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Influence of mycorrhizal fungi on fate of *E. coli* O157:H7 and *Salmonella* in soil and internalization into Romaine lettuce plants ☆

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ABSTRACT

The objectives of this study were to determine the influence of a symbiotic arbuscular mycorrhizal (AM) fungus on persistence of *Salmonella* and enterohemorrhagic *Escherichia coli* O157:H7 (EHEC) within soil, and survival within Romaine lettuce. Romaine seedlings were grown with or without AM fungi. Soil surrounding plants was inoculated with ca. 8 log CFU/plant of either *Salmonella enterica* or *E. coli* EHEC composites. Samples (soil, root, and shoot) were analyzed on days 1, 8, 15 and 22 for *Salmonella* and EHEC by direct plating and selective enrichment. Twenty-four hours after inoculation, populations of *Salmonella* and EHEC, respectively, were 4.20 and 3.24 log CFU/root, 2.52 and 1.17 log CFU/shoot, and 5.46 and 5.17 log CFU/g soil. By selective enrichment, samples tested positive for *Salmonella* or EHEC at day 22 at rates of 94 and 68% (shoot), 97 and 56% (root), and 100 and 75% (soil), respectively, suggesting that *Salmonella* has a greater propensity for survival than EHEC. *Salmonella* populations in soil remained as high as 4.35 log CFU/g by day 22, while EHEC populations dropped to 1.12 log CFU/g in the same amount of time. Ninety-two percent of all Romaine leaves in our study were positive for internalized *Salmonella* from days 8 to 22 and remained as high as 1.26 log CFU/shoot on day 22 in AM fungi + Romaine plants. There were no differences ($P > 0.05$) between the survival of either pathogen based on the presence or absence of mycorrhizal fungi. Results of this study suggest that AM fungi do not affect the internalization and/or survival of either *S. enterica* or *E. coli* O157:H7 in Romaine lettuce seedlings. Our results should provide Romaine lettuce farmers confidence that the presence and/or application of AM fungi to crop soil is not a contributing factor to the internalization and survival of *Salmonella* or *E. coli* O157:H7 within Romaine lettuce plants.

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1. Introduction

Romaine lettuce (*Lactuca sativa* L. var. *longifolia*, aka, *Lactuca sativa* cv. Romana) is native to the eastern Mediterranean Basin and western Asia, where cultivation of lettuce dates back to illustrations in ancient Egyptian tombs ca. 4500 B.C. Ancient Greeks and Romans both consumed lettuce as food and for its purported therapeutic medicinal properties. Romaine lettuce sales in the U.S. continue to increase commensurate with a rise in fresh produce consumption (Lynch et al., 2009). Today, Romaine lettuce is one of the top six vegetable crops grown for domestic use in the U.S., and the 5 pound per capita consumption in 1993, has increased to more than 11 pounds in 2010 (Spalding, 2011). Nevertheless, foodborne illnesses associated with the consumption of contaminated leafy greens appear to be growing, along with the market for lettuce sales (Nygard et al., 2008). The persistence of enteric human pathogens, such as *Salmonella* and enterohemorrhagic *Escherichia coli* (EHEC) in soil is a significant food safety issue. Lettuce contamination with foodborne pathogens is costly to the fresh produce industry in terms of product recalls, liability, potential litigation, increased product testing, etc. The U.S. Centers for Disease Control and Prevention reported that two multistate outbreaks of EHEC infections attributed to the consumption of Romaine lettuce occurred in both 2010 and 2011 (CDC, 2010, 2012). Another outbreak involving Romaine lettuce and *E. coli* O157:H7 in 2012 affected people in both California and Canada (Andrews, 2012). Other foodborne outbreaks have also been associated with various leafy greens foods (Marder et al., 2014).

Survival of human bacterial pathogens in soil matrices is also a key area of concern. Various factors involved in the persistence of foodborne pathogens in plant soil have been reported (Gagliardi and Karns, 2002; Islam et al., 2004a, 2004b; Klerks et al., 2007; Mootian et al., 2009).

