic stressors on plant–microbe interactions in soil ecotoxicology studies and risk assessment. A case study exploring the use of root-organ cultures to evaluate the effects of 12 common veterinary and human-use pharmaceuticals on the arbuscular mycorrhizal fungus, *Glomus intraradices* grown on *Daucus carota* root-organ cultures is presented. The bioassays were conducted over a 28-day exposure period at concentrations up to 1000 µg l⁻¹. Root length and the fungal endpoints of hyphal growth and spore production were evaluated weekly during the study. Sulfamethoxazole and atorvastatin were the most phytotoxic compounds with EC₅₀ values of 45 µg l⁻¹ and 65 µg l⁻¹, respectively. Three compounds exhibited selective mycotoxicity, whereby the fungal symbiont was adversely affected at concentrations significantly less than that calculated for root length. The EC₅₀ for *G. intraradices* hyphal length was 45 µg l⁻¹ for doxycycline, while carbamazepine and 17- α -ethynyl estradiol targeted spore production with EC₅₀ values of 113 and 116 µg l⁻¹, respectively. The assay results indicate that the root lengths responded quickly to the presence of phytotoxic pharmaceuticals in the culture medium. Hyphal length is a sensitive endpoint after 21 days exposure, while spore production requires 28 days exposure before significant differences could be detected. Root-organ cultures provide an effective means to evaluate chemical stressors on arbuscular mycorrhizal fungi and can be used to screen for root-based phytotoxicity.

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1. Introduction

Veterinary and human-use pharmaceuticals in the environment have received increasing attention from the scientific community since the late 1990s, when it was recognized that they were ubiquitous in the environment and that these compounds may be pseudo-persistent due to continuous or repeated input (Halling-Sørensen et al., 1998; Daughton and Ternes, 1999). Research to date has largely focused on the environmental fate, occurrence, and effects of these compounds in aquatic systems. However, the terrestrial environment also receives considerable pharmaceutical inputs, particularly agricultural systems. In addition, certain pharmaceuticals have been found to accumulate in soils (Thiele-Bruhn, 2003). Veterinary pharmaceuticals enter terrestrial systems through the common practice of amending soils with manure from intensive livestock operations or directly from grazing and free-ranging livestock. Human pharmaceuticals and personal care prod-

ucts may enter soils through the application of biosolids collected from the grit chamber, settling tanks, and waste activated sludge of municipal wastewater treatment systems.

Where toxicity testing has been conducted on soil systems, the focus has been on higher organisms such as plants (Migliore et al., 2003; Kumar et al., 2005) and soil invertebrates (Baguer et al., 2000) or on the effects of antibiotics on bacterial soil function and antibiotic resistance (Kümmerer, 2004). Soil fungi have been virtually ignored, despite being critical to ecosystem function as decomposers, mutualists, pathogens and a food-source for many other soil organisms. One argument for their exclusion from toxicological testing is that it is assumed that all fungi are 'r'-type strategists, with many species ready to occupy vacated niches should the appropriate environmental conditions arise (Finlay et al., 1997; Kapustka, 1999). However, some specialized fungi provide ecological functions that are not duplicated by any other organism, within the fungal or any other kingdom. One such fungal group are the arbuscular mycorrhizal fungi (AMF), which form a permanent, obligate symbiotic relationship with the roots of over 80% of all plant species (Trappe, 1987; Wang and Qiu, 2006).

* Corresponding author. Tel.: +1 519 824 4120x53926; fax: +1 519 837 3861.
E-mail address: dhillis@uoguelph.ca (D.G. Hillis).

Mycorrhizal fungi are responsible for enhanced uptake of macro- and micro-nutrients as well as access to nutrient pools typically not available to the root systems of plants, such as nitrogen (Hodge et al., 2001) and phosphorus (Tarafdar and Marschner, 1994), directly from decomposing organic matter. In addition, AMF improve host plant tolerance to stressors such as drought (Augé, 2001), pathogenic organisms (Whipps, 2004), toxic organic compounds (Corgié et al., 2006), and metals such as zinc (Audet and Charest, 2006) or cadmium (Rivera-Becerril et al., 2005). The extensive below-ground mycelial network produced by mycorrhizal fungi has been found to supply nutrients to seedlings and support seedling establishment (van der Heijden, 2004). Finally, soil aggregation and structure benefit from AMF by the exudation of the very stable glycoprotein, glomalin, which acts as an adhesive, holding soil particles together (Haddad and Sarkar, 2003). The combination of these benefits provided by AMF contribute to plant health and soil fertility (Jeffries et al., 2003) with plant–soil microbe interactions explaining a significant proportion of variation in the abundance of a plant species in a community (Klironomos, 2002). Ultimately, AMF have a significant influence on plant biodiversity, ecosystem variability, and productivity (van der Heijden et al., 1998; Klironomos et al., 2000).

The numerous, measurable and tangible benefits contributed by mycorrhizal fungi has lead to recognition of their potential use in sustainable agriculture (Hart and Trevors, 2005). However, for AMF to contribute to agroecosystem productivity, healthy populations and communities of fungi and other microbes must be available for plants to host. Very little is known about the influence of anthropogenic compounds applied intentionally in the form of pesticides and fertilizers or unintentionally such as pharmaceuticals or personal care products in soil amendments on the structure and function of AMF. Some contaminants, such as polycyclic aromatic hydrocarbons (Kirk et al., 2005; Verdin et al., 2006) and pesticides (Wan and Rahe, 1998; Wan et al., 1998) have been shown to have effects on AMF, indicating that pharmaceuticals, many of which specifically target bacteria and fungi, may have effects as well.

