Persistence and ecological implications of maize-expressed transgenic endo-1,4-β-D-glucanase (E1) in agricultural soils

by

Adam Joseph Kenny

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Program of Study Committee:
Jeffrey Wolt, Major Professor
Michael Thompson
Joel Coats

Iowa State University

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GLOSSARY

Biofeedstock – a plant-based material used in biorefineries as an energy source.

Dissipation – the biotic or abiotic inactivation of a biomolecule that prevents detection and functionality.

EEC (estimated environmental concentration) – a realistic, conservative exposure estimate based on the expression level or concentration (exudate) of the protein, mass of residue or volume loaded, and the applicable quantity of receiving environment (soil).

Endogenous – produced from within, native.

Enzyme Activity Assay – a method for detecting functional enzymes by incubating with a substrate and quantifying some assessment endpoint of catalysis (e.g. colorimetric, dye, fluorogenic, viscosity, mass-balance).

Exogenous – added from a foreign source.

Mesophilic – characterized as developing or functioning at moderate environmental temperatures where most organisms exist (generally 20-45^{o}C).

Persistence – detection of a biomolecule over time in a given environmental system (related to dissipation).

Thermophilic – "heat-loving" – characterized as developing or functioning at high temperatures not sustainable to most organisms (generally 45-120^{o}C).

Transgenic – an organism that expresses a heterologous gene, often contrasted with traditional breeding.

CHAPTER 1. GENERAL INTRODUCTION AND LITERATURE REVIEW

TRANSGENIC BIOFEEDSTOCK CROPS

Bioenergy

Extractable petroleum production is estimated to peak in the next decade, assuming it has not already [1], and the search for alternative transportation energy is well underway. One alternative route the US Department of Energy (DOE) supports is use of lignocellulosic biomass for conversion to ethanol fuel [2]. Biofuel from cellulose is desirable because cellulosic materials represent a large potential feedstock, energy security needs and ecological concerns from carbon dioxide emission (relating to climate change) are addressed, and most feedstock resources do not compete with food and feed (e.g. corn stover, timber) [3-6]. It is important to note that cellulosic biofuel is viewed as a temporary, transition fuel with aims to alleviate fossil fuel dependency. Biofuels from lignocellulosic materials are considered 2nd generation biofuel technology, whereas 3rd and 4th generation technologies utilizing algae for biodiesel and other organisms for bio-hydrogen/electricity use are under development for more permanent solutions [7]. For the moment, however, lignocellulosic ethanol is appealing because it conforms to the current liquid fuel framework [8].

Although ethanol fuel production from plant sugars has over 100 years of history, the realization that fossil fuels are non-renewable and depleting rapidly has sparked renewed interest in plant ethanol use. The 2005 'Billion Ton Study' [2] from the United States DOE and Department of Agriculture (USDA) recognized the capability of using renewable biomass to replace ~20% of US transportation fuel needs by 2030. Lignocellulosic corn stover was noted as being "a major untapped resource" from conventional agricultural practices and is estimated to comprise nearly 40% of agricultural feedstocks and 30% of all proposed feedstocks. Assuming no-till agricultural management practices, nearly two-thirds of stover on productive Midwestern soils can be sustainably allocated for ethanol production with the remaining one-third left in the field to prevent soil erosion and sustain nutrient availability [9].

Lignocellulosic Ethanol Production

Lignocellulose is generally defined as structural plant polymers of cellulose surrounded by and intertwined with highly impervious lignin and hemicellulose layers. Lignin and hemicellulose in higher plants are evolutionary products designed to increase rigidity and

prevent deconstruction, so access to internal cellulose is understandably difficult. Once these protective layers are disrupted, however, carbon polysaccharides can be enzymatically degraded to yield high-energy glucose. In ethanol production from biofeedstocks, glucose is then fermented by yeast microbes to produce ethanol (alcohol fuel).

A prototypical outline of lignocellulosic biofuel conversion from start to finish (including associated costs) can be found in the 2002 technical report from the NREL [10]. In chronological sequence, general production steps are:

1. Collection of stover and fractionation
2. Pretreatment (acid/heat hydrolysis)
3. Enzymatic saccharification
4. Fermentation
5. Distillation, product recovery and clean-up

Despite recognition that more than a billion tons of biomass may be presently available for ethanol conversion, the associated production costs present a significant barrier. The major feasibility issue, labeled the 'recalcitrance problem,' relates to steps 2 and 3 of production – acid/heat pretreatment of the biomass and enzyme loading for subsequent depolymerization of internal cellulose [7-8, 11-14]. These two processes go hand in hand as the ability of pretreatment processes dictates how easily cellulases may access the carbon substrate. Alvira et al. [15] and Houghton et al. [16] provide detailed information on pretreatment technologies and enzymatic conversion technologies, respectively.

Transgenics

Although progress has been made in lowering the associated production costs, there is a growing realization that genetically engineered (GE) feedstock crops are needed to improve processing and make cellulosic bioenergy cost-effective [12-13]. Gressel insightfully noted that food crops have been cultivated for thousands of years to reach the efficiency and production levels of today and that it is ignorant to believe that the production of efficient feedstock crops could happen any sooner without genetic engineering to speed up the process [7]. Considering the time-sensitive urgency to replace petroleum-based fuel (on a decadal scale), genetically engineering crops to have traits that are desirable for biofuel production is a sensible strategy. A number of transgenic solutions have been proposed to address the recalcitrance problem including: repressing lignin biosynthesis [17-20], advances in pretreatment technology [15],

culturing of microorganisms with highly active ligninolytic enzymes [21], and *in planta* expression of cellulase enzymes [12-13, 22]. Of particular relevance here are feedstocks that express cell-wall degrading enzymes such as E1 endoglucanase [pdb:1ece]. Expressing industrial bioprocessing enzymes in feedstocks is not an entirely novel idea as a 1st generation biofuel corn product already exists in the market that expresses a thermostable alpha-amylase intended to break down starch from grain [23].

THERMOSTABLE ENDO-1,4-β-D-GLUCANASE (E1)

Isolation and Characterization

Acidothermus cellulolyticus is an actinobacterial thermophile that was first isolated from hot springs in Yellowstone National Park in 1986. Since then, this microorganism and the cellulases it produces have been sought for use in cellulosic biofuel conversion [24]. Within a decade of discovery, three endocellulases from *A. cellulolyticus* had been purified and characterized by a group from the National Renewable Energy Lab (NREL) [25-27]. The low molecular-weight E1 (~60kDa) enzyme was the most suitable for industrial application and has been the focus of many biofuel advancements in the past decade [e.g. 28-32]. E1 is a thermostable endoglucanase that has a temperature and pH optima of 83^{o}C and 5, respectively [25]. Endoglucanase is one of three components in the cellulase complex. Cellulases are biocatalytic systems made up of endoglucanases (EC 3.2.1.4), exoglucanases (EC 3.2.1.91), and β-glucosidases (EC 3.2.1.21) that work synergistically to depolymerize cellulose. The currently accepted mechanism of cellulase action begins with endoglucanase hydrolyzing internal β-1,4 glycosidic bonds within amorphous regions to produce new chains ends. Exoglucanase, also recognized as cellobiohydrolase (CBH), processively attacks chain ends to yield glucose or celliobiose. β-glucosidase completes any residual breakdown needed and prevents product-inhibition from cellobiose [33].

Introducing thermostable enzymes (e.g. E1) into the cellulosic biofuel conversion process is desirable because they exhibit higher conversion efficiencies, maintain activity at the temperatures/pH characteristic of the fermentation process, and have decreased conformational entropy which leads to increased stability [22, 34]. Additionally, using higher temperatures during conversion processes can prevent batch contamination, decrease solution viscosity for improved mixing, and better solubilize substrates [35].

Industrial Application

Although cellulosic ethanol has the potential to address US DOE goals of energy independence and carbon-neutrality, production costs are prohibitively expensive [36]. As mentioned, the addition of thermostable cellulases is expected to increase efficiency and decrease cost of ethanol production from biofeedstocks. Endoglucanases, exemplified by E1, are particularly favorable as they initiate the endo-exo synergistic effect in cellulose depolymerization [37]. E1 is a highly active, thermostable enzyme that could replace mesophilic endoglucanases that are currently added to batch fermentations after carbon substrates have been pretreated. In order to address efficiency needs, however, high biological expression of E1 is required. Of the numerous means proposed for E1 production, *in planta* expression allows for the highest accumulation, lowest energy cost, and greatest scale-up potential [22].

Using plants as 'green bioreactors' could improve the production of proteins by utilizing solar energy and the photosynthetic capability of plants to inexpensively manufacture biomolecules that also have high scale-up potential. Additionally, plant expression usually results in correct protein folding, prevents human pathogen contamination, and may allow for direct use without extensive purification [38]. In one study, *in planta* expression of E1 also resulted in activity on internal cell walls during plant growth and hence improved the processability of host feedstocks [39].

A large number of plants have been transformed to express E1 including *Arabidopsis thaliana* [29], *Solanum tuberosum* (potato) [40], *Lemna minor* (duckweed) [31], *Oryza sativa* (rice) [43], *Nicotiana tabacum* (tobacco) [30, 39, 41-42, 44], and *Zea mays* (corn) [28, 39, 45-47]. The cost of producing cellulases needed for biofeedstock digestion could be greatly reduced by harvesting them from transgenic plants and adding them during the production process. To further improve economic efficiency, E1 could be expressed directly in the biofeedstock of choice – specifically, maize stover [e.g. 28]. Ideally E1 would be released from the plant cell during acid/heat pretreatment whereupon it would degrade the carbonaceous cell wall and release fermentable sugars. However, current pretreatment technologies may be too severe for E1 to persist and remain active. Teymouri et al. [44] showed that a mild pretreatment with ammonia fiber expansion (AFEX) decreased residual cellulase activity by more than 65

percent. Thus the usefulness of E1 could be greatly improved should a less severe pretreatment technology be implemented during conversion.

FATE OF EXTRACELLULAR PROTEINS IN SOIL

Soil Enzymes

Extracellular soil enzymes are generally released by microbes into the harsh interstitial matrix where they either become complexed with clay and humic particles or cell debris or are degraded by microbes and proteases. Enzyme sorption to organo-minerals usually happens in a matter of hours. Once adsorbed, enzymes usually lose a portion (if not all) of their functionality [48]. The severity of activity lost will depend on conformational changes relating to the rigidity of the protein tertiary structure and exposure of the active sites. In the environment, complexed proteins are generally considered to be irreversibly bound, however, extraction buffers and mechanical procedures have been developed for bench-scale laboratory studies to release enzymes back into the solution phase [49]. Soil-enzyme complexation is thought to be a function of the surface properties of soil particles because a pH-dependent relationship exists where adsorption is strongest at a protein's isoelectric point (pI). Protonation and deprotonation reactions at the solid-solution interface of clay and humic substances develop a negatively charged surface that reacts strongly with neutral-charged proteins [50]. Along with pH, other criteria noted for playing a role in the sorption of enzymes are hydrogen bonding, van der Waal forces and general soil properties such surface area and cation-exchange capacity [51]. Given the variable nature of interactions between soils and enzymes, three possible fates can occur regarding their persistence and functionality (Table 1). Briefly, enzymes are either complexed or biodegraded and, if complexed, will either retain functionality and persist for some amount of time or become inactive or denatured. Denatured or non-functioning enzymes will not affect ecological processes whereas persistent and active proteins might.

Transgenic Protein Expression

Similar to soil microbes, crops grown for protein production will incidentally release some amount of protein to the environment. The probable release pathway of plant-expressed heterologous proteins to the soil environment is through (1) post-harvest decomposition of residues, (2) root exudation or (3) root sloughing. Once released, the fate of exogenous proteins may be viewed similarly to extracellular enzymes.

Since the inception of genetically engineered crops in 1996, cultivation has expanded rapidly with 90% of corn, soybean, and cotton grown in the US containing at least one transgene in 2011 [52]. The dominant transgenic crops have insect resistant and/or glyphosate-tolerant traits that ensure plant protection from pests and prevention of toxicity from herbicides, respectively. Although these genetic enhancements benefit agriculture and society, they have required assessment to ensure their safety to humans and the environment. Crops expressing the insecticidal crystalline protein (ICP) from *Bacillus thuringiensis* (Bt) have been extensively studied with respect to environmental safety, especially regarding effects to non-target organisms [53]. The estimated environmental concentration (EEC) for maize-expressed transgenic proteins introduced into agricultural soils range from 20-350 ng g^{-1} soil (Table 2). The fate of transgenic proteins released to soil requires assessment to determine the risk to non-target organisms and ecosystem services. These risks will vary with each unique plant transformation and therefore need to be considered on a case-by-case basis [54].

Environmental Exposure

Environmental loading estimates are valuable parameters for determining exposure in environmental/ecological risk assessments, yet environmental loadings of most transgenic proteins are difficult to estimate due a lack of consistent reporting throughout the literature. For example, expression of ICPs has been reported as ng mg^{-1} total protein, ng mg^{-1} dry weight, and ng mg^{-1} fresh weight all within the same table [55]. Estimates have also been reported on a percentage basis of total soluble protein [56]. Fairly consistent reports can be found in the comprehensive GM Crop Database (http://cera-gmc.org/index.php?action=gm_crop_database), but still lack completeness. Proper estimates are further complicated because

- Moisture content of stover at grain harvest can range from 12-75% depending on the stover component (stalk, leaf, cob, husk) [10, 57]
- Stover components do not have equal mass shares, typically 50-20-15-15 for stalk-leaf-cob-husk [10]
- Heterologous expression levels vary among the maize tissue components and throughout the growing season [58].

Transient moisture contents and expression levels make interpreting sparse data labeled 'fresh weight' quite difficult. Ideally all transgenic maize intended for environmental release could be presented in a characteristic tabular format similar to Table 3. The environmental risk

of transgene proteins released to the environment is a product of the formulated problem and exposure, and thus an accurate exposure estimate is necessary to lead the risk assessment in a way that is useable to decision-makers [54].

It is estimated that where genetically engineered maize expressing E1 is grown, it will add 17-120 g ha^{-1} y^{-1} of thermostable endoglucanase in post-harvest residues to receiving soils.[1] Graham et al. [9] considered stover collection after grain harvesting with respect to sustainable residue allocation that ensures enough stover is left in the field to prevent soil erosion and maintain crop productivity (i.e. retention of soil water and nutrients). From a sustainable agronomic management viewpoint they determined that stover for biofuel production would likely come from highly productive soils (producing >2 Mg stover ha^{-1}) and cost less than $30 Mg^{-1}.

Persistence and Ecological Effects

To date, thermostable E1 has not been studied in soil systems. Two other proteins that have been expressed in maize and characterized for fate and behavior are the ICPs and heat-labile enterotoxin subunit B (LTB) proteins.

Bt Cry Proteins. Cry proteins are naturally expressed in the soil bacteria *Bacillus thuringesis* and act by binding to receptors within the midgut of insects causing cell lysis and eventual starvation and death [59]. Field-grown maize currently expressing heterologous ICPs is estimated at 74% of all corn grown in the US when Bt-only and stacked gene varieties are combined [52] and includes eight unique Cry proteins [60]. The prevalence of such wide environmental release spurred a number of research articles that aimed to determine their persistence and ecological effects [62-68]. Because expression may occur in all components of maize tissue, Cry proteins enter the soil environment through multiple release pathways. The persistence of bacterial and crop-expressed Cry protein in soil experiments has shown little consistency likely due to differences in microbial activity/composition, adsorptive particles, pH, tissue composition relating to mineralization (C:N, lignin), crop and soil management techniques, and the multiple analytical assays, but for typical receiving environments these proteins tend to lose activity in a matter of days [62]. It is also interesting to note that the type of Cry protein can play a significant role in persistence and retention of insecticidal activity [69]. Generally Cry proteins do not persist in the soil for extended periods of time. Icoz and

[1] See Appendix A. Exposure Assessment.