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Arbuscular mycorrhizal (AM) fungi, which form a natural symbiotic relationship with the roots of most crop plants, have been investigated for their role in the survival and internalization of human pathogens (Gurtler et al., 2013a). AM fungi are known to affect rhizosphere biodiversity, improve disease and drought resistance, and bolster crop growth by supplying the plant with phosphorous (Smith and Read, 2008). For these reasons, AM fungi should be especially important in organic farming. More specifically, in establishing its mutualistic relationship with plants, AM fungal hyphae, assisted by plant cell wall-degrading enzymes, penetrate the roots of the host plant to form arbuscules within root cortical cells (Akiyama and Hayashi, 2006; Blee and Anderson, 1998; Garcia-Garrido et al., 1999, 2000; Genre et al., 2008; Jakobsen and Nielsen, 1983; Sharda and Koide, 2008). This process has recently been shown to provide a natural conduit through which saprophytic fungi can traverse (De Jaeger et al., 2010).

Previous studies (Gurtler et al., 2013a) with leek (*Allium porum* L.) demonstrated the ability of *E. coli* O157:H7 inoculated into soil at an initial level of 8 log CFU/plant to vascularly internalize and survive within leek roots. In that study a *Salmonella* composite was demonstrated to survive within the leek roots and shoots for up to 22 days with or without mycorrhizal fungi. Although EHEC survived in Myc+ leek shoots up to 22 days, in the absence of AM fungi, EHEC was not detected in the shoots on either day 15 or day 22. These results suggested a potential role of mycorrhizal fungi in facilitating longevity of an otherwise relatively short-lived pathogen in plant tissues.

The exact mechanisms involved in AM fungi modulating the invasion and persistence of human and plant pathogens in crops are not easily discernible. Mechanistically, AM fungi behave in a number of ways, which could potentially affect pathogen persistence. Firstly, the fungi are able to stimulate the growth of endogenous soil bacteria, antagonistic to plant pathogens (Linderman, 2000). For example, a synergistic relationship between AM fungi and microbiological biocontrol agents such as *Trichoderma harzianum*, *Pseudomonas fluorescens*, *Verticillium chlamydosporium* Kamyschko ex Barron, and *Burkholderia cepacia* Palleroni & Holmes, Rifai has been identified (Dalpé and Monreal, 2004). All fungi could likewise impact the survival of human pathogenic bacteria in the soil. Secondly, AM fungi may potentially modulate the production of root exudates and other defense secretions such as glucanases, chitinases, flavonoids, phytoalexins, peroxidases, hydroxyproline-rich glycoproteins and phenolic compounds (Dalpé and Monreal, 2004; Harrier and Watson, 2004; Nagahashi and Douds, 2011). Thirdly, AM fungi enable water to be extracted from smaller pores within the soil by increasing connections with soil particles through the binding mechanism of the hyphae, enhancing osmotic adjustment, improving stomatal conductance, and influencing root hydraulic conductivity (Augé, 2000). Lastly, the colonization of roots by AM fungi could provide a conduit through which human pathogenic bacteria gain entry to the root (as noted above). These and other associated factors demonstrate the very complex relationship and underlying interactions promoted by the fungi within the rhizosphere. Any one, or all, of these activities could theoretically affect pathogen growth and/or persistence.

Many studies have examined the internalization of human pathogens within crop plant tissue (e.g., maize, thale cress, peanut, alfalfa, barrel clover, spinach, parsley, iceberg, Romaine and Ruby Red lettuce, arugula, basil, tomato, green onions, wheat, rice, radish sprouts, alfalfa sprouts, mung bean sprouts, radish, cress, barley, cabbage, and crisphead lettuce) (Erickson, 2012; Hirneisen et al., 2012; Lynch et al., 2009), none have addressed the role that AM fungi may play in these outcomes. The objectives of the present study, therefore, were to determine the influence of AM fungi on the persistence of *Salmonella* and EHEC within soil as well as the effects of the fungus on internalization and survival of the pathogens within Romaine lettuce roots and leaves. Our hypothesis is that AMF would affect Romaine lettuce much as it did leeks in increasing the internalization and longevity of the foodborne pathogens in the lettuce.