The use of root-organ cultures to evaluate the ecotoxicological impact of anthropogenic compounds on arbuscular mycorrhiza was pioneered by Wan et al. (1998). A comprehensive review of the culturing methods and uses of root-organ cultures suggested that the method could be further refined to become a standard toxicological test (Fortin et al., 2002). As there have been no other attempts to use this in vitro system as a toxicological bioassay, we adapted the root-organ culture method (Wan et al., 1998), to further standardize and refine the culturing and assessment procedure and to include plant–AMF interactions as a response. In this study, the effects of a selection of veterinary and human-use pharmaceuticals commonly found in manure or biosolids were evaluated on (Fisherbrand, Cat. No. 09-719C) AMF, *Glomus intraradices* Schenck & Smith, grown on a root-organ culture of *Daucus carota* L. Some precedent has been set for the use of root-organ cultures in measuring the effects of stressors on AMF including light quality and quantity as well as differing CO₂ concentrations (Nagahashi and Douds, 2005). The effect of naturally occurring chemicals, such root exudates and flavanoids, have also been documented (Bécard et al., 2004; Nagahashi and Douds, 2005). The objective of this study was to evaluate the toxicological potential of a suite of pharmaceuticals regularly found in biosolids and manure, on plants, arbuscular mycorrhizal fungi and plant–mycorrhizal interactions using a root-organ culture system.

2. Methods

2.1. Cultures, preparation and growth conditions

G. intraradices were grown on root inducing transformed DNA (Ri T-DNA) *D. carota* root-organ cultures (ROC) propagated from

an established culture 'B' (IRBV'95) maintained at the University of Guelph, Guelph, ON. The cultures were propagated by placing plugs of mature ROC (8–12 weeks old) on new minimal medium (M-medium) as described by Bécard and Fortin (1988). The cultures were maintained in a growth chamber set at 24 h dark and 26 °C. In preparation of each experiment, a mature and healthy root-organ culture with vigorous extraradical mycelial growth was selected and divided into six 2.5 cm diameter by 0.5 cm deep plugs and placed onto fresh M-medium. After 8–12 weeks of growth, these six F2 plates were used to conduct the experiment, so that all experimental units were derived from the same F1 culture.

2.2. Experimental design

The experiment was conducted as a static, non-renewable test with the pharmaceutical treatments added during the pouring of M-medium. The M-medium used for the experiments was adjusted to pH 5.5 ± 0.2. The 12 pharmaceuticals were 17- α -ethynyl estradiol (Sigma-Aldrich, St. Louis, MO; CAS# 57-63-6), atorvastatin (Rugao Foreign Trading Corp., Shanghai, China; CAS#134523-00-5), carbamazepine (Sigma-Aldrich, St. Louis, MO; CAS# 298-46-4), chlortetracycline (Medisca Pharmaceutique Inc., Montreal, QC; CAS# 64-72-2), doxycycline (Wiler-PCCA, London, ON; CAS# 10592-13-9), irgasan (Fluka Biochemika, Buchs, Switzerland; CAS# 3380-34-5), levofloxacin (Zhejiang Pharma, Shanghai, China; CAS#100986-85-4), monensin (Elanco Animal Health, Indianapolis, IN; CAS# 22373-78-0), oxytetracycline (PCCA, Houston, TX; CAS# 2058-46-0), sulfamethoxazole (Sigma-Aldrich, St. Louis, MO; CAS# 723-46-6), tetracycline (PCCA, Houston, TX; CAS# 64-75-5) and tylosin (Medisca Pharmaceutique Inc., Montreal, QC; CAS# 74610-55-2). After sterilization in an autoclave (121 °C for 20 min), the bulk solution of M-medium was separated into 6 aliquots of 160 ml each and allowed to cool in a water bath to 58 °C. To each M-medium aliquot, 1 ml of a pharmaceutical solution was added based on a back-calculated serial dilution to create M-medium with nominal concentrations at 10, 30, 100, 300 and 1000 $\mu\text{g l}^{-1}$. Pharmaceuticals were brought into solution using water or methanol, based on K_{OW} , with 1 ml of the applicable solvent added to the control aliquot of the M-medium. The use of methanol as a solvent, when necessary, has been shown not to exhibit toxicological effects in root-organ cultures at concentrations up to 2.5% by volume (Wan et al., 1998). Methanol concentrations in the final prepared media did not exceed 0.625%. The stock solutions of pharmaceuticals were added to the M-medium with a syringe, through a 0.2 μm filter a representative to maintain sterility and then the solution was agitated for 10 s to ensure thorough mixing of the pharmaceutical and M-medium. A volume of 25 ml of treated aliquot was poured onto six 150 mm × 10 mm plastic petri dishes. The treated M-medium was covered with an opaque black plastic container and allowed to set for 24 h. Experiments were initiated when six plugs (2.5 cm diameter × 0.5 cm width) were taken from a single established culture and one plug was applied to each pharmaceutical treatment. This process was performed six times to produce a randomized complete block design of six blocks of six replicates, with blocking for the F2 parent culture.

The *D. carota* endpoint, root length, and the *G. intraradices* endpoints, spore production and hyphal length, were evaluated at 3, 7, 10, 14, 21, and 28 days post treatment. Root and hyphal length were assessed using the gridline intersect method described by Tennant (1975). Spore production was assessed by counting the total number of spores produced within a plate. Where spore densities were greater than 500 spores/plate, half or quarter of the plate was counted, with a minimum of 200 spores counted in the representative portion.