Stotzky have summarized data on the fate and effects of Cry proteins in soil pertaining to macrofauna (earthworm, woodlouse, pillbug, sowbug, collembolan, nematode, and mite), microorganisms (bacteria, fungi, protozoa, and algae), microbe-mediated processes (C,N,P cycling), and residence time (half-life) [62]. Proteins were detected using either an insect bioassay or ELISA (enzyme-linked immunosorbent assay). The overwhelming consensus is that Cry proteins released to soil have little to no significant effect on soil biota or microbe-mediated processes.

LTB Proteins. A gene coding heat-labile enterotoxin subunit B (LTB) has been expressed in corn grain for oral administration of a vaccine protein that prevents diarrhea. As this protein is expressed in grain, the environmental release of maize-expressed LTB was based on estimates of kernels left in the field post-harvest [70]. Studies by Kosaki et al. have shown soluble forms of bacterial LTB and its closely homologous CTB persist in soil for 1-2 days [71]. Persistence of maize-expressed LTB (tissue-amended soil) had a DT50 (time to 50% of dissipation) of approximately 35-90 days. An ecological study of maize-expressed LTB showed no observable effects to springtails or earthworms in soil microcosm studies based on mortality rates.

SOIL CARBON AND CELLULASES

The consequences of climate change are predicted to be far-reaching and generally detrimental to life on Earth, and carbon dioxide (CO_2) from human activities is considered an important contributor [6]. Soils have a proportionately large store of carbon with more than three times that of the atmospheric and four and half times that of the biotic pool [72]. Land-use changes in the past 200 years have generally depleted soil carbon stores with over 20% of carbon from cultivated areas being transferred to atmospheric pools [73]. Given that CO_2 is the most significant green-house-gas from anthropogenic sources and that soils can store a substantive amount of carbon, there have been many efforts to not only prevent the loss of soil carbon, but to actively sequester it as well [74].

Soil enzymes are biological catalysts that play a significant role in the elemental fluxes of carbon, nitrogen, phosphorus, and sulfur. Glucoside hydrolases (EC 3.2) represent a large class of enzymes and are especially important for carbon cycling as they are responsible for hydrolyzing substrates into sugars for microbial consumption. Cellulases (EC 3.2.1.4), in particular, are key glucoside hydrolases for they break down cellulose, the world's most

abundant biopolymer and largest constituent of plant matter. The biochemical reactivity of soil cellulases can be an important parameter for assessing the effects of different agronomic management practices and has been calculated using a number of extraction and detection methods [61, 75-79]. For one of the more popular methods used, Deng and Tabatabai [78] found that cellulases (endoglucanases) from productive Iowa soils could convert up to 220 mg glucose/kg soil/day from a carboxymethyl cellulose substrate. Combining microbe-derived activity and the activity from plant-derived E1 at its estimated loading, overall soil endoglucanase activity could hypothetically be doubled.

PROBLEM FORMULATION AND RESEARCH OBJECTIVES

Genetically engineering biofeedstock crops for improved conversion efficiency is a sensible strategy to meet energy needs in the near future. Specifically, lignocellulosic maize stover has been transformed and expresses a thermostable endoglucanase (E1) to decrease production costs and increase efficiency. Transgenic E1-maize would be grown on productive Midwestern soils where stover can be economically and sustainably allocated for cellulosic biofuels. Decomposition of post-harvest residue left on fields would release E1 into the soil at a concentration (~120 g ha^{-1}) that approximately doubles the endogenous endoglucanase activity (Fig 1).

E1-endoglucanase was originally isolated from hot springs and has optimal activity within a pH range characteristic of representative soil types. Thermostable enzymes such as E1 are also known to have rigid, stabilizing structures and may be resistant to physicochemical denaturing in the soil environment. Should E1 enzymes persist, they could affect specific ecosystem services such as carbon cycling. Soil is an important carbon sink and sequestration is viewed as a potential strategy to decrease atmospheric CO_2 and mitigate the effects of climate change. Bioenergy from lignocellulosic maize is a carbon-neutral alternative to petroleum fuels, but may impact the environment and, thus, society if well-intended technologies disrupt ecosystem services. This research addresses the potential effects of E1 by investigating its persistence and overall effect on soil carbon mineralization.

Specific Objectives

1. Optimize an extraction and detection method (enzyme assay) for thermostable E1 from soil matrices.

2. Determine the level and persistence of bacterial and plant-derived E1 activity in three representative soils and compare with the persistence of a mesophilic cellulase.
3. Determine the effect of E1 on soil carbon mineralization by amending soil microcosms with E1 or Wild-type maize tissue and measuring CO_2-C release.

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Table 1. General fate of extracellular proteins released to the soil environment from plants. Ecological implications are dependent upon enzyme stability.

Stability	Description	Implications
Persistent, Functional	Sorbs to stabilizing solid (e.g. clay surface) and remains biologically active for some period of time.	Functioning may or may not be ecologically significant. For example, long term activity could impact nutrient cycling, microbial functioning, and other non-target organisms. Alternatively, activity could represent an insignificant fraction of whole soil activity. Short- and long-term monitoring suggested.
Persistent, Not Functional	Sorbs to stabilizing solid, but loses most or all functionality likely due to conformational changes and effect on active site.	A detectable yet non-functioning enzyme would not likely pose an ecological concern. However, there may be the case where residual proteins could become active after some time. Long-term monitoring suggested.
Not Persistent, Not functional	Degrades via microbe-mediated processes or as a result of chemical and/or physical denaturing	Rapid dissipation of the protein would not pose an ecological concern.

Table 2. Heterologous protein expression in maize stover and the estimated environmental concentration based on residue left on fields after harvest. Estimates are for Bt Cry proteins and thermostable E1 (endo-β-1,4-D-glucanase).

Event	[a] Protein expression in Tissue ($\mu g\ g^{-1}$)	[b] Estimated environmental concentration ($ng\ g^{-1}$ soil)
[c] Cry 1Ab (Mon810)	9.4	18.8
[c] Cry 1Ab (Bt11)	12.5	25.6
[c] Cry 1F (TC1507)	176	352
[d] E1 (Spartan I)	33.0	66.0

[a] Tissue expression was converted to reflect tissue weight (~36% moisture) at harvest compared to fresh tissue weight (plant maturity). Stover components were weighted (50-20-15-15 mass ratio, stalk-leaf-cob-husk) and expression values averaged. The weighted average moisture of stover at grain harvest was 36% where at physical maturity it was 67%. Expression was essentially doubled with the exception of E1 (Spartan Corn) which had already accounted for tissue moisture. Percentages are wet-weight, estimated from Pordesimo et al. [57].

[b] Estimate based on residue amended evenly with top 15 cm of soil profile (2 mg tissue/g soil). Reported per gram oven-dry soil. See also Apendix A. Exposure Assessment.

[c] Data [55]

[d] Data [28]

Table 3. Tabular template for improving exposure assessment of transgenic proteins expressed in maize (Example using Cry1Ab from Bt11) [55]. Expression will vary across the growing season for individual maize components and subcomponents.

Grain		Root		[a] Stover		
Moisture (%)	Expression (μg g^{-1})	Moisture (%)	Expression (mg g^{-1})		Moisture (%)	Expression (mg g^{-1})
40	0.4	6-30	[b] 6		36-72	8.3-16.6
				stalk	50-75	[b] 6
				leaf	30-74	10
				cob	14-52	16.2
				husk	12-47	[b] 6

[a] Stover moisture and expression values are ranges based on 50-20-15-15 ratio of stover components (stalk-leaf-cob-husk). Typical moistures at plant physiological maturity and harvest were used [57].

[b] Average expression value, no specific data [55].

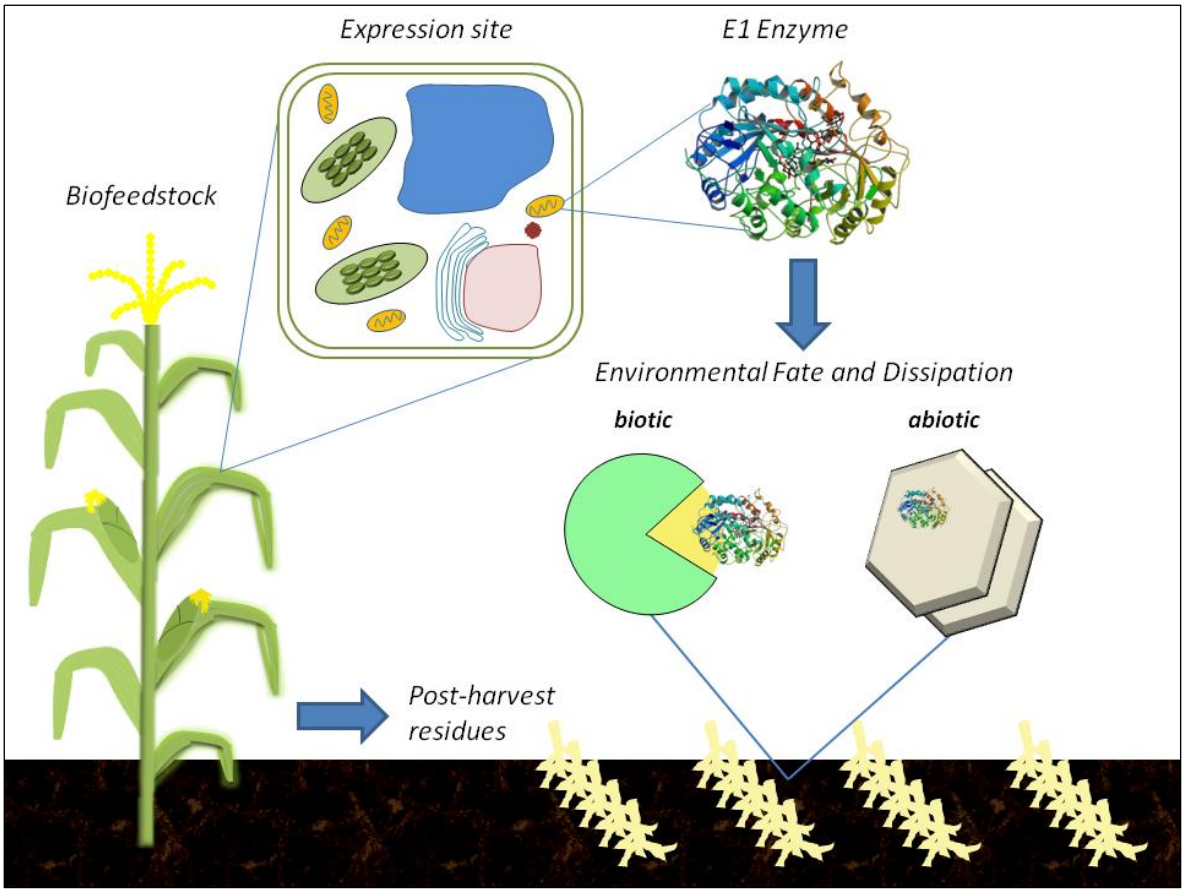

Figure 1. General overview of transgenic maize-expressed E1 release to the soil environment from post-harvest residue decomposition. Dissipation is dependent upon clay and humic colloid content and reactivity with E1 and microbial robustness resulting in biodegradation. E1 structure from Protein Data Bank (PDB: 1ECE) [80].

Chapter 2. Persistence and Ecological Implications of Maize-expressed Transgenic Endo-1,4-β-D-glucanase (E1) in Agricultural Soils

ABSTRACT

Plant expression of thermostable endoglucanase (E1) [pdb:1ECE] has been proposed for improved conversion of lignocellulose to ethanol for fuel production. Since E1 was isolated from hot springs and has thermostable properties, it may have increased stability when released to agricultural soils during post-harvest residue decomposition. Endoglucanases are a subclass of cellulases and act by cleaving β-1,4 glycosidic bonds in cellulose polymers at amorphous sites. In addition, they are important in carbon cycling and can be useful indicators of possible ecological perturbations in soil. It is estimated that where genetically-engineered maize expressing E1 is grown, it will add 17-120 g ha^{-1} y^{-1} of thermostable endoglucanase in post-harvest residues to receiving soils. The persistence and reactivity of residual E1 is being investigated in soil microcosm studies for soils amended with bacterial and plant-solubilized E1 as compared to soil endogenous activity and to activity added from a mesostable form of endoglucanase (*Aspergillus* and *Trichoderma* spp.). An optimized analytical method involving a carboxymethyl cellulose (CMC) substrate and dinitrosalicylic acid (DNS) detection method effectively assayed endoglucanase activity in amended and unamended soils and was used for determining E1 persistence in representative soils. Effects on soil carbon mineralization were determined by comparing CO_2 evolution from soils amended with E1 and Wild-type corn tissue. Extraction and recovery of the mesostable comparator, bacterial E1, and plant-soluble E1 shows near-complete dissipation of activity within a 24-hour period. Carbon mineralization studies indicate no significant difference between soils amended with either the transgenic E1 or Wild-type maize tissue. The combined result from persistence and CO_2 evolution studies suggest that maize expressing up to 30 g Mg^{-1} of E1 in stover (120 g ha^{-1} environmental load) would not persist in representative soils or significantly impact carbon cycling.

INTRODUCTION

Growing dependence on foreign oil, coupled with extensive fossil-carbon use has accelerated the need to produce low-cost, domestic bioenergy feedstock crops. Specifically, maize cellulosic residues represent a major potential biofeedstock source from conventional agricultural production [1]. Currently the conversion production strategy lacks the efficiency needed to make cellulosic feedstocks economically viable. In order for carbon polymer sugars to become available for processing into ethanol, the biofeedstock must undergo dilute acid pretreatment at temperatures generally in excess of 75^{o}C to eradicate the recalcitrant lignin and hemicellulose layers that shield the carbon substrate. Pretreatment is then followed with exogenous cellulase additions to release fermentable glucose sugars. Cellulases are biocatalytic systems made up of endoglucanases (EC 3.2.1.4), exoglucanases (EC 3.2.1.91), and β-glucosidases (EC 3.2.1.21) that work synergistically to depolymerize cellulose, the world's most abundant biopolymer. The currently accepted mechanism of cellulase action begins with endoglucanase hydrolyzing internal 1,4 glycosidic bonds within amorphous regions to produce new chain ends. Exoglucanase, also recognized as cellobiohydrolase (CBH), processively attacks chain ends to yield glucose or celliobiose. β-glucosidase completes any residual breakdown needed and prevents product-inhibition from cellobiose [2].

A significant drawback to effective biofuel production from cellulosic materials is the expense required when cellulase enzymes are added after pretreatment. In fact, the use of cellulases generated from large microbial bioreactors accounts for approximately one-third to one-half of the entire production cost for cellulose-derived ethanol [as cited in 3]. Although significant advances have been made to lower these production costs, the most sensible and likely strategy requires genetic transformation of the source biofeedstock for better utilization of the cellulosic material [4,5].

The genetic engineering of bioenergy feedstocks is being explored for a variety of cellulosic crops (*Zea mays* (corn), *Panicum virgatum* (switchgrass), *Miscanthus* spp). These crops may be genetically engineered to improve processibility through *in planta* expression of bioprocessing enzymes [5-10] and by altering the lignin content and composition [11-14]. Improved cellulose conversion efficiencies for maize stover can be achieved through *in planta* production of bioprocessing enzymes so that following biomass pretreatment and cell lysis, plant-made cellulases become active and cleave cellulosic polymers into fermentable sugars. A

case in point is highly thermostable endo-1,4-β-D-glucanase from *Acidothermus cellullyticus* (E1) [pdb:1ECE] that has been expressed at high levels in maize green tissue [3]. Thermostability is highly desired in the biofuel conversion process as enzymes are subjected to high temperatures and variable pH. Additionally, saccharification at higher temperatures can prevent batch contamination, decrease solution viscosity for improved mixing, and better solubilize cellulose substrates [15]. E1-endoglucanase has an optimal temperature at 83^{o}C and has shown marked stability in both acidic and basic environments.