2. Materials and methods

2.1. Soil preparation

Potting soil was prepared in-house for this study by combining a mixture of 0.75:1:1:0.75 [V/V] autoclave-sterilized soil, sand, vermiculite, and turface (calcined clay, Applied Industrial Materials, Corp., Deerfield, IL) (SSVT). The soil component used in SSVT was collected from the Rodale Institute Experimental Farm in Kutztown, PA, with a final SSVT total carbon content of 0.6%. The Pennsylvania State University Agricultural Analytical Services Laboratory conducted routine soil fertility analysis on a pooled, thoroughly mixed sample of the soil component of the SSVT used in this experiment, which yielded the following results: pH = 6.9; available phosphorus (as P_2O_5) = 362 mg/kg soil; and potassium (as K_2O) = 400 mg/kg soil. Soil was autoclave-sterilized primarily to inactivate naturally-occurring mycorrhizal fungi for growth of non-mycorrhizal controls as well as to allow for complete and exclusive colonization by our inoculated AM fungus spores.

2.2. Mycorrhizal fungus preparation

AM fungus spores of *Glomus intraradices* Schenck and Smith (DAOM 181602) were cultivated in vitro in two-chambered, split Petri plates with Ri T-DNA transformed carrot roots, as described in Gurtler et al. (2013a) according to the method of St-Arnaud et al. (1996).

2.3. Romaine plant preparation and G. intraradices spore inoculation

Seeds used to grow Romaine seedlings in this study were purchased from Johnny's Selected Seeds (Lettuce, Green Romaine/COS Winslow, ME) certified organic by Maine Organic Farmers and Gardner's Association (MOFGA). Romaine lettuce, *L. sativa* cv. Romana (organic, pelleted seeds) were sown into conical plastic pots (66 cm^3, RLC-4, "Pinecell", Stuewe and Sons, Corvallis, OR). AM fungus and non-AM fungus pots were separated to avoid cross-contamination. The SSVT was sterilized to exclude colonization by indigenous mycorrhizal fungi. One Romaine seed was placed ca. 3.2 mm beneath the surface of SSVT in each non-AM fungus pot. In the AM fungus treatment, pots were 50% filled with SSVT and 300 *G. intraradices* AM fungus spores (1 ml solution) were pipetted onto the surface and the pot filled with SSVT. The pots were then watered with deionized water and one Romaine seed was placed ca. 3.2 mm under the SSVT. Each tray was placed in a growth chamber with light:dark cycles of 16:8 h at 25:18°C to allow germination and seedling growth up to six weeks to allow for colonization of roots by *G. intraradices* (see Fig. 1). The seedlings were fertilized weekly with 10 ml of Hoagland's nutrient solution without phosphorus (Hoagland and Arnon, 1938) until used for the study. Just prior to initiating experiments with the pathogens, a subsample (n = 3) of seedlings was harvested for measurement of shoot dry weight (80 °C) and phosphorus content. Roots were retained for staining to determine extent of AM fungus colonization. Shoot tissue was ground to pass a 20 mesh sieve, digested in H_2SO_4/H_2O_2, and analyzed for *P* via the method of Murphy and Riley (1962).

2.4. Destructive measurements and assessment of AM fungi/root colonization

Root staining was performed via the method of Phillips and Hayman (1970) to observe the colonization of Romaine lettuce by AM fungi to ensure plants were colonized prior to inoculation with pathogens. Briefly, roots were immersed in 10% [wt/v] potassium hydroxide (KOH) and heated for 30 min at 90 °C. The KOH was poured off, roots were rinsed with water twice, and then 1% hydrochloric acid (HCl) was added sufficient to cover the roots. Tubes were then held with the solution inside for five minutes, the HCl was poured off, and roots were stained with the Diazo dye, Trypan blue (Phillips and Hayman (1970)). Tubes were heated for five minutes at 90 °C, Trypan blue was eluted and roots were