2.3. Statistical analysis

Root length, hyphal length, and spore production were analyzed using 'proc GLM' of SAS version 9.1.3 (SAS Institute, 2003). The three endpoints were evaluated on day 14, 21 and 28, using a one-way analysis of variance (ANOVA) to identify significant effects with a Type I error rate (α) of 0.05. To test the assumptions of an analysis of variance, the data set was subjected to an analysis of residual error for each endpoint. First, the residual error of the dependent variable (endpoint) was plotted against the predicted values of the endpoint, pharmaceutical concentration and block, to ensure that errors were independent, homogeneous, and randomly distributed. The Shapiro-Wilk's W test was used to determine if the raw observations followed a normal distribution (Shapiro and Wilk, 1965). If a normal distribution could not be obtained, then the dataset was subjected to a natural logarithm (ln) transformation. Finally, studentized residuals were calculated using the 'proc mixed' function to test for outliers using Lund's test (Bowley, 1999). When a significant effect on an endpoint was determined, a lowest observable effects concentration (LOEC) for that endpoint was computed using the Dunnett's adjustment ($\alpha = 0.05$), which allows for unplanned comparisons between all means and the control.

2.4. Calculation of effective concentration values

Following the ANOVA, the raw data was subjected to linear and non-linear regression analyses using SigmaPlot, Version 9 (Systat Software Inc., 2004). Each dataset was subjected to six different reparameterized negative-response regression models which have been previously described (Brain et al., 2006; Environment Canada, 2005). The six models were derived from linear, exponential, logistic (3-parameter), logistic (4-parameter), gompertz, and hormetic concentration-response curves. The regression model that exhibited the highest adjusted r -squared was selected to calculate the effective concentrations at 50%, 25% and 10% levels. If convergence could not be obtained after applying the six regression models or all the tested models resulted in adjusted r^2 of less than 0.30, the dataset was deemed to have no concentration-response within the concentration range tested. Unless otherwise noted, the day 28 LOECs and EC_x values were used to describe the endpoint responses.

3. Results

3.1. *D. carota* root length

Root length (Table 1) was relatively quick to respond to the phytotoxic effects of the tested pharmaceuticals. This is exemplified by sulfamethoxazole and atorvastatin, which caused a reduction in root growth early in the experiment and LOECs calculated at day 14 were the same as day 28. By day 21 of the experiments, the phytotoxic effects of the pharmaceuticals tested exhibited little change in the calculated LOECs and EC_x values, with the exception of tylosin, which did not cause a significant root growth until day 28. Six of the twelve tested compounds exhibited a significant negative concentration-response by day 28 (Table 1). Sulfamethoxazole was most phytotoxic, with a LOEC of $10 \mu\text{g l}^{-1}$ and an EC_{50} of $45 \mu\text{g l}^{-1}$. Atorvastatin and levofloxacin followed sulfamethoxazole as the most phytotoxic compounds, with LOECs of $300 \mu\text{g l}^{-1}$ and EC_{50} s of 100 and $178 \mu\text{g l}^{-1}$, respectively. Phytotoxic responses were also caused by chlortetracycline and tylosin in the concentration range tested. Irgasan caused a temporary reduction in root elongation at $1000 \mu\text{g l}^{-1}$, with a significant decrease at 14 days with recovery by day 21 as indicated by the non-significant LOEC

value and lack of concentration response. A significant stimulatory response to the *D. carota* root-organ culture length was detected for tetracycline, at $1000 \mu\text{g l}^{-1}$.

3.2. *G. intraradices* hyphal length

Hyphal growth (Table 2) was found to be a relatively insensitive endpoint at day 14, with no significant differences observed until at least day 21. The day 28 hyphal length measurement was the most sensitive. At this time, the data did not require transformation to meet the ANOVA assumptions and the LOECs calculated were all lower than those measured at day 21, with the exception of sulfamethoxazole, which had in the same LOEC at both day 21 and day 28. Five of the twelve tested compounds caused a significant negative concentration-response, as indicated by the LOECs (Table 2). Four of these five compounds (sulfamethoxazole, atorvastatin, levofloxacin, and chlortetracycline) also caused phytotoxic responses in the root-organ cultures. Based on the EC_{50} s, these four compounds reduced hyphal length in the same relative order of potency as seen for root length: sulfamethoxazole > atorvastatin > levofloxacin > chlortetracycline. In contrast, doxycycline, which had no significant effect on root length at the largest concentration tested, resulted in the smallest LOEC observed for this endpoint at a concentration of $100 \mu\text{g l}^{-1}$, which corresponded to an EC_{50} of $45 \mu\text{g l}^{-1}$.

3.3. *G. intraradices* spore production

As is normal in the growth pattern of spores, there was little spore production in the first 14 days and therefore production values could not be normalized for statistical analysis, except in a few cases. Spore production was also relatively insensitive at day 21 with no statistically significant reductions in spore production observed until day 28 (Table 3). At this observation period, significant reductions in spore production were noted for sulfamethoxazole, levofloxacin, doxycycline, carbamazepine and 17- α -ethynyl estradiol (Table 3). The non-linear regressions for spore production often resulted in EC values much smaller than that predicted by the calculated LOECs due, in part, to the large variability in this endpoint. Spore production was a more sensitive mycorrhizal endpoint than hyphal length for carbamazepine and 17- α -ethynyl estradiol with LOECs at $1000 \mu\text{g l}^{-1}$ and $300 \mu\text{g l}^{-1}$, while hyphal length exhibited no significant reductions to these two compounds. Doxycycline reduced spore production at the highest treatment of $1000 \mu\text{g l}^{-1}$, which was a less sensitive endpoint for this compound than mycorrhizal hyphal length.