Scope and Justification

It is estimated that where genetically engineered maize expressing E1 is grown, it will add 17-120 g ha^{-1} y^{-1} of thermostable endoglucanase in post-harvest residues to receiving soils.[1] The wide-scale agricultural release of E1-expressing maize will require environmental assessment to better understand the potential impact of residues left in the field [16]. Especially important in this regard is the evaluation of effects on ecosystem services (e.g. carbon mineralization and biogeochemical cycles). It has been well documented that enzymes in the soil environment are direct biological instigators of elemental cycling and indicators of overall soil tilth [17-21]. The importance of soil glucoside hydrolases cannot be overemphasized as the soil carbon pool is more than three times that of the atmosphere pool and four and half times that of the biotic pool [22]. Furthermore, E1 was originally isolated from hot springs and has thermostable properties that may confer increased stability when released to agricultural soils during post-harvest residue decomposition [23]. Enzyme function and stability have been linked to numerous soil physicochemical properties such as texture (especially clay) and organic matter (OM) content. Clay and humic substances are thought to protect enzymes/proteins from degradation due to enhanced stability via steric hindrance and conformational changes upon organo-mineral sorption that make them less susceptible to proteolysis and physicochemical denaturing [24]. Ironically, the sorption process can also disrupt the tertiary structure of proteins to the extent that they are no longer biologically active, however, there is also evidence that activity may be retained after sorption. Regardless of the residual functionality, once enzymes are adsorbed to soil particles they are generally considered to be irreversibly bound [24].

[1] See Appendix A. Exposure Assessment.

The novel expression, release to a foreign environment, and potential effects to ecosystem services require better knowledge of E1 fate in the soil environment. We have incorporated standard, albeit modified, protocols that aim to address the research objectives and provide decision-makers with useful approaches to understanding the environmental fate and behavior of transgenic proteins.

Objectives

As a first step in the assessment of residue effects, I have optimized a method for extracting and detecting E1 from soil. Using this optimized method I compared the reactivity and persistence of endoglucanases from various sources (bacterial E1, E1-maize soluble protein, endogenous soil enzymes, and mesophilic endoglucanases) when amended to representative agricultural soils. Lastly I have measured carbon dioxide (CO_2) evolution to compare whole respiration rates from soils that were unamended or amended with either transgenic (E1) or non-transgenic (Wild-type) plant materials. The CO_2 evolution study provides indirect data on persistence of whole tissue-derived E1 that has been amended to soils and also indicates whether E1 presence in soil affects mineralization of labile carbon.

MATERIALS AND METHODS

Test Materials

Transformed (E1) and untransformed (Wild-type) maize were developed at Michigan State University under the direction of Dr Mariam Sticklen in the Department of Crop and Soil Sciences. Transgenic tissue was 2nd generation corn resulting from the breeding of plants that produced E1-endoglucanase in the mitochondria (1.5-E1-10b x 1.5-E1-10c). Non-transgenic near-isoline tissue (Wild-type) was used as a control. Upon receipt of dried plants, tissues were pulverized in liquid nitrogen and stored at -20^{o}C until used (Table 1). Total carbon and total nitrogen analysis was performed on both tissues at the Iowa State Soil and Plant Analysis Laboratory (Ames, IA).

The mesophilic cellulase comparator (*Aspergillus* and *Trichoderma* spp.) was obtained from MP Biomedical (Solon, OH). Bacterial E1 from *Streptomyces lividans* was provided by the National Renewable Energy Laboratory (Golden, CO). The bacterial E1-endoglucanase and mesophilic comparator were characterized to determine the temperature (15-60^{o}C) and pH (3-

8.5) optima using the carboxymethyl cellulose (CMC) and dinitrosalicylic acid (DNS) analytical technique (see methods).

Plant total soluble protein (TSP) was obtained by geno/grinding (SPEX CertiPrep, Metuchen, NJ) 100 mg of the previously milled tissue in 1 ml of extraction buffer (50 mM sodium acetate (NaAc), 10 mM EDTA, 0.1% Triton X-100, pH 5). Crude soluble extracts were centrifuged and an aliquot was subjected to ammonium-sulfate precipitation using an initial saturation concentration of 100% (0^{o}C). Crude extracts and ammonium sulfate solution were combined at 0.4:1 ratio (70% final saturation concentration of ammonium sulfate), briefly vortexed, and then stored in a -20^{o}C freezer for 1 hour before centrifugation at 20,800 x *g* (14,000 rpm) for five minutes. A protein pellet formed and was reconstituted in a storage solution to an equivalent of 0.2 g/ml (original tissue). Activity of plant soluble protein from E1-maize and Wild-type maize was determined with the optimized detection assay and compared against activity from E1 standards to estimate expression levels. All stock enzyme materials were stored in 1% bovine serum albumin (BSA), 50 mM NaAc, pH 5 in polypropylene vials for optimum stability.

Test Systems

All preliminary work was performed using an air-dried Nicollet loam surface soil. Definitive studies utilized surface soils from the Hanlon-Spillville-Coland alluvial catena which were sampled from the ISU Hinds Irrigation Plots.[2] For definitive studies, field-moist samples of each soil were freshly collected prior to initiation and sieved to pass 2-mm mesh openings. Each soil sample was maintained moist at a workable consistency in the dark at 4° C until used. These materials were used within 60 days of sampling. Characterization of sieved soils was conducted by Midwest Laboratories Inc. (Omaha, NE) using standard methods (Table 2).

Detection Optimization

E1-endoglucanase is a unique enzyme that is novel to agroecosystems and therefore a number of detection optimizations were undertaken to improve detection. Excluding immunoassay detection, all optimization procedures employed the mesophilic comparator

[2] Hanlon: course-loamy, mixed, superactive, mesic Cumulic Hapludoll
Spillville: fine-loamy, mixed, superactive, mesic Cumulic Hapludoll
Coland: fine-loamy, mixed, superactive, mesic Cumulic Hapludoll
Nicollet: fine-loamy, mixed, superactive, mesic Aquic Hapludoll

cellulase (as E1 materials were fairly limited) and Nicollet storage soil. Initially E1 was detected using a highly specific enzyme-linked immunoassay (ELISA) and antibodies from the National Renewable Energy Lab (NREL) [25]. An alternative detection method using enzyme activity has also been used extensively to detect plant-expressed proteins from soil (e.g. phytase) [26] and was tested here. Activity assays work by measuring end-product release and then back-calculating the concentration using a correlation curve from appropriate standards. Measuring the enzyme activity of endoglucanases that have been extracted from soils proved to be the best detection method and was used for method optimization and persistence studies. One-half gram Nicollet soil samples and soluble mesophilic preparations of 150 ng were used for all optimization analyses. Optimization activity assays were performed at 40^{o}C with 72 hour hydrolysis unless noted otherwise.

Substrate and Detection. Endoglucanase activity has been reported using carboxymethyl cellulose (CMC) as well as the fluorogenic substrate 4-methylumbelliferone β-D-cellobioside (MUC) as substrates [27-29]. Using MUC is problematic as dissolved OM can quench fluorescence and may also be hydrolyzed by cellulases other than endoglucanase [30-31]. CMC is cleaved primarily by endo-acting cellulases and has been used to measure E1 in clean systems as well as general endoglucanase in soil extracts. Therefore, CMC was used in all enzyme assays. The products of CMC hydrolysis from endoglucanase are glucose monomers. I tested two types of assays that either (1) detected sugar residues that were dyed with Remazolbrilliant Blue R and then solubilized when CMC was cleaved or (2) detected glucose directly using a reducing sugar assay solution. Blue-dyed CMC (Azo-CMC, Sigma-Aldrich, St Louis, MO) was initially screened for background effects from the activity of purified plant-soluble matrix from both transgenic and non-transgenic tissue. Reducing sugar detection was tested for resistance to matrix effects by comparing the linearity of glucose standards in the presence and absence of extraction and substrate background solutions. Briefly, glucose standards were added at the specified detection range (ng/ml or μg/ml) to the background matrix (1:1 optimized extraction buffer and 2% CMC substrate solution, pH 5) and added 1:1 to the alkaline ferricyanide reagent [32] or dinitrosalicylic acid (DNS) reagent [33]. The reducing sugar DNS assay (μg/ml range) proved to be the most robust detection reagent and was used for the remainder of optimization and persistence studies. For routine use the DNS reagent was mixed at 1:1 ratio (400 μl total) with the CMC hydrolysis solution and heated

to 95^{o}C for 5 minutes. The colored solution was centrifuged at 20,800 x *g* for 1 minute and a 200 µl aliquot was immediately measured at 540 nm in microplate wells using a BioTek PowerWave spectrophotometer (Winooski, VT). Also, a sodium azide (NaN_3) microbial inhibitor was added to the CMC substrate according to [35] and glucose release was compared to control samples.

Extraction. Cellulase enzymes have typically been extracted from soil using a sodium acetate (NaAc) buffer around pH 5, however, several standard extraction solutions were tested to compare recovery. Extractions solutions were NaAc [6], Tris-EDTA-NaCl (TEN) [25], phosphate buffered saline with Tween 20 (PBST) [as reported in 42], PALM [47], and deionized water. Extraction solutions were compared by spike-recovery of the mesophilic comparator enzyme from soil using the geno/grinding rigorous extraction technique (see below) after incubation at 25^{o}C for five hours. Previous extraction techniques of enzymes or proteins from soil have applied either a passive or rigorous approach to aid in detection. The passive and simultaneous extraction-detection method was attempted first by incubating soil samples (unamended or amended with mesophilic comparator) with 600 µl of 2% CMC (50 mM NaAc, 0.05% sodium azide (NaN_3), pH 5) and measuring glucose release with the DNS reagent [35]. A no-soil control unamended or amended with the mesophilic comparator was also assayed.

A more rigorous two phase extraction-detection approach was also tested as it has been used in more recent soil enzyme assays [36]. Both vortexing and bead-beating (geno/grinding at 100 strokes min^{-1}) were compared for rigorous extraction efficiency. Here soil samples (unamended or amended with mesophilic comparator) were extracted with 600 µl of the optimized extraction buffer (50 mM NaAc, 10 mM EDTA, 0.01% Triton X-100, pH 5) for 5 minutes and then centrifuged at 20,800 x *g*. Aliquots (300 µl) were incubated with a 2% CMC substrate solution (1:1) and recovery was determined with the DNS reagent. To prevent enzyme denaturation during the bead-beating procedure I tested for activity recovery over time with separate enzyme-solution vials being shaken with 4.5 mm beads at 100 strokes min^{-1} for 1, 3, 5, 10, and 20 minutes in the geno/grinder. Assays also used the appropriate temperature and pH as characterized under Test Materials. The final extraction-detection protocol for persistence studies was as follows:

1. Add 600 µl of NaAc extraction solution to 0.5 gram soil samples (oven-dry equivalent) in 2 ml extraction tubes.
2. Rigorously extract using geno/grinding for 5 minutes.
3. Centrifuge at 20,800 x *g* for 5 minutes.
4. Combine 300 µl aliquot with 300 µl of 2% CMC (w/NaN_3) substrate solution.
5. React enzyme extract and substrate for 72 hrs at either 40^{o}C (mesophilic comparator) or 65^{o}C (thermophilic E1), pH 5.
6. Combine 200 µl aliquot of reaction solution with 200 µl of DNS reagent and incubate at 95^{o}C for 5 minutes.
7. Centrifuge at 20,800 x *g* for 1 minute and pipette 200 µl aliquot of colored product solution to 96-well microplates and measure spectrophotometrically at 540 nm.

Persistence

During studies of enzyme persistence, test systems utilized 0.5 g soil subsamples (oven-dry equivalent) in 2 ml microcentrifuge tubes. Test materials (enzymes) were diluted from stock, applied to surface soil samples at environmentally relevant concentrations, and mixed in with a stainless steel spatula. Amendment preparations of 150 ng, 30 ng, and 40 ng were used in all persistence studies of the mesophilic comparator, bacterial E1, and plant soluble E1, respectively. This represents fortification of test soils at concentrations of 300, 60, and 80 ng g^{-1}, respectively, and equivalent to 600, 120, and 160 g ha^{-1}. Preparation quantities were based on activity equivalence so that each released a similar amount of glucose at their respective temperature optimum (i.e. they hydrolyzed CMC into glucose at equal rates). Plant-soluble E1 was amended at 10x concentration to produce a measureable result. The 10x loading (160 g ha^{-1}), however, was only slightly above the high-end estimate of E1 that would be released to the soil environment (120 g ha^{-1}). Preliminary (probe) studies used the air-dried Nicollet series and mesophilic comparator enzyme and were followed up by using bacterial and plant-soluble E1. Definitive studies used bacterial and plant-soluble E1 with all three fresh soils (Hanlon-Spillville-Coland). Separate soil samples were treated with water and used as controls. Following fortification, soil moisture was adjusted with deionized water and the samples were incubated in the dark at a constant temperature (Ambient Temperature Incubator BOD50A16, Thermo-Scientific, Waltham, MA). Excluding probe studies, the base water potential and temperature used in dissipation studies were 100 kPa and 25^{o}C, respectively. The

100 kPa moisture for Hanlon, Spillville, and Coland was 12.6, 17.3, and18.1%, respectively.[3] Each study included six sampling time points that utilized the optimized detection assay (above). Sampling times extending over ~2 ½ half-lives of the test materials and were determined on the basis of a probe study. At each sampling time triplicate vials for each treatment were sampled for analysis of activity.

Ecological Implications: CO_2 Evolution

To measure mineralization effects from E1-endoglucanase, freshly sampled Hanlon, Spillville, and Coland soils were amended with E1 or Wild-type maize tissue or unamended in a fully factorial design in three replications (3x3x3 = 27 samples). For amended samples, 20 mg of ground tissue was added to 10 g soil (oven-dry equivalent) whereas unamended samples were simply 10 g soil. Soils were added to 120 ml bottles and brought up to 100 kPa water potential with the addition of deionized water and then sealed with rubber stoppers. Gas samples were analyzed with a LI-7000 CO_2/H_2O infrared gas analyzer (LI-COR, Lincoln, NE). For each time of sampling, stoppers were pierced with a syringe and head space was well-mixed before 0.4 ml of gas was withdrawn for analysis. Head space was then replaced with laboratory air under light positive pressure, stoppers were placed back on vials and incubation was continued until the next sampling interval. Sampling and incubation occurred over a 45-day period. LI-COR software yielded an integral for each sample which was converted to CO_2 (based on gas standards) and then again converted to reflect CO_2-C released per gram soil. Gas samples were analyzed every day until CO_2 release slowed to where samples could be taken every few days to fit the detection range from gas standards. CO_2 standards were calibrated before every use and moisture was added to samples twice during the experiment to make up for small amounts of water vapor lost during analysis.

Statistical Analysis

Comparisons presented graphically throughout represent the 95% CI calculated on the basis of triplicate analysis, unless otherwise indicated.

An empirical, biphasic exponential decay model [$f(x)=a*\exp(-b*x)+c*\exp(-d*x)$] was fit to persistence data and describes the dissipation of extractable enzyme materials from soil over time (SigmaPlot V.10) where a and c determine the asymptotes of the curve, b and d are first order rate constants, and x denotes time in hours. Each decay phase, a and c, was

[3] Calculated using van Genuchten pedotransfer functions. See Table 2.

constrained by c = 100-a, while b and d were constrained to > 0. A biphasic model was used to better characterize the dissipation of enzyme materials and was adapted from Wolt et al. [37] who used this type of model to describe the degradation of pesticides in soil.

For CO_2 evolution studies, a least squares full factorial method was used to examine interactions between variables (Time, Replication, Treatment, Soil). All cross terms indicated significant interactions, therefore, Tukey's Honestly Significant Difference (HSD) test was applied to determine significant effects of Treatment by Soil as defined at the α=0.05 level (JMP V.10) (Appendix C).

RESULTS

Characterization of Test Materials

The bacterial E1 exhibited highest activity between 60-70^{o}C while the mesophilic comparator was most active around 40^{o}C (Figure 1). Both enzymes displayed optimal activity around pH 5 (Figure 2), however, E1 retained fairly high activity across a much broader range (pH 5-9). Tissue characterization data reveal similar C:N ratios and soluble protein content from E1 and Wild-type tissue. Transgenic maize was positive for E1 activity and expression was estimated at 4 ng mg^{-1} tissue (Fig 3). Combining our expression data with that of Mei et al. [3], an estimated environmental concentration of 10-60 ng E1 g^{-1} soil (17-120 g ha^{-1} soil loading) was calculated (Table 3). Wild-type maize did not exhibit any measurable endoglucanase activity.