3.4. Plant-mycorrhiza interactions

In general, interactions between the *G. intraradices*, and the carrot root organ host were classifiable into the following categories:

No response

A statistically significant response ($\alpha = 0.05$) was not detected in the root length or either of the mycorrhizal endpoints at the concentration range tested. Of the 12 pharmaceuticals tested, two compounds, monensin and oxytetracycline, showed no response.

Generalized toxicity (Narcosis)

A statistically significant negative response ($\alpha = 0.05$) was detected in which both root length and the most sensitive mycorrhizal endpoint for that compound exhibited the same concentration-dependent response. Chlortetracycline and levofloxacin produced LOECs of $1000 \mu\text{g l}^{-1}$ and $300 \mu\text{g l}^{-1}$, respectively, for a mycorrhizal endpoint and root length.

Table 1The effects of 12 pharmaceuticals on *Daucus carota* root length 14, 21, and 28 days after exposure

Compound	Day	LOEC ($\mu\text{g l}^{-1}$)	<i>p</i> -Value	Model	Adjusted R^2	EC ₅₀ ($\pm$ SE) ($\mu\text{g l}^{-1}$)	EC ₂₅ ($\pm$ SE) ($\mu\text{g l}^{-1}$)	EC ₁₀ ($\pm$ SE) ($\mu\text{g l}^{-1}$)
Atorvastatin	14	300	0.0409	Log (4-Par)	0.96	259.4 (1.6)	121.8 (1.8)	47.4 (3.8)
	21	1000	0.0039	Log (4-Par)	0.95	208.6 (1.6)	107.7 (1.8)	60.3 (2.5)
	28	300	0.0005	Log (4-Par)	0.94	172.9 (1.5)	94.4 (1.7)	55.4 (2.2)
Carbamazepine	14	NSD	0.8320	NR	NC	>1000	>1000	>1000
	21	NSD	0.5523	Linear	0.37	>1000	542.6 (68.5)	12.4 (5.4)
	28	NSD	0.5638	Linear	0.38	>1000	>1000	19.4 (7.5)
Chlortetracycline	14	NSD	0.0726	Hormesis	0.69	575.0 (1.5)	471.5 (1.5)	335.0 (1.6)
	21	1000	0.0072	Hormesis	0.68	552.8 (1.5)	315.6 (1.6)	176.6 (2.1)
	28	1000	0.0328	Hormesis	0.93	950.6 (1.4)	587.3 (1.3)	455.2 (1.3)
Doxycycline	14	NSD	1.0000	NR	NC	>1000	>1000	>1000
	21	NSD	0.9999	NR	NC	>1000	>1000	>1000
	28	NSD	1.0000	NR	NC	>1000	>1000	>1000
17- α -Ethinyl estradiol	14	NSD	0.1233	Hormesis	0.88	>1000	269.9 (1.9)	142.6 (2.0)
	21	NSD	0.2523	Hormesis	0.65	>1000	371.6 (1.8)	211.0 (2.0)
	28	NSD	0.0975	Hormesis	0.71	>1000	421.8 (1.9)	210.1 (2.2)
Irgasan (Triclosan)	14	1000	0.0192	Gompertz	0.49	>1000	872.0 (2.4)	600.8 (18.9)
	21	NSD	0.1848	NR	NC	>1000	>1000	>1000
	28	NSD	0.1786	NR	NC	>1000	>1000	>1000
Levofloxacin	14	NSD	0.1919	Linear	0.66	>1000	89.9 (3.7)	6.0 (1.7)
	21	300	0.0109	Log (3-Par)	0.80	451.8 (1.8)	80.6 (2.1)	24.6 (2.5)
	28	300	0.0001	Log (3-Par)	0.77	289.4 (1.5)	74.1 (1.7)	35.4 (1.8)
Monensin	14	NSD	0.9310	NR	NC	>1000	>1000	>1000
	21	NSD	1.0000	NR	NC	>1000	>1000	>1000
	28	NSD	1.0000	NR	NC	>1000	>1000	>1000
Oxytetracycline	14	NSD	0.3799	NR	NC	>1000	>1000	>1000
	21	NSD	0.3615	NR	NC	>1000	>1000	>1000
	28	NSD	0.4229	NR	NC	>1000	>1000	>1000
Sulfamethoxazole	14	10	0.0043	Linear	0.93	62.7 (1.3)	7.9 (1.2)	2.3 (1.1)
	21	30	0.0058	Linear	0.94	61.2 (1.4)	7.8 (1.2)	2.3 (1.1)
	28	10	0.0300	Linear	0.96	45.4 (1.3)	6.7 (1.1)	2.1 (1.1)
Tetracycline	14	NSD	0.7269	NR	NC	>1000	>1000	>1000
	21	1000 ^a	0.0207	NR	NC	>1000	>1000	>1000
	28	1000 ^a	0.0266	NR	NC	>1000	>1000	>1000
Tylosin	14	NSD	0.9799	NR	NC	>1000	>1000	>1000
	21	NSD	0.8645	NR	NC	>1000	>1000	>1000
	28	1000	0.0449	Gompertz	0.38	>1000	296.9 (3.3)	42.0 (7.7)

LOECs were calculated using analyses of variance (ANOVA) with a Dunnett's adjustment for treatment-control comparisons ($\alpha = 0.05$). EC_x values are based on the non-linear regression model with the greatest adjusted R^2 .

NC = not calculated; NR = no relationship determined with the non-linear regression model at the concentration range tested; NSD = no significant difference, indicating no concentration-response; Par = parameter.

^a The endpoint showed a significant dose-response increase from the control.

Stimulation

A statistically significant ($\alpha = 0.05$) increase was observed for root length or either of the mycorrhizal endpoints relative to the control. Only tetracycline resulted in a stimulation response, whereby hyphal length was significantly increased at 300 $\mu\text{g l}^{-1}$ and root length was significantly greater at 1000 $\mu\text{g l}^{-1}$ relative to the control.

Phytotoxicity

A statistically significant negative response ($\alpha = 0.05$) in root length at a concentration less than that calculated for either mycorrhizal endpoint. Sulfamethoxazole had the smallest calculated LOEC for any of the measured endpoints (10 $\mu\text{g l}^{-1}$ for root length), whereas the mycorrhizal endpoints were not adversely affected until 300 $\mu\text{g l}^{-1}$. Atorvastatin significantly reduced root growth at 300 $\mu\text{g l}^{-1}$, while hyphal growth did not significantly decrease until 1000 $\mu\text{g l}^{-1}$. Tylosin did not produce a significant decrease in either of the mycorrhizal endpoints but produced a LOEC of 1000 $\mu\text{g l}^{-1}$ for the root-organ cultures. A temporary decrease in root length was noted for irgasan at 1000 $\mu\text{g l}^{-1}$ with no effect observed on the mycorrhizal endpoints.

Mycotoxigenicity

A statistically significant negative response ($\alpha = 0.05$) to a mycorrhizal endpoint at a concentration less than that calculated for the root length. Doxycycline, carbamazepine and 17- α -ethinyl estradiol represent this category (Fig. 1). These three compounds did not cause a significant reduction in root length. However doxycycline significantly reduced mycorrhizal hyphal length at 100 $\mu\text{g l}^{-1}$, while 17- α -ethinyl estradiol and carbamazepine significantly decreased spore production at 300 and 1000 $\mu\text{g l}^{-1}$.

4. Discussion

4.1. Temporal considerations of the bioassay

Elongation of roots responded quickly to the presence of phytotoxic pharmaceuticals in the culture medium. After day-14, the calculated LOECs and EC values often did not change between weekly measurements. Other compounds however, produced effects at only one time interval of the three measured, such as irgasan (day 14) or tylosin (day 28). This indicates that measurements at multiple times for this non-destructive test are of value and warranted.

Table 2The effects of 12 pharmaceuticals on *Glomus intraradices* hyphal length 14, 21, and 28 days after exposure

Compound	Day	LOEC ($\mu\text{g l}^{-1}$)	p-Value	Model	Adjusted R^2	EC ₅₀ ($\pm$ SE) ($\mu\text{g l}^{-1}$)	EC ₂₅ ($\pm$ SE) ($\mu\text{g l}^{-1}$)	EC ₁₀ ($\pm$ SE) ($\mu\text{g l}^{-1}$)
Atorvastatin	14	NSD	0.3057	Linear	0.54	154.3 (6.1)	12.4 (2.5)	2.7 (1.4)
	21	NSD	0.1943	Linear	0.54	153.6 (4.4)	12.4 (2.1)	2.7 (1.3)
	28	1000	0.0121	Linear	0.76	65.4 (2.1)	8.1 (1.4)	2.7 (1.3)
Carbamazepine	14	NSD	0.9999	NR	NC	>1000	>1000	>1000
	21	NSD	0.8999	NR	NC	>1000	>1000	>1000
	28	NSD	0.1187	Linear	0.32	388.2 (12.6)	19.7 (3.5)	3.3 (1.7)
Chlortetracycline	14	NSD	0.2373	Gompertz	0.74	885.7 (3.0)	232.0 (4.8)	83.3 (9.9)
	21	NSD	0.0605	Gompertz	0.80	397.5 (2.3)	122.0 (3.8)	46.9 (5.7)
	28	1000	0.0437	Gompertz	0.91	147.0 (2.8)	35.8 (4.3)	11.6 (5.2)
Doxycycline	14	NSD	0.0882	Linear	0.46	274.9 (3.1)	12.8 (2.0)	2.8 (1.3)
	21	1000	0.0174	Linear	0.37	87.1 (2.9)	9.3 (1.7)	2.4 (1.2)
	28	100	0.0384	Linear	0.75	45.0 (1.7)	6.7 (2.0)	2.1 (1.1)
17- α -Ethinyl estradiol	14	NSD	0.6292	Hormesis	0.80	354.2 (4.1)	169.2 (3.0)	120.2 (3.0)
	21	NSD	0.4936	Hormesis	0.64	312.6 (3.9)	100.3 (2.8)	60.2 (2.9)
	28	NSD	0.1437	Hormesis	0.51	>1000	241.9 (3.4)	129.1 (3.1)
Irgasan (Triclosan)	14	NSD	0.1013	NR	NC	>1000	>1000	>1000
	21	NSD	0.4313	NR	NC	>1000	>1000	>1000
	28	NSD	0.8202	NR	NC	>1000	>1000	>1000
Levofloxacin	14	NSD	0.3962	Gompertz	0.60	526.5 (2.7)	145.8 (4.9)	46.0 (9.2)
	21	NSD	0.3483	Hormesis	0.83	460.3 (7.1)	71.3 (4.1)	32.3 (4.0)
	28	300	0.0263	Hormesis	0.99	114.8 (1.6)	54.3 (1.7)	36.1 (1.9)
Monensin	14	NSD	0.7886	NR	NC	>1000	>1000	>1000
	21	NSD	0.4898	NR	NC	>1000	>1000	>1000
	28	NSD	0.8646	Linear	0.31	>1000	53.1 (18.5)	4.9 (3.2)
Oxytetracycline	14	NSD	0.2831	NR	NC	>1000	>1000	>1000
	21	NSD	0.2523	NR	NC	>1000	>1000	>1000
	28	NSD	0.4102	NR	NC	>1000	>1000	>1000
Sulfamethoxazole	14	NSD	0.1298	Gompertz	0.38	502.9 (3.3)	147.0 (6.6)	121.2 (6.0)
	21	300	0.0479	Linear	0.66	74.9 (2.1)	8.7 (1.5)	2.4 (1.2)
	28	300	0.0023	Linear	0.93	45.1 (1.5)	6.7 (1.1)	2.1 (1.1)
Tetracycline	14	NSD	0.4128	NR	NC	>1000	>1000	>1000
	21	300 ^a	0.0063	NR	NC	>1000	>1000	>1000
	28	300 ^a	0.0384	NR	NC	>1000	>1000	>1000
Tylosin	14	NSD	0.9993	Hormesis	0.98	>1000	521.1 (3.9)	326.9 (3.0)
	21	NSD	0.9707	Hormesis	0.97	>1000	904.9 (4.3)	549.5 (2.9)
	28	NSD	0.2535	Hormesis	0.68	555.0 (8.6)	104.1 (4.6)	50.9 (3.5)