Detection Optimization

The blue-dyed substrate (Azo-CMC) worked very well in a clean system and a standard curve was easily developed. However, the dyed-substrate was not robust enough to distinguish between enzyme activity and matrix effects from the plant soluble protein solution. Even after a long incubation period this detection method suffered from low sensitivity, and both E1 and non-E1 soluble protein had absorbances that were barely above the limit of detection.

The sugar-reducing assays (Alkaline-ferricyanide and DNS) both worked well in detecting enzyme activity, but the Alkaline-Ferricyanide (Alk-Ferr) reagent was too sensitive to background effects and the reference curve was not useable after the extraction/substrate solution was added to glucose standards (Fig 4). The DNS assay was highly robust and only exhibited a slight change in absorbance values after extracts were added to glucose standards

(Fig 5). Although the DNS assay is not as sensitive to glucose production, it provided a readily useable reagent for detecting endoglucanase activity from complex plant and soil matrices. Additionally, the DNS reagent had longer color stability and required less work to prepare.

The NaAc buffer was the most effective extraction solution with over 35% more recovery than phosphate buffered saline with Tween 20 (PBST). The Tris-EDTA-NaCl (TEN) buffer as well as the PALM and Water extractant solutions exhibited significantly lower recovery relative to NaAc and PBST with only a fraction of endoglucanase activity detected (Fig 6). NaAc extraction also displayed reasonably low variability (9.8% relative coefficient of variation) and had the added convenience of having the same pH required in the activity assay. Adding sodium azide (NaN_3) to the CMC solution increased glucose accumulation during incubation with CMC and therefore improved detection (Fig 7).

The passive extraction-detection method was able to measure whole soil activity (substrate + soil incubated together), but lacked a positive control and hence did not provide useable information for the detection of E1 in soil. This is seen in Fig 8 as both amended and unamended soils had indistinguishable activities whereas the no-soil mesophilic enzyme released significantly high amounts of glucose. During the first 10-15 hours of enzyme-soil incubation the use of geno/grinding (bead-beating) nearly doubled endoglucanase recovery from soil compared to simply vortexing (Fig 9). The impact of geno/grinding on denaturing of the protein in solution shows a rather unusual pattern in that over the first three minutes activity of the enzyme seems to increase beyond 100% with activity peaking around 135% of original activity. After five minutes of extraction at 100 strokes min^{-1} there was a general decrease in activity that may be attributed to protein denaturation (Fig 10). Denaturing from heat was unlikely as the tubes themselves were not warm to the touch after geno/grinding.

Persistence

In preliminary studies where soluble enzyme test materials were amended to the Nicollet test system, the bacterial E1 and mesophilic comparator exhibited near-identical dissipation with a Time to 50% Dissipation (DT50) of ~2.5 hours and little to no extractable activity after a period of ~24 hours (Fig 11). Plant-soluble E1 was slower to dissipate as more than 50% remained after a 24-hour period, however, this result was atypical compared to subsequent findings in the definitive persistence studies.

Definitive studies where E1 from bacterial and plant-soluble sources was added to fresh soil microcosms resulted in little variation across soils and E1 source. The DT50 of ~1.3 hours for all definitive bacterial E1 samples indicate that the fresh, microbially robust soils had a slightly quicker dissipation rate compared to the air-dried Nicollet soil (Fig 12). E1 TSP had a surprisingly short persistence across all fresh soils as less than 40% of E1 was recoverable within a 30 minute incubation period (Fig 13). In comparing the dissipation trends from bacterial E1 and plant soluble E1 there was a more rapid decline in extractable activity of the E1 TSP (Fig 14). Controls in all persistence studies yielded no extractable activity. Taken together, the preliminary and definitive data demonstrate that bacterial- and plant-derived E1 persist for a short time (minimal recovery after 24-30 hours of contact with soil).

Ecological Implications: CO_2 Evolution

Within each test soil, the rate of mineralization and cumulative CO_2 evolved from those amended with either E1 maize or Wild-type maize was not significantly different. Although not statistically significant, both Hanlon and Spillville soils had 7-8 % more CO_2 evolved during the 45-day incubation period when amended with E1 maize than Wild-type maize (Figs 15 & 16). Conversely, the Coland soil showed almost 20% higher total mineralization when amended with the Wild-type maize tissue (Fig 17). Control soils exhibited 3-5 times less CO_2 evolved than amended counterparts. As an indirect test of E1 persistence, the long-term release and reactivity of plant-expressed E1 was insignificant as determined by no effect observed on carbon mineralization rates or total carbon mineralized from E1 tissue amended soils.

Comparing total CO_2 evolved within each amendment across soil types provided important information regarding treatment effects. In unamended soils the silt loam Spillville series had the highest total mineralization with almost double the amount released from Hanlon and over 50% more than Coland. When amended with Wild-type tissue, Spillville soils released significantly greater CO_2 than Hanlon soils. When amended with E1 tissue, Spillville soils released significantly greater CO_2 than Coland soils (Fig 18). The discernible difference in CO_2 evolution across soil types can be viewed as a positive control for effects and further validates the observation of no-effect from treatment (E1 and Wild-type tissue amendments).

DISCUSSION

The novel expression of E1 in maize and its release to the environment requires an ecological assessment of potential impacts. In this study I addressed three questions to better understand the fate and behavior E1 in soil: (1) How can E1 be detected in soil matrices? (2) How persistent is E1 in the soil environment when comparing dissipation from various sources and to a mesophilic comparator? (3) What is the ecological impact of E1 entering agroecosystems during residue decay and what is the relation to carbon cycling?

Test Materials and Soils

In this study maize tissue expressing E1 specifically in the mitochondria was utilized because it has shown promise as a good sub-cellular compartment for plant expression of proteins (M. Sticklen, pers. comm.). However, E1 has also been localized in the endoplasmic reticulum, apoplast, and chloroplast of plant cells as well as expressed throughout plant tissue [5, 10]. Besides optimizing the sub-cellular compartmentalization of E1, our collaborators at Michigan State have also recently developed maize that expresses the entire suite of cellulase enzymes needed to hydrolyze cellulose. The product, Spartan III corn, expresses the E1 endoglucanase, an exoglucanase (CBHI) from *Trichoderma reesei*, and a β-glucosidase from the rumen of cows [38]. The mesophilic comparator enzyme was used to examine the dissipation of a 'typical' cellulase enzyme in the soil environment. This particular enzyme was chosen because it was synthesized from organisms that are ubiquitous in soil (*Aspergillus* and *Trichoderma* spp.) and fit the activity profile of a mesophilic enzyme. Levels of E1 expression in our test material (4 ng mg^{-1} tissue) were within the range of E1 maize expression in the literature (0.3-33 ng mg^{-1} tissue) [5, 29].

Soils in the definitive study were chosen for their characteristic representation of sites where E1 maize would be grown and to examine differences across texture and organic matter content. Also, soils were sampled from an alluvial catena so that soil-forming factors were similar and specific differences could be interpreted more easily. Sustainable stover collection generally requires that highly productive soils be used for cultivation and thus our test soil systems reflect characteristic Midwestern soils where E1 maize would be planted [39]. Based on soil survey data collected across the entire state of Iowa for the past 50 years (>10K entries), the majority of soils that would support E1corn growth would have 3-5% OM, neutral to slightly acidic pH, and 20-30% clay content [40]. The Spillville and Coland soils fit this

description well. The Hanlon soil, however, had low OM (1.9%) and clay content (16%) and relatively high pH (7.6). If E1 were persistent in Iowa soils, it would likely be more evident in Hanlon due to its lower reactivity and especially its high pH which would favor E1 activity over that of endogenous soil enzymes.

Substrate and Detection. Using an enzyme activity assay required that a substrate with high specificity for endoglucanase be used. This study also attempted to use realistic, conservative estimates (60 ng g^{-1} equivalent to 120 g ha^{-1}) of protein loading to improve the usefulness of results for regulatory decision-makers. The relatively low concentration of E1used in this study made it desirable to implement a highly sensitive detection assay to prevent lengthy incubations. Therefore, use of the MUC substrate would have been ideal as it has highly sensitive reaction products. However, detection is compromised when extracting from soil for two reasons. First, organic matter has a quenching effect on fluorescence and can cause a significant reduction in sensitivity that would require each soil to be calibrated and increase the likelihood of error [30]. Secondly, MUC is hydrolyzed by the entire suite of cellulase enzymes and hence lacks the specificity needed for E1 detection in soil [31].

The blue dyed Azo-CMC substrate (Sigma-Aldrich, St. Louis) also would have been ideal to use as it measured a dye released only after enzyme activity, but this technique suffered from major background interference and low detectability overall. Sugar reducing assays are another way to detect catalytic products and work by utilizing redox reactions that can be measured colorimetrically. Monomeric glucose has an exposed aldehyde group which can be moderately reducing in the presence of a particular reagent. For example, an alkaline-ferricyanide (Alk-ferr) reagent contains ferric iron (Fe^{3+}) that is reduced to ferrous iron (Fe^{2+}) while the aldehyde group is oxidized to form carboxylate [32]. Similarly, the 3,5-dinitrosalicylic acid (DNS) reagent is reduced to 3-amino,5-nitrosalicylic acid and again the aldehyde group becomes oxidized to form carboxylate [33]. In both of these reactions, the subsequent heating of vials at 95^{o} C can yield measurable color differences that may then be correlated with glucose concentration (enzyme activity end-product).

The Alk-ferr reagent was highly sensitive to the presence of glucose production (ng/ml range), but only worked in a clean system free of matrix effects. The DNS assay, however, is highly robust and has been verified in the literature as having a wide working linear range and low interference from proteins compared to other reducing sugar assays [41]. For dissipation

studies a wide linear range is ideal so that depleting materials can be detected over time. One drawback to using the DNS assay is that it is has low sensitivity and requires a long incubation time (72 hours) in order to release sufficient amounts of glucose and fall in the linear detection range (μg/ml). One way to boost the glucose signal and simultaneously prevent contamination is to add a microbial inhibitor as Deng and Tabatabai [35] demonstrated with toluene. Here 0.05% sodium azide (NaN_3) was added to the CMC substrate solution and indeed there was a boost in signal that also allowed for prolonged incubation [34]. There was a general trend in detection assays where increasing sensitivity increased susceptibility to matrix effects and error.

One particularly challenging and interesting aspect of finding a suitable detection assay was sorting through the ambiguous literature on endoglucanase and cellulase in general. For example, many assays that purport to detect activity of cellulase may actually be using carboxymethyl cellulose substrate [35], which is highly specific for endoglucanases or crystalline cellulose substrate [http://www.worthington-biochem.com/cel/assay.html], which is highly specific for exoglucanases. Work towards an agreeable cellulase nomenclature has been difficult over the last 20 years as the mechanism and specificity of different cellulases has been altered with emerging knowledge. The synergism and feedback between cellulases is very complex and there are no perfect substrates to test for true cellulase activity using enzyme assays. Whether soluble, insoluble, crystalline, dyed, fluorogenically tagged, etc. every substrate will favor particular cellulases, exclude others, and misrepresent whole cellulase ativity. The inability of activity assays to reflect natural cellulose hydrolysis necessitates the use of supplemental studies that measure carbon mineralization.

Extraction. Deng and Tabatabai [35] detected cellulase activity from soil using a simultaneous passive extraction and substrate hydrolysis technique using whole soil samples. More recently Vancov and Keen [36] incorporated a two-phase technique where cellulase enzymes are mechanically extracted with bead-beating, followed by a separate incubation hydrolysis step. The two-phase extraction-detection method has only recently been used and specifically in the detection of other transgenic proteins from soil [e.g. 42, 43]. Results from the passive extraction indicated that although soil cellulase activity was detectable, E1 activity could not be detected and so there was no positive control. This could be due to sorption mechanisms that may still be occurring since the extraction and hydrolysis step were

combined, leaving large uncertainty in measurements. Rigorous extraction performed the best and geno/grinding (bead-beating) proved to extract better than simply vortexing. The accompanying graphic (Fig 6) suggests that a rigorous extraction may be more important in the early phases of enzyme dissipation and could indicate that sorption is the primary reason for initial rapid inactivation of enzymes when added to soil. Modern extraction solutions also typically contain a chelating agent and mild, non-ionic detergent to improve protein recovery (45). Here 10 mM EDTA was used to decrease the reactivity of metals in solution and 0.01% (w/v) Triton X-100 detergent to aid in extracting bound proteins and keep them in the solution phase.

The effect of a rigorous extraction (geno/grinding) on enzyme stability shows an interesting pattern where activity actually increases over the first few minutes and then begins to decline from 5-20 minutes of extraction. When proteins are stored in vials one suggestion is to either have a highly concentrated batch (>1 mg/ml) or to add a carrier protein (e.g. bovine serum albumin - BSA) so that proteins stay in solution and do not become coagulated or adhered to the wall of the vial or to each other. Based on the use of low concentration, conservative protein loadings, the bead-beating procedure could be 'freeing' enzymes that were bound to one another or the vial wall during the first few minutes of bead-beating. If enzymes are sorbed to each other or glass or plastic surfaces their active sites could be temporarily blocked. Another idea as to why there is an increase in activity before denaturing begins could be that enzymes are being fragmented and that some of them become better prepared for substrate action. Regardless of the reason for the spike in activity, Fig 7 shows a decline in functionality after 5 minutes of geno/grinding at 100 strokes min^{-1} that is presumably due to shear forces denaturing the enzyme.

Persistence

Due to the high reaction temperature of E1 incubations (65^{o}C), thermostable E1 activity was selectively assayed by eliminating any endogenous endoglucanase activity. Results from persistence studies show bacterial and plant-soluble E1rapidly dissipate within a matter of hours due to degradation or sorption. It is possible that E1 becomes bound to colloidal material and retains activity, however, any residual effects were not significant. Plant-soluble E1 was amended at approximately 10-fold higher than our estimated environmental concentration (Appendix A) to increase detection. Low E1 expression in characterized E1 tissue could be an

artifact of losses obtained during plant protein extraction and purification. The mesophilic comparator displayed nearly identical persistence in soil and provides evidence that E1 does not persist in the soil environment any differently than a typical mesophilic enzyme. The persistence of these enzymes is a function of the biochemical reactivity of the soil as a whole. Endoglucanases released to the environment are either rapidly adsorbed to soil particulates or degraded by microbes and proteases. The forces acting upon the test materials (biotic and/or abiotic) are hard to tease apart and will depend on the overall microbial activity and the percentage of clay and humic substances. Thus, regardless of the disabling mechanism, the general ability to detect a biomolecule from soil over time can be labeled persistence or, conversely, dissipation [42, 45-46].

Dissipation studies on other transgenic proteins in soil have primarily used immunoassay, bioassay, or enzyme assay for detection [e.g. 44, 27]. All three methods use indirect evidence to support detection and have their own merits. Immunoassay (e.g. ELISA, enzyme-linked immunosorbent assay) is highly specific and can be high-throughput, but requires initial support in developing antibodies and can also mistakenly detect non-functioning or fragmented proteins making it difficult to interpret ecological effects. Bioassays have mostly been used in quantifying the persistence of Cry proteins and are based on mortality of insects that ingest the insecticidal proteins. Bioassays are highly sensitive and can be very useful with the proper controls, but are very labor and time intensive [43]. Activity assays are useful because they detect enzymes or proteins based on retained and measurable functionality, but are often labor and time intensive as well. Of the transgenic proteins that have been studied for environmental persistence, most soluble protein amendments have shown similar dissipation rates to E1 with a generally rapid loss of detection [42, 47]. One study did find that purified Cry1ab proteins persisted as long 234 days [48], but this appears to be an anomalous result as all other dissipation data on Cry proteins indicate less than 30 days of persistence.