LOECs were calculated using analyses of variance (ANOVA) with a Dunnett's adjustment for treatment-control comparisons ($\alpha = 0.05$). EC_x values are based on the non-linear regression model with the greatest adjusted R^2 .

NC = not calculated; NR = no relationship determined with the non-linear regression model at the concentration range tested; NSD = No significant difference, indicating no concentration–response; Par = parameter.

^a The endpoint showed a significant dose–response increase from the control.

The results from this study suggest that the hyphal length is relatively insensitive until > 21 days post exposure. Spore production was even less sensitive, with significant differences typically not appearing until 28 days. The first attempts to use a comparable method to evaluate the effects of a chemical on AMF were based on a 14-day growth period (Wan and Rahe, 1998; Wan et al., 1998). Our results suggest that the assay time should extend for at least 28 days to ensure adequate spore production to detect differences.

4.2. *D. carota* root-organ cultures

The 28-day EC₅₀s for sulfamethoxazole, atorvastatin, levofloxacin, and chlortetracycline, calculated at 45, 173, 289, and 951 $\mu\text{g l}^{-1}$, respectively, for *D. carota* root length are similar to the effective concentrations determined in another study using the floating aquatic macrophyte, *Lemna gibba*, where exposure also primarily occurs at the root-matrix interface. EC₅₀s for wet weight for sulfamethoxazole, levofloxacin, and chlortetracycline were 81, 185, 219 $\mu\text{g l}^{-1}$, respectively (Brain et al., 2004). Atorvastatin produced a wet weight EC₅₀ of 135 $\mu\text{g l}^{-1}$ for *L. gibba* (Brain et al., 2006). The EC₅₀s for these different plants were in the same rank

and within an order of magnitude of each other, suggesting that the root-organ cultures exhibit a predictive and reliable response when the pharmaceutical does not specifically target part of the photosynthetic process.

Assessing responses using in vitro culture is beneficial when little is known about the toxicity of a substance and baseline data to prioritize compounds for higher levels of testing are required. In vitro cultures eliminate the heterogeneity inherent in soil systems, making the results directly comparable with each other. However, this benefit is also the major criticism associated with in vitro systems because of questions surrounding bioavailability; degradation and fate of pharmaceutical compounds are directly influenced by soil properties, such as organic matter content and texture (ratio of clay, sand, and silt). For example, measurable differences in plant growth, root growth and nutrient status of pinto bean (*Phaseolus vulgaris* L.) were observed at a concentration of 10 mg l^{-1} chlortetracycline and oxytetracycline when grown in liquid culture (Batchelder, 1981). In a second study, the bean plants were grown in a sandy loam and phytotoxic effects were observed at 160 mg/kg , however, in a clay loam, no effects on bean plant growth were observed up to 160 mg/kg (Batchelder, 1982). Cultured plants systems, such as

Table 3The effects of 12 pharmaceuticals on *Glomus intraradices* spore production 14, 21, and 28 days after exposure