Ecological Implications: CO_2 Evolution

No observed effect on whole carbon mineralization from soils amended with E1-maize suggests that E1 will not significantly impact carbon cycling. Assuming that E1 is released during residue decomposition, it would become active until it was degraded microbially or lost functioning via sorption to soil colloids (dissipation). However, no observed effect of E1 amendment on whole carbon mineralization could mean that endoglucanases, in general, are

not rate-limiting catalysts in natural, whole tissue degradation. The collective action of all enzymes involved in deconstruction of whole plant tissue (ligninases, hemicellulases, pectinases, cellulases, etc.) could limit the impact of having abundant amounts of endoglucanase (plant-derived E1 + endogenous) in the soil environment. Additionally, E1 is merely a component of the cellulase complex and may not be functionally important if other cellulase enzymes are not present at appropriate levels. Evidence from persistence studies, however, supports that E1 dissipates rapidly and that this would be the primary cause for no observed effects. Mineralization was significantly affected by soil type (Spillville > Coland > Hanlon) and provides convincing evidence that tissue treatment had no effect on soil carbon mineralization. The effect from soil can provide a positive control for detection of effects and since no difference was observed between soils amended with E1 or Wild-type maize, there is greater confidence in the result.

Biodegradation is a particularly plausible fate for tissue-amended E1 in soil. By considering the degradative action of fungal hyphae (filamentous branching between soil colloids and organic residues), it can be speculated that E1 would be released at some distance from the surface of clay and humic particles and that microbes and proteases would have the opportunity to metabolize it first. Also, shredders (termites, sow bugs, etc.) are considered important during initial decomposition of residue and may digest and destroy E1 before colloidal surfaces play any significant role. In this regard it is also possible that tissue-expressed E1 could survive microbial digestion and be excreted as part of a fecal pellet.

Limitations and Uncertainties

This study utilized maize that expresses E1-endoglucanase in plant cell mitochondria, but there have been many other expression sites that may affect the nature of dissipation. As mentioned, recent advances have also allowed for multiple cellulolytic enzymes to be expressed in maize stover and this may be important for whole cellulase action on natural substrates. There may also be unique soil types that require special consideration when planting E1 maize. For example, soils with high pH, low organic matter and clay content, or low overall microbial activity may have longer tissue residence times, less (a)biotic reactivity and hence greater overall persistence.

Persistence of tissue amendments was indirectly observed throughout CO_2 evolution studies and bridged with plant-soluble protein dissipation. Persistence studies on maize

incorporated into soil microcosms were not possible due to the high glucose background from tissue. The high level of glucose present in these tissue-amended extracts (>400 μg/ml) likely had inhibitory effects on endoglucanase activity and further prevented persistence studies from being performed. Glucose inhibition feedback is not as significant as that from cellobiose, but high concentrations can stall enzymatic conversion and has been documented in the ethanol industry as well [49]. Interestingly, process engineers have addressed this problem by using simultaneous saccharification and fermentation (SSF) where sugars are fermented upon release, thus preventing product feedback inhibition [50].

GENERAL CONCLUSIONS

Clean, renewable energy will be needed in the near future to address dwindling petroleum reserves and mitigate anthropogenic-induced climate change from carbon dioxide (CO_2). To meet these objectives in a timely manner, maize has been genetically engineered to produce a bioprocessing enzyme which may reduce financial and energy costs associated with lignocellulosic ethanol conversion. The novel expression of thermostable E1-endoglucanse in maize green tissue requires an environmental safety assessment to better understand the fate and implications of plant-expressed E1 in representative soil environments. The major study of this thesis examined the persistence and ecological effects from the standpoint of an environmental risk assessment. That is, a likely problem was formulated, an exposure assessment was performed to scope the problem, and exposure characterization data was generated to provide decision-makers and risk managers with practical information. Harvestable stover for biofuel production is likely to come from highly productive Midwestern soils. Based on residue stover left on fields after harvest, E1 from maize is conservatively anticipated to release 120 g ha^{-1} into representative soils. The objectives were to successfully optimize a method for detecting E1 from soil matrices, determine the persistence of E1 in soil, and evaluate any effects to carbon cycling.

Considerable time was needed to properly detect E1 from soil. The final extraction and detection procedure was optimized to include a suitable substrate, robust detection reagent, optimized extraction buffer, rigorous extraction technique, long incubation time, and microbial inhibitor. Following method optimizations, E1 could be detected from soil matrices at environmentally relevant concentrations, but not when maize tissue was present (due to high

glucose background). Persistence studies showed bacterial and plant-expressed E1, as well as the mesophilic comparator, dissipated rapidly within a 24-30 hour period from all soils. Effects from E1 on carbon cycling were analyzed using CO_2 evolution comparisons for 45 days. There was no significant difference between soils that had been amended with E1 maize or Wild-type maize. However, the soil type played a significant role in the amount of CO_2 released which further verified the interpretation that E1 maize had a negligible effect on carbon cycling.

The combined result from persistence and CO_2 evolution studies is that maize expressing up to 30 g Mg^{-1} of E1 (120 g ha^{-1} environmental load) would not persist in representative soils or significantly impact carbon cycling. However, the transformed maize used in this study only expressed one component of the cellulase complex, endoglucanase. Maize that expresses the entire suite of cellulase enzymes has been grown under laboratory conditions and could be the final product that goes to market. Therefore, future studies may wish to develop and incorporate rapid immunoassays (e.g. ELISAs) and continue testing for effects to carbon cycling via CO_2 evolution studies.

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Table 1. Test enzymes and sources.

Test Material	Source	Expression System	Study
Mesophilic comparator cellulase	MP Biomedical	*Aspergillus niger*, *Trichoderma reesei*	Persistence, preliminary
Bacterial E1-endoglucanase	NREL (Nat'l Renew Energy Lab)	*Streptomyces lividans*	Persistence, definitive
E1 TSP (total soluble protein)	Extracted from E1-maize (MSU)	*Zea mays*	Persistence, definitive
Wild-type plant tissue	Sticklen Lab	*Zea mays*	CO_2 evolution, definitive
E1 plant tissue	Sticklen Lab	*Zea mays*	CO_2 evolution, definitive

Table 2. Soil Characteristics.

		Texture %						[e] Water Content %		
Series	[a] Textural Class	sand	silt	clay	[b] OM %	[c] pH	[d] CEC (cmol kg^{-1})	33 kPa	100 kPa	1500 kPa
[f] Nicollet	Loam	48	34	18	2.9	6.7	16.8	16.5	-	11.52
[g] Hanlon	Sandy Loam	56	28	16	1.9	7.6	14.7	14.2	12.6	8.61
[h] Spillville	Silt Loam	24	50	26	3.4	6.8	22.1	24.7	17.3	14.73
[h] Coland	Loam	34	40	26	3.7	7.0	23.9	23.4	18.1	15.69

[a] Textural determination – hydrometer

[b] Organic Matter (OM) – loss on ignition (LOI)

[c] 1:1 soil-water extract

[d] Cation Exchange Capacity – sodium acetate method (EPA 9081)

[e] Water content – pressure plates for 33 and 1500 kPa. 100 kPa estimated using van Genuchten pedotranfer functions [51]

[f] Fine-loamy, mixed, superactive, mesic Aquic Hapludoll

[g] Course-loamy, mixed, superactive, mesic Cumulic Hapludoll

[h] Fine-loamy, mixed, superactive, mesic Cumulic Hapludoll

Table 3. Maize tissue properties including E1 expression and estimated environmental concentration (EEC).

	C:N	[a] Total Protein (%)	[a] Total Soluble Protein (TSP) (%)	[a] Functional E1 (% TSP)	[b] E1 - EEC (ng g^{-1} soil)
Transgenic	23 : 1	10.10 ± 0.06	0.09 ± 0.01	0.547 ± 0.01	9.6 - 59.4
Non-Transgenic	18 : 1	8.82 ± 0.04	0.11 ± 0.03	nd	0

[a] Average ± the standard deviation from triplicate analysis

[b] See Appendix A. for calculation.

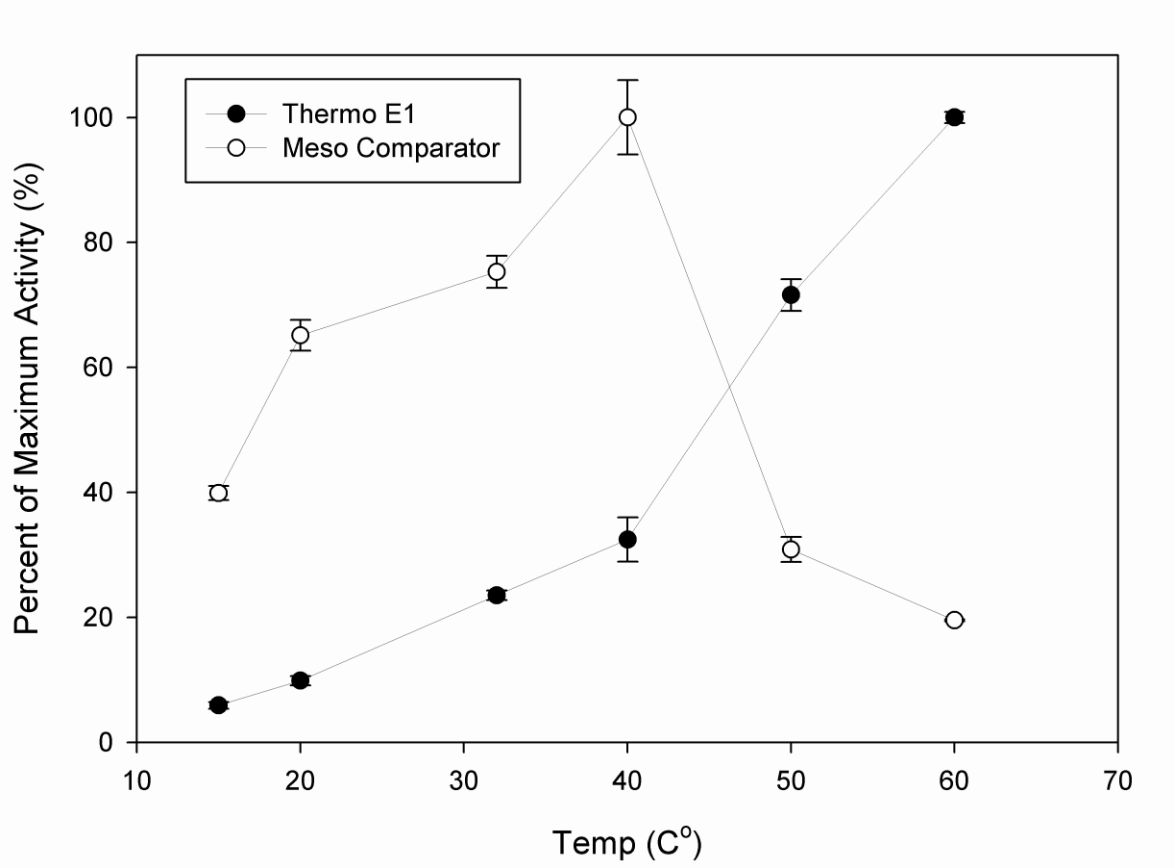

Fig 1. Enzyme temperature optima. Activity analysis was performed with a carboxymethyl cellulose (CMC) substrate and dinitrosalicylic acid (DNS) detection reagent. Bacterial E1 (Thermo E1) has optimum activity at >60^{o}C (83^{o}C reported [24]). The mesophilic comparator (Meso Comparator) displays optimum activity at 40^{o}C. Each data point represents the average of three replications ± 95% CI.

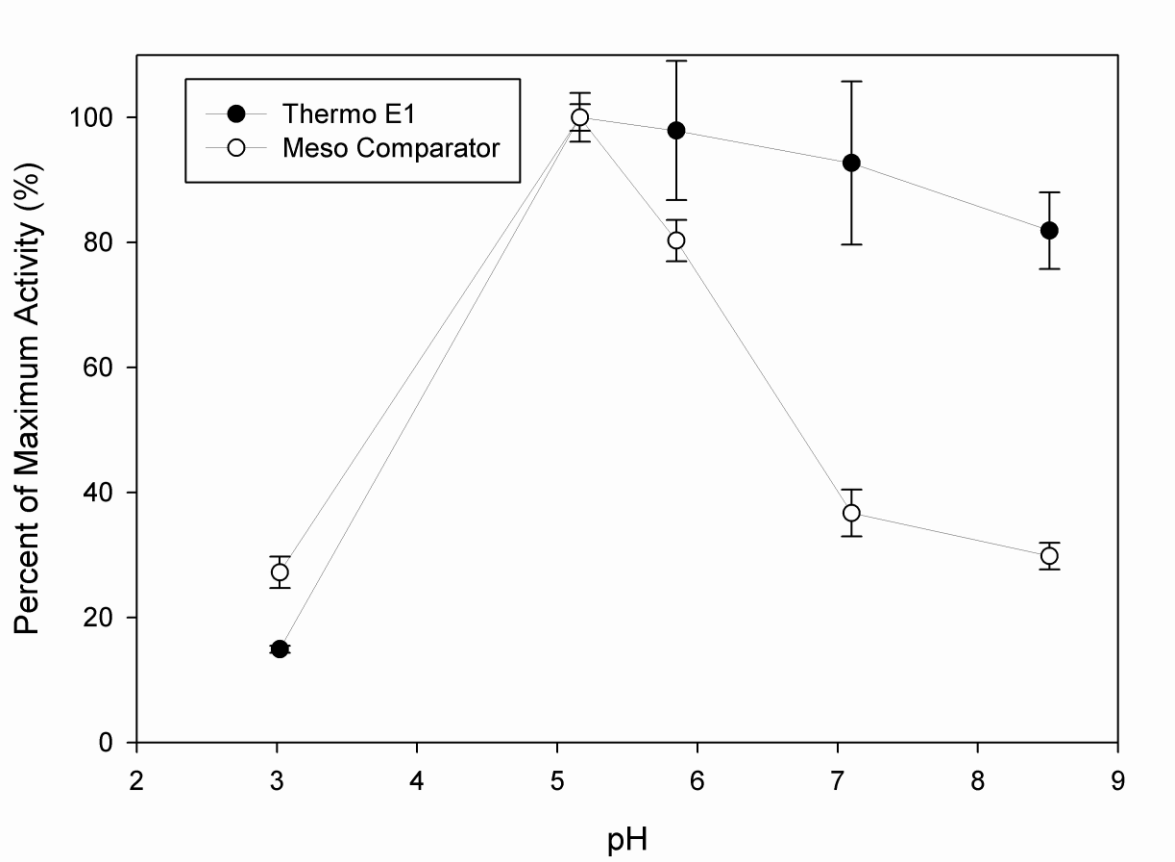

Fig 2. Enzyme pH optima. Activity analysis was performed with a carboxymethyl cellulose (CMC) substrate and dinitrosalicylic acid (DNS) detection reagent. Bacterial E1 (Thermo E1) and its mesophilic comparator (Meso Comparator) have optimal activity around pH 5. Bacterial E1 is uniquely characterized by sustained activity over neutral to basic pH. Each data point represents the average of three replications ± 95% CI.

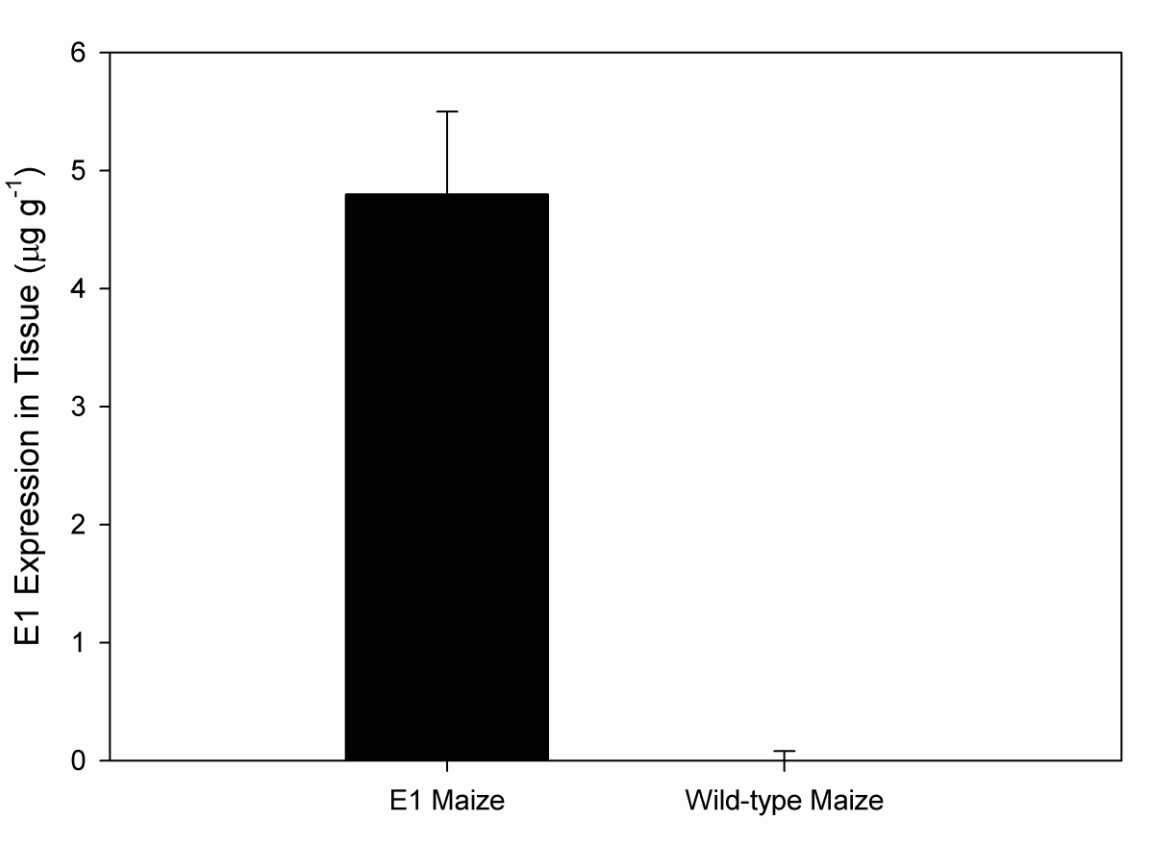

Fig 3. E1expression in maize tissue. Expression was calculated from endoglucanase activity of plant soluble protein and correlation with pure E1 activity standards. Milled tissue was extracted with optimized sodium acetate (NaAc) buffer at 100 strokes min^{-1} in geno/grinder. Proteins from crude extracts were 'salted out' using ammonium sulfate precipitation and protein pellets were reconstituted in a storage solution. Activity analysis was performed with a carboxymethyl cellulose (CMC) substrate and dinitrosalicylic acid (DNS) detection reagent. Activity from E1 maize confirms the presence of E1. Wild-type protein activity was at or below the limit of detection, resulting in no expression. Each bar represents the average of triplicates ± 95% CI.

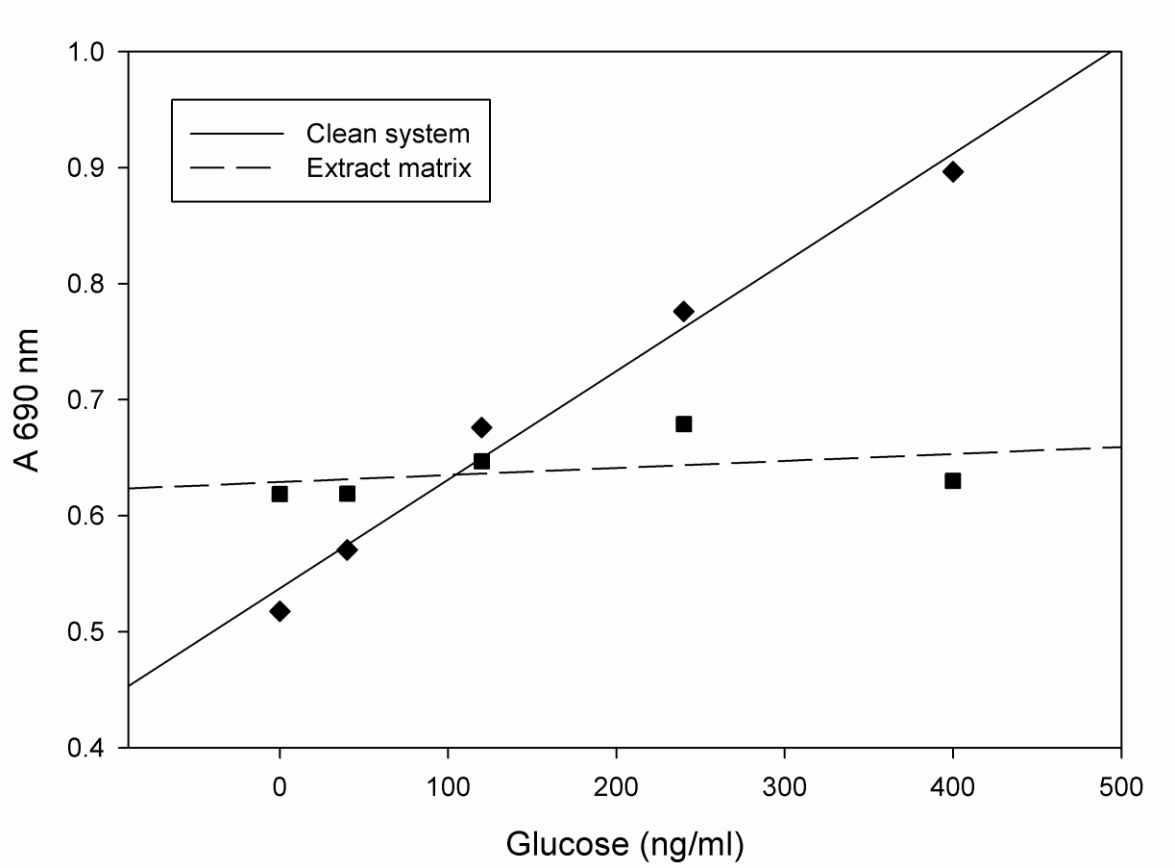

Fig 4. Glucose detection using the Alkaline-Ferricyanide reducing sugar assay. Glucose standards were developed in a clean system (deionized water) and extract matrix (CMC substrate and soil extract solution). Glucose detection using the Alkaline-Ferricyanide assay was highly sensitive (ng/ml range), but was not resistant to matrix effects. Each data point represents the average of three replications.

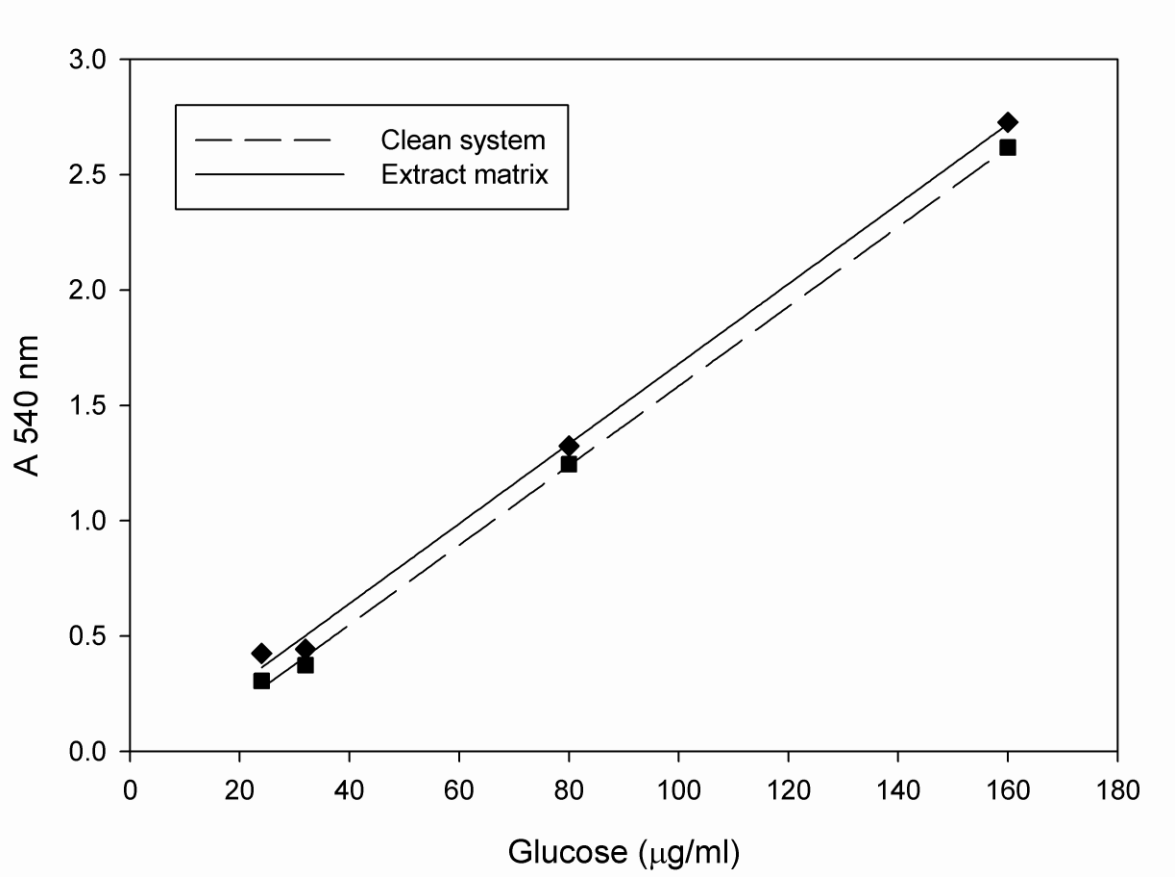

Fig 5. Glucose detection using the dinitrosalicylic (DNS) reducing sugar assay. Glucose standards were developed in a clean system (deionized water) and extract matrix (CMC substrate and soil extract solution). Although detection sensitivity was low using the DNS assay (μg/ml range), background interference was negligible. Each data point represents the average of three replications.

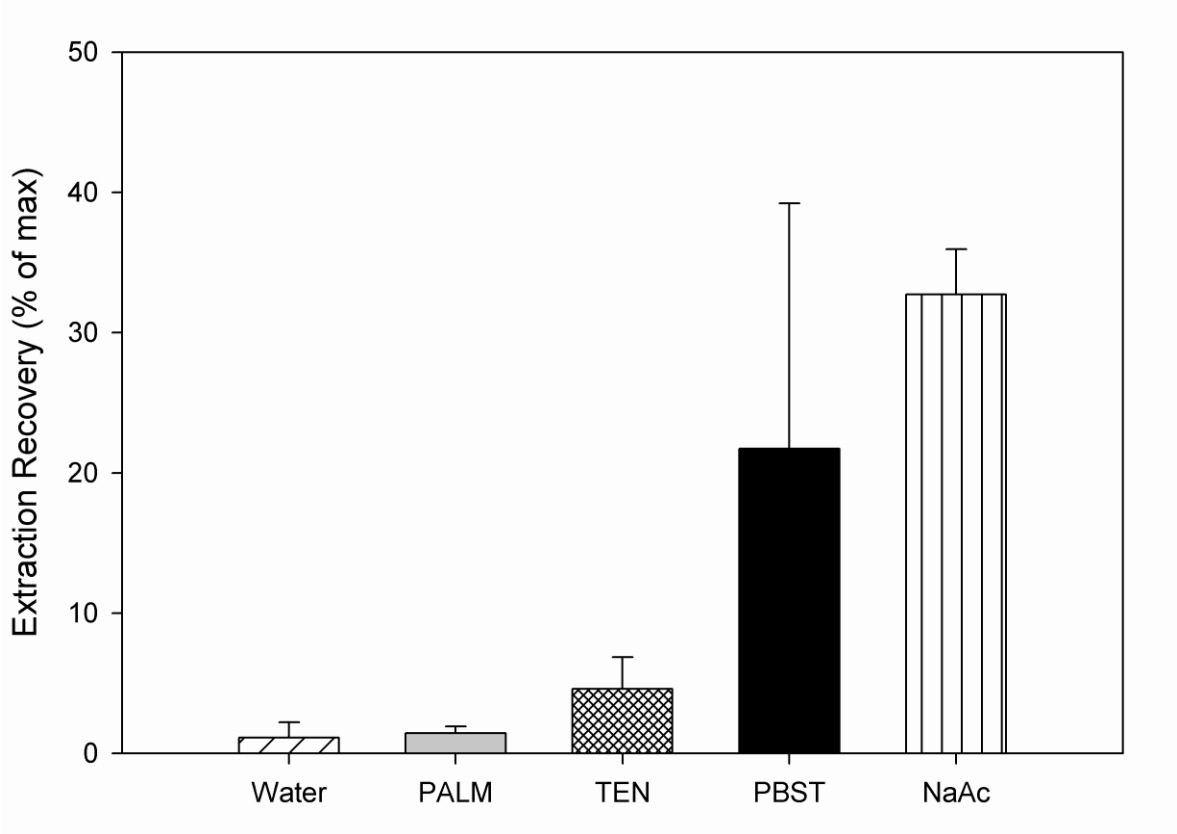

Fig 6. Extraction buffer efficiency. Samples were shaken at 100 strokes min^{-1} in geno/grinder before activity analysis with carboxymethyl cellulose (CMC) and dinitrosalicylic acid (DNS). The NaAc extractant exhibited much higher extraction recovery over all other buffers with the possible exception of PBST, which suffered from high variability. Abbreviations: (NaAc) sodium acetate, (PBST) phosphate buffered saline solution with Tween, (TEN) Tris-hydrochloric acid with ethylenediaminetetraacetic acid (EDTA) and sodium chloride (NaCl), (PALM) [47]. Each bar represents the average of triplicates ± 95% CI.

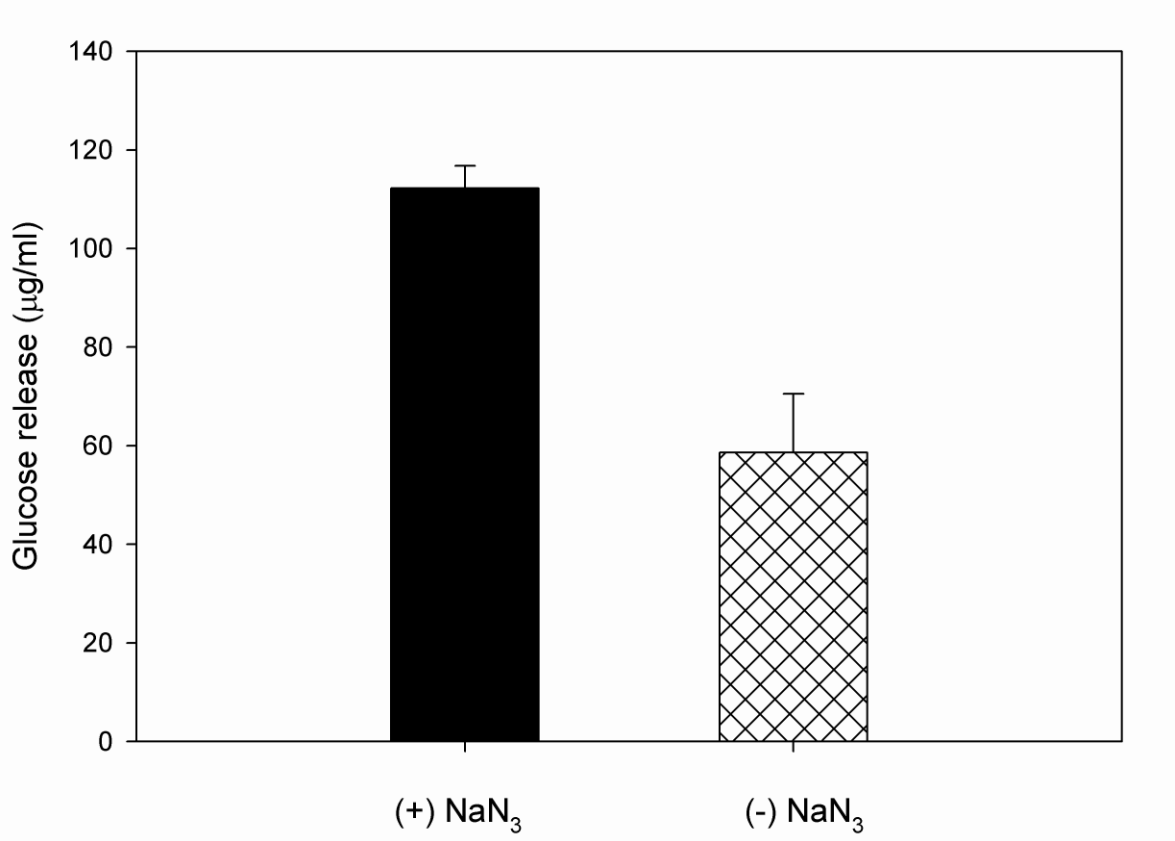

Fig 7. Effect of microbial inhibitor on glucose detection. One-half gram Nicollet soil samples were amended with 150 ng mesophilic comparator enzyme and extracted with the optimized sodium acetate (NaAc) buffer. Extracts were reacted with carboxymethyl cellulose (CMC) with (+) and without (-) 0.05% sodium azide (NaN_3). Glucose detection was significantly boosted when NaN_3 was added to the substrate solution. Each bar represents the average of triplicates ± 95% CI.