Compound	Day	LOEC ($\mu\text{g l}^{-1}$)	p-Value	Model	Adjusted R^2	EC ₅₀ ($\pm$ SE) ($\mu\text{g l}^{-1}$)	EC ₂₅ ($\pm$ SE) ($\mu\text{g l}^{-1}$)	EC ₁₀ ($\pm$ SE) ($\mu\text{g l}^{-1}$)
Atorvastatin	14	–	–	NR	NC	>1000	>1000	>1000
	21	NSD	0.8134	NR	NC	>1000	>1000	>1000
	28	NSD	0.1519	Linear	0.76	41.8 (2.3)	6.5 (1.5)	2.1 (1.2)
Carbamazepine	14	–	–	NR	NC	>1000	>1000	>1000
	21	NSD	0.1639	Linear	0.53	43.6 (3.2)	6.6 (1.8)	2.1 (1.3)
	28	1000	0.0442	Linear	0.31	113.4 (8.1)	10.6 (2.8)	2.6 (1.5)
Chlortetracycline	14	NSD	0.6594	NR	NC	>1000	>1000	>1000
	21	NSD	0.5402	Hormesis	0.64	358.6 (23.7)	310.7 (3.0)	283.8 (2.8)
	28	NSD	0.8059	Hormesis	0.43	734.5 (4.8)	368.6 (3.6)	257.5 (4.1)
Doxycycline	14	–	–	NR	NC	>1000	>1000	>1000
	21	NSD	0.0769	NR	NC	>1000	>1000	>1000
	28	1000	0.0170	Linear	0.61	36.9 (1.8)	6.1 (1.4)	2.1 (1.1)
17- α -Ethinyl estradiol	14	–	–	Hormesis	0.85	168.6 (5.0)	86.6 (3.9)	63.2 (4.1)
	21	NSD	0.4564	Hormesis	0.89	36.9 (2.6)	23.3 (2.7)	18.7 (2.7)
	28	300	0.0458	Hormesis	0.91	115.9 (2.5)	55.0 (2.3)	38.1 (2.5)
Irgasan (Triclosan)	14	–	–	NR	NC	>1000	>1000	>1000
	21	NSD	0.4564	NR	NC	>1000	>1000	>1000
	28	NSD	0.2364	NR	NC	>1000	>1000	>1000
Levofloxacin	14	NSD	0.7797	NR	NC	>1000	>1000	>1000
	21	NSD	0.1814	Hormesis	0.91	100.0 (3.1)	45.5 (2.8)	31.9 (2.9)
	28	300	0.0338	Hormesis	0.99	75.4 (1.7)	38.7 (1.7)	27.5 (1.8)
Monensin	14	NSD	0.5630	NR	NC	>1000	>1000	>1000
	21	NSD	1.0000	NR	NC	>1000	>1000	>1000
	28	NSD	0.9986	NR	NC	>1000	>1000	>1000
Oxytetracycline	14	–	–	NR	NC	>1000	>1000	>1000
	21	NSD	0.0579	NR	NC	>1000	>1000	>1000
	28	NSD	0.4168	NR	NC	>1000	>1000	>1000
Sulfamethoxazole	14	300	0.0229	Linear	0.85	24.0 (1.6)	4.9 (1.3)	1.9 (1.1)
	21	1000	0.0105	Linear	0.78	40.7 (1.9)	6.4 (1.4)	2.1 (1.1)
	28	300	0.0120	Linear	0.74	39.7 (1.7)	6.3 (1.3)	2.1 (1.1)
Tetracycline	14	–	–	NR	NC	>1000	>1000	>1000
	21	NSD	0.0737	Hormesis	0.50	>1000	>1000	>1000
	28	NSD	0.1648	Hormesis	0.31	>1000	>1000	>1000
Tylosin	14	–	–	Hormesis	0.84	284.6 (9.3)	142.9 (5.9)	104.1 (6.4)
	21	NSD	0.9017	Hormesis	0.54	>1000	>1000	945.6 (6.5)
	28	NSD	0.3500	NR	NC	>1000	>1000	>1000

LOECs were calculated using analyses of variance (ANOVA) with a Dunnett's adjustment for treatment-control comparisons ($\alpha = 0.05$). EC_x values are based on the non-linear regression model with the greatest adjusted R^2 .

NC = not calculated; NR = no relationship determined with the non-linear regression model at the concentration range tested; NSD = no significant difference, indicating no concentration–response; Par = parameter.

root-organ cultures, can therefore be used as first tier, toxicity tests. Consequently, root organ cultures should not be used to directly predict the phytotoxic responses of plants to contaminants in soil, however, they may be used to predict the order of sensitivity to plants exposed to a series of compounds and may provide a useful tool to explore root-based phytotoxic modes of action.

4.3. *G. intraradices* endpoints

Where general toxicity to both the plant host and fungal symbiont occur, or phytotoxicity dominates the interaction, the effects of the pharmaceutical would correlate with a decrease in mycorrhizal functioning. A reduction in plant host vitality is most important in this situation as the mycorrhizal fungi are dependent on the plant to provide carbon resources for growth. In addition, pharmaceuticals exhibiting a generalized or phytotoxic response appeared to inhibit hyphal growth and spore production at the same concentrations, that is, neither mycorrhizal endpoint was more sensitive than the other during a primarily phytotoxic response, for example, sulfamethoxazole. Sulfamethoxazole had an EC₅₀ for *G. intraradices* hyphal length and spore production of 45.1 and 39.7 $\mu\text{g l}^{-1}$, respectively (Tables 2 and 3).

For compounds specifically displaying antifungal properties, mycorrhizal populations may be affected before any change in the above-ground plant system would be observed. This is because phytotoxic effects, due to selective mycotoxicity, would likely manifest themselves indirectly through decreased nutrient or water availability or the increased influence of other biotic (pathogens) or abiotic stressors (heavy metals). In addition, where compounds expressed mycotoxicity, the magnitude of differences observed in effective concentrations between the mycorrhizal endpoints appeared to depend on the pharmaceutical mode of action. This results in one mycorrhizal endpoint often being more sensitive than the other. For example, doxycycline caused a marked reduction in mycorrhizal hyphal growth at 100 $\mu\text{g l}^{-1}$, however, spore production was only reduced at 1000 $\mu\text{g l}^{-1}$. For carbamazepine and 17- α -ethynyl estradiol, no significant difference was detected in mycorrhizal hyphal length, but spore production was reduced at 1000 and 300 $\mu\text{g l}^{-1}$, respectively, indicating that the latter was a more sensitive endpoint.