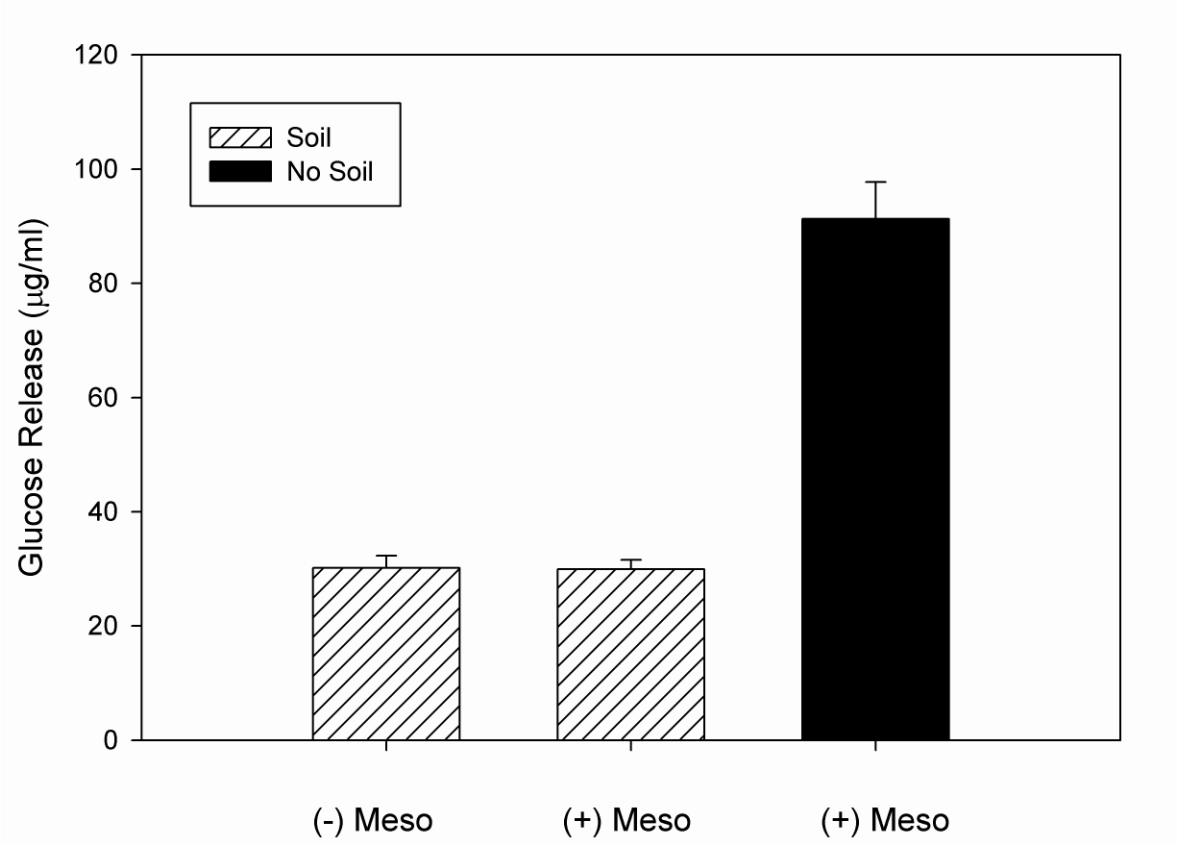

Fig 8. Passive enzyme extraction. One-half gram soil samples were assayed for activity by incubation with 600 µl of carboxymethyl cellulose (CMC) substrate and sodium acetate (NaAc) extraction buffer. Reaction products were measured using the dinitrosalicylic acid (DNS) reagent. Mesophilic enzyme activity (Meso) was not detectable in soil using the passive extraction and detection method from Deng and Tabatabai [35]. Each bar represents the average of triplicates ± 95% CI.

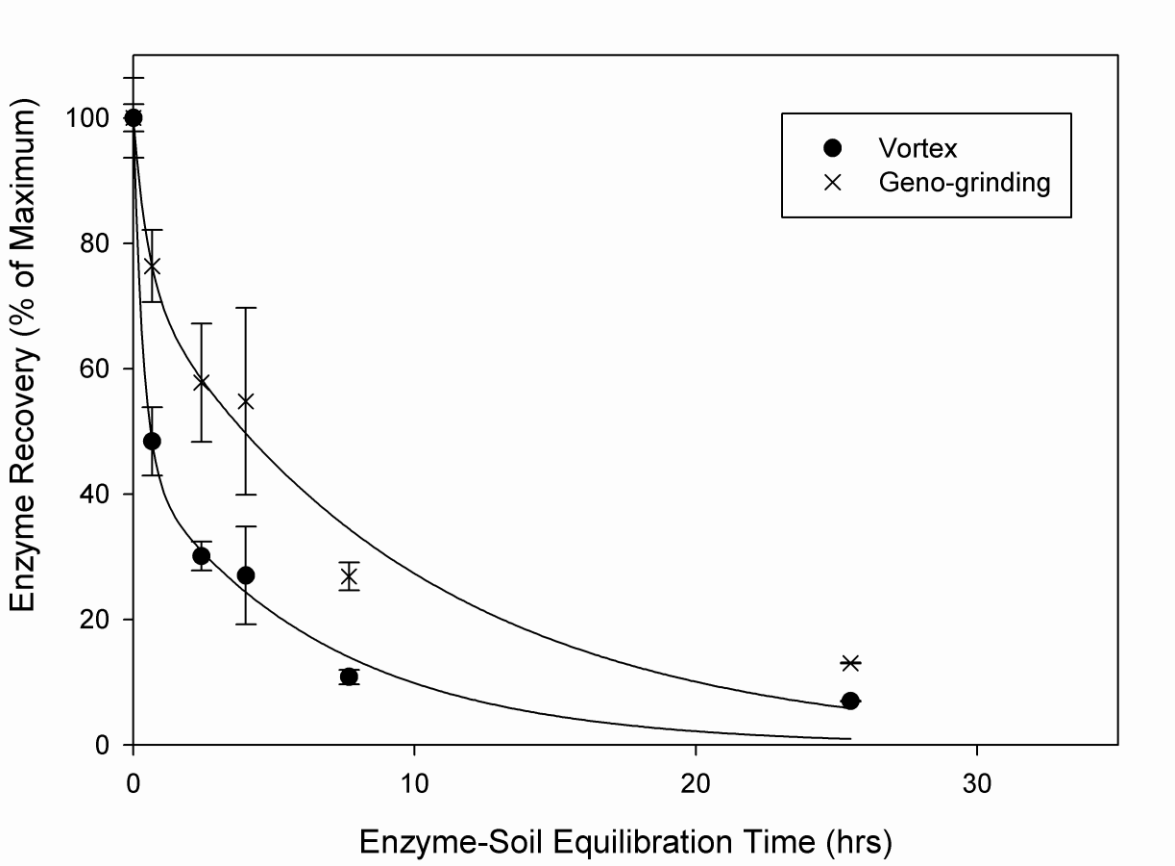

Fig 9. Effect of rigorous extraction on enzyme recovery. Half-gram Nicollet soil samples were amended with 150 ng mesophilic comparator and extracted over time by vortexing or geno/grinding for 5 minutes. Activity from each sampling point was analyzed with a carboxymethyl cellulose (CMC) substrate and dinitrosalicylic acid (DNS) detection reagent. Geno/grinding proved to be the more efficient extraction technique. Each data point represents the average of triplicates ± 95% CI.

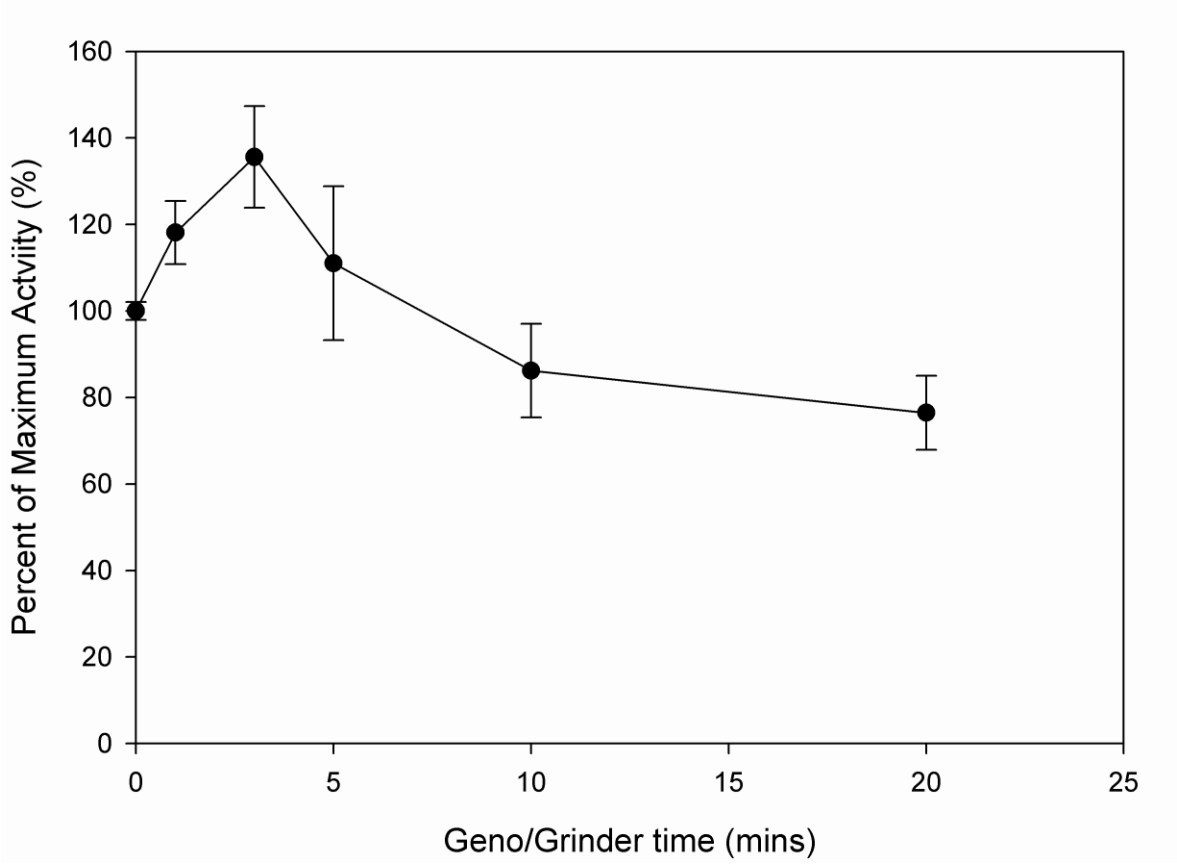

Fig 10. Enzyme stability during rigorous extraction. Mesophilic enzyme (150 ng) was added to vials and extraction beads. Samples were shaken at 100 strokes min^{-1} in geno/grinder before activity analysis with carboxymethyl cellulose (CMC) and dinitrosalicylic acid (DNS). Mesophilic enzyme recovery was acceptable for up to 5 minutes of extraction. Each data point represents the average of triplicates ± 95% CI.

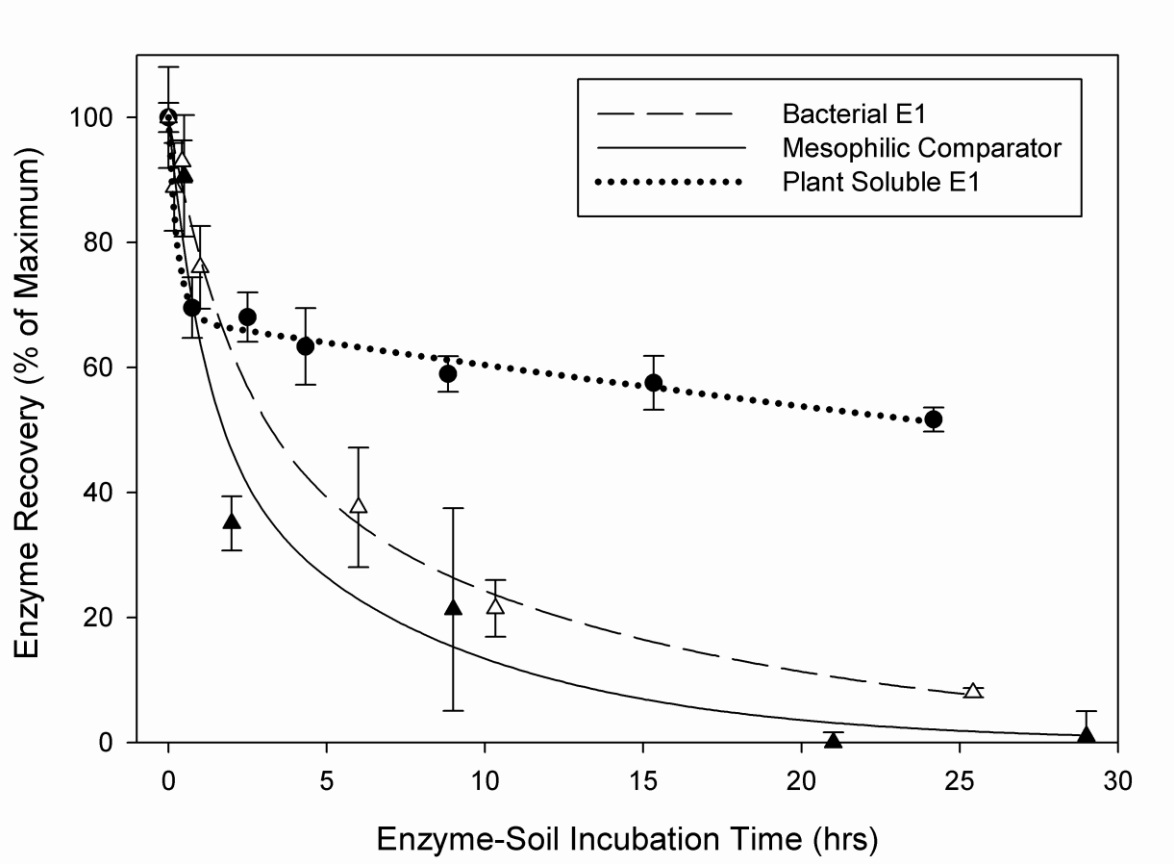

Fig 11. Endoglucanase dissipation probe study. Each sample was extracted by geno/grinding in a NaAc buffer for 5 minutes followed by activity analysis with carboxymethyl cellulose (CMC) and dinitrosalicylic acid (DNS). Bacterial E1, its mesophilic comparator, and plant-soluble E1 were spiked at 60 ng g^{-1}, 300 ng g^{-1}, and 80 ng g^{-1} soil, respectively. Each data point represents the average of triplicates ± 95% CI. Curves represent least squares best fit (of means) to a biphasic model of the form $f(x)=a*\exp(-b*x)+c*\exp(-d*x)$ where a and c determine the asymptotes of the curve, b and d are first order rate constants, and x denotes time in hours. Constraints were: 100=a-c, a = 50, b>0, d>0.