4.4. Possible mechanisms of AMF toxicity

The natural estrogen, 17- β -estradiol, has been shown to exert stimulatory effects on *G. intraradices* hyphal growth at small

Fig. 1. Structural responses of *D. carota* root length and *G. intraradices* hyphal length and spore production in response to three selectively mycotoxic pharmaceuticals represented as percent of control ($\pm$ SE). Mean values significantly different from the control ($p = 0.05$) are indicated with the symbol *.

concentrations (Poulin et al., 1997). A stimulatory effect of the synthetic estrogen, 17- α -ethynyl estradiol, was also observed at small concentrations in the current study. However, at higher concentrations, *D. carota* root length and both *G. intraradices* endpoints were depressed. This response may be due to interference with molecular signaling between plant and fungus and may also explain the effect of carbamazepine on spore production. Carbamazepine has been reported to lower endogenous estrogens and the efficacy of contraceptive steroids in humans (Morrell, 2003). The plant flavonoids involved in the molecular signals that occur between the plant and AMF in the symbiosis are estrogenic (Poulin et al., 1997). It is plausible, therefore, that carbamazepine and 17- α -ethynyl estradiol are disrupting the molecular cross-talk between plant and AMF by interfering with flavanoid receptors and the signals needed to initiate spore production.

The tetracycline family of compounds are selectively anti-bacterial and have few effects on eukaryotes (Chopra and Roberts,

2001) because of selective binding to the 30S subunit of bacterial ribosomes, resulting in the inhibition of protein synthesis (Liu et al., 2002). At high concentrations it has been suggested tetracycline toxicity on plants is induced directly through the chelating of metal nutrients (Nickell and Gordon, 1961; Brain et al., 2008). At the low concentrations tested in this experiment it is therefore not surprising that most of the tetracyclines were relatively innocuous to root-organ cultures and fungi. Tetracycline stimulated plant growth, which in turn likely resulted in the observed stimulation in mycorrhizal growth. Oxytetracycline caused no response at the concentrations tested and chlortetracycline produced a measurable reduction in both root length and hyphal length at only the highest concentration tested. The significant reduction in hyphal growth at 100 $\mu\text{g l}^{-1}$ doxycycline is surprising as this compound is not considered to be an antifungal agent. In fact, doxycycline has been found to promote the overgrowth of fungi due to the suppression of normal microbiota (Liu et al., 2002). Tetracyclines have been reported to inhibit protein synthesis in mitochondria due to binding of the 70S ribosome (Chopra and Roberts, 2001) and the semi-synthetic, doxycycline, has been reported to have a wider spectrum of biological activity (Cunha, 1997). The apical compartment of the fungal hyphae includes a zone dense in mitochondria just behind the growing apex (Deacon, 1997) and interference in the normal functioning could possibly explain the reduced hyphal growth.

4.5. AMF endpoint sensitivity

Spore production appeared to be a more variable endpoint than hyphal growth (Tables 2 and 3). However, both endpoints are valuable in assessing the potential impacts of contaminants on the mycorrhizal life-cycle. On the one hand, hyphae are deemed to be a short-term propagule, which will colonize plants in the immediate vicinity of a currently colonized plant. Mycorrhizal hyphae are also the primary reason for improved plant health through the symbiotic association as they access a much larger soil volume in search of limiting nutrients, such as phosphorus, and the small diameter hyphae increase the capillary action of water transfer during drought. On the other hand, spores are long-term propagules, which are produced by healthy fungi but also provide a defense mechanism to ensure long-term survival in response to a stressor. It has been noted that increased spore production is a typical response of mycorrhizae to stressors, such as heavy-metal contaminated soils (Regvar et al., 2006). Due to differences in sensitivity and the different purpose of hyphae and spores in mycorrhizal fungi life-strategies, it is suggested that both endpoints should be measured when evaluating toxic responses of mycorrhiza to a contaminant.

4.6. AMF species selection and plant-AMF interactions

It is important to note that this study evaluated a single species of plant root-organ culture with a single species of AMF. There are approximately 150 species of AMF currently identified, although, as an Order, most display little in terms of specificity towards host selection (Smith and Read, 1997). However, the plant benefits realized due to the mycorrhizal associations are not equal among different plant-fungal species combinations (Gustafson and Casper, 2006). Because of this, mycorrhizal fungi may help govern plant community structure and successional trajectories, with both plant productivity and plant diversity shown to increase with increasing diversity of mycorrhizal fungi (Kernaghan, 2005). Thus, interspecific differences between mycorrhizal fungi should be explored to evaluate differences in their sensitivity to contaminants. In addition, a wide variety of plants that have been transformed into successful root-organ cultures allowing for investigation of

interspecies plant differences in response to pharmaceuticals as well as the different plant–fungal combinations and the maintenance of the symbiosis in response to a chemical stressor.

5. Conclusions

There is increasing recognition of the importance of beneficial soil microbes such as AMF as a biological tool in sustainable agriculture and in reducing our dependence on intensive agricultural practices such as irrigation, pesticide and fertilizer additions. Soil amendment with manure has been shown to increase mycorrhizal colonization of plants (Gryndler et al., 2006). However, the benefits associated with increased plant–mycorrhizal symbiosis may not be observed where fungicidal pharmaceuticals are present in the amendment at biologically active concentrations. The results of this study suggest that some pharmaceuticals, commonly found in wastewater treatment biosolids and livestock manure, exhibit antifungal properties towards AMF. The pharmaceutical concentration required to cause effects in AMF were, however, typically much higher than environmental concentrations. Finally, our study has shown that plant–AMF bioassay systems can provide a useful approach to assess the toxicity of contaminants. Moreover mycorrhizal fungi provide one example where functional redundancy is not always maintained at the microbial level and therefore warrants closer examination with regard to its use as an indicator organism in ecotoxicology and soil health.

Acknowledgements

I would like to thank the Natural Sciences and Engineering Research Council of Canada for research funding. Richard Brain and Mark Hanson contributed to the experimental design and statistical analysis. Stuart Campbell aided with propagation techniques for the root–organ cultures.

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