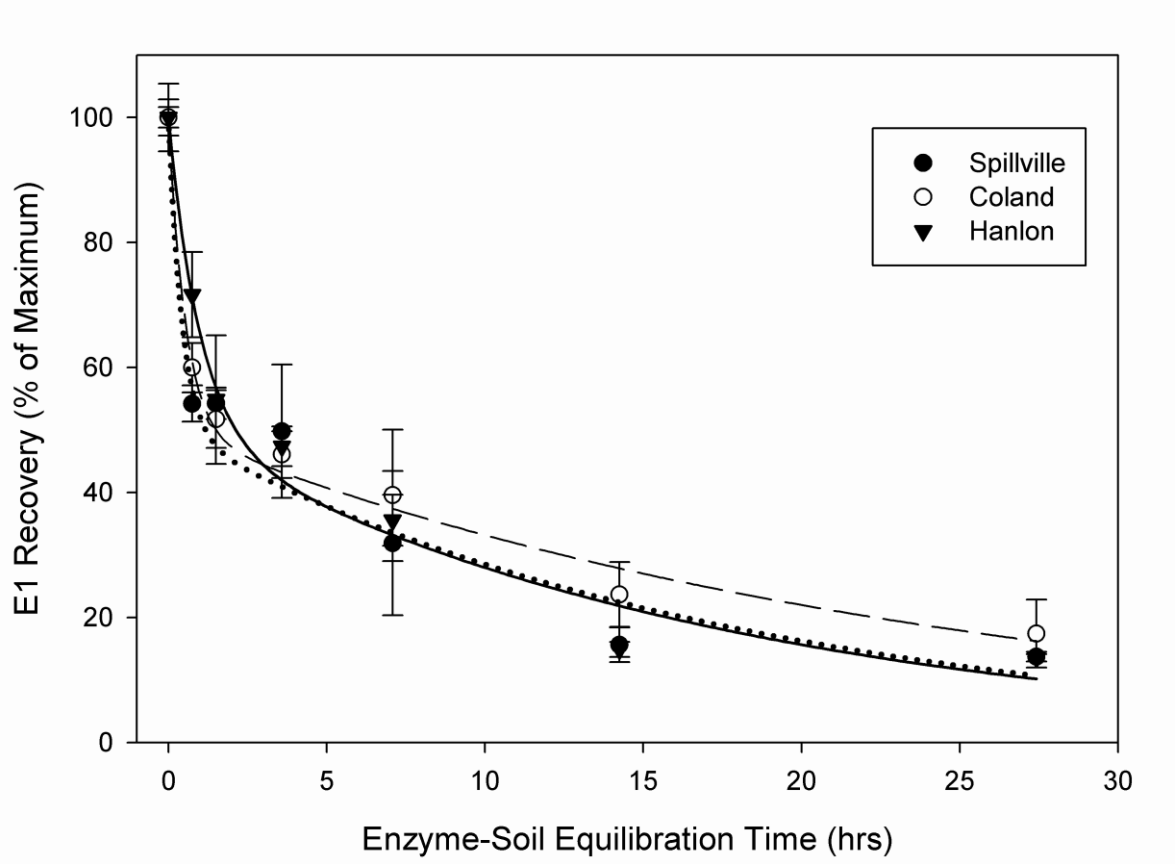

Fig 12. Bacterial E1 dissipation. Bacterial E1 was spiked at 60 ng g^{-1} soil. Each sample was extracted by geno/grinding in a NaAc buffer for 5 minutes followed by activity analysis with carboxymethyl cellulose (CMC) and dinitrosalicylic acid (DNS). Bacterial E1 persisted for a little more than 24 hours in all soil types and there was no difference in recovery at each sampling time point between soils. Each data point represents the average of triplicates ± 95% CI. Curves represent least squares best fit (of means) to a biphasic model of the form $f(x)=a*\exp(-b*x)+c*\exp(-d*x)$ where a and c determine the asymptotes of the curve, b and d are first order rate constants, and x denotes time in hours. Constraints were: 100=a-c, a = 50, b>0, d>0.

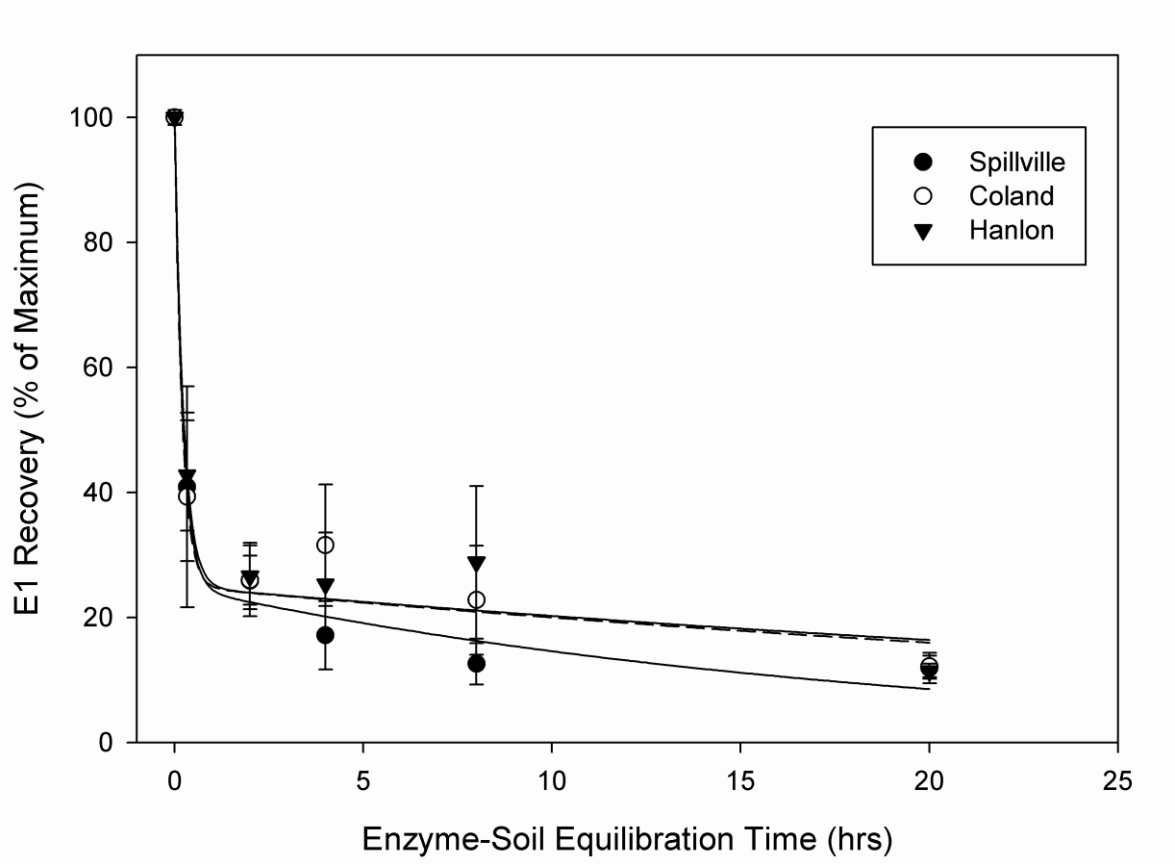

Fig 13. Dissipation of plant-soluble E1. Plant-soluble E1 was spiked at 80 ng g^{-1} soil. Each sample was extracted by geno/grinding in a NaAc buffer for 5 minutes followed by activity analysis with carboxymethyl cellulose (CMC) and dinitrosalicylic acid (DNS). Plant-soluble E1 was amended at 80 ng g^{-1} soil. Plant soluble E1 persisted for <24 hours in all soil types. Each data point represents the average of triplicates ± 95% CI. Curves represent least squares best fit (of means) to a biphasic model of the form $f(x)=a*\exp(-b*x)+c*\exp(-d*x)$ where a and c determine the asymptotes of the curve, b and d are first order rate constants, and x denotes time in hours. Constraints were: 100=a-c, a = 50, b>0, d>0.

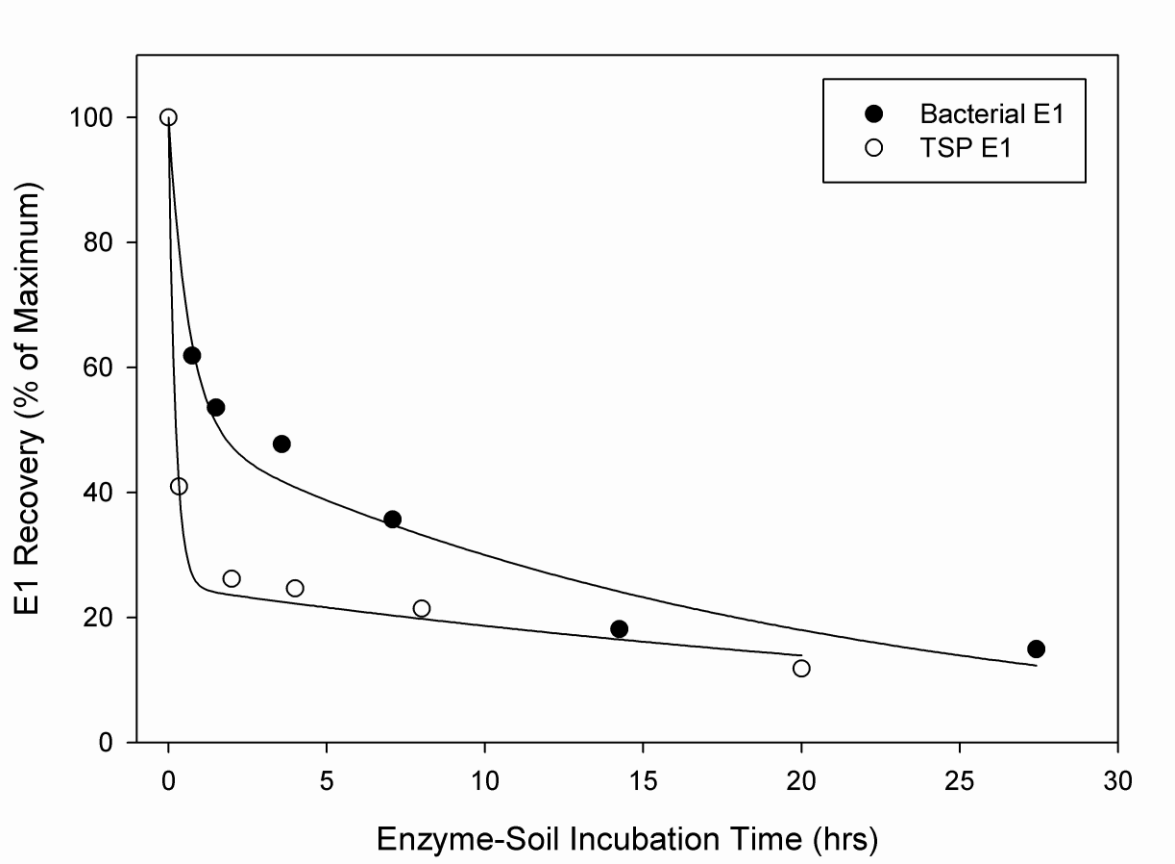

Fig 14. E1 persistence – bacterial vs plant-expressed. Bacterial E1 and plant-soluble E1 were spiked at 60 ng g^{-1} and 80 ng g^{-1} soil, respectively. Each sample was extracted by geno/grinding in a NaAc buffer for 5 minutes followed by activity analysis with carboxymethyl cellulose (CMC) and dinitrosalicylic acid (DNS). Each data point represents the average of triplicates ± 95% CI. Curves represent least squares best fit (of means) to a biphasic model of the form *f*(x)=a*exp(-b*x)+c*exp(-d*x) where a and c determine the asymptotes of the curve, b and d are first order rate constants, and x denotes time in hours. Constraints were: 100=a-c, a = 50, b>0, d>0.

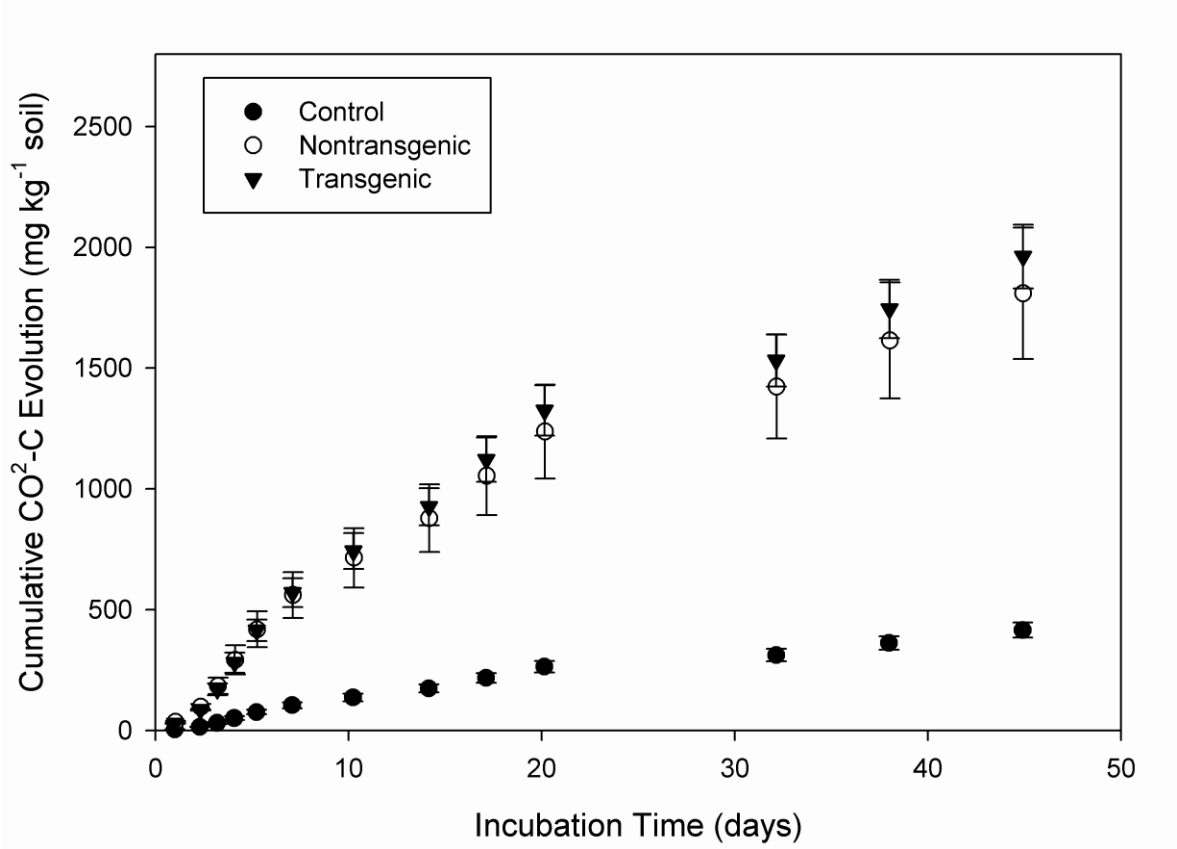

Fig 15. CO_2-C evolution from Hanlon surface soils. Ten-gram soil samples were amended with 20 mg of tissue (equivalent to 3.58 Mg ha^{-1}) unless left unamended and CO_2-C evolution determined with a LI7000 infrared gas analyzer. There was no observed difference in CO_2-C release from Hanlon soils amended with E1 maize and Wild-type maize. Each data point represents the average of triplicates ± 95% CI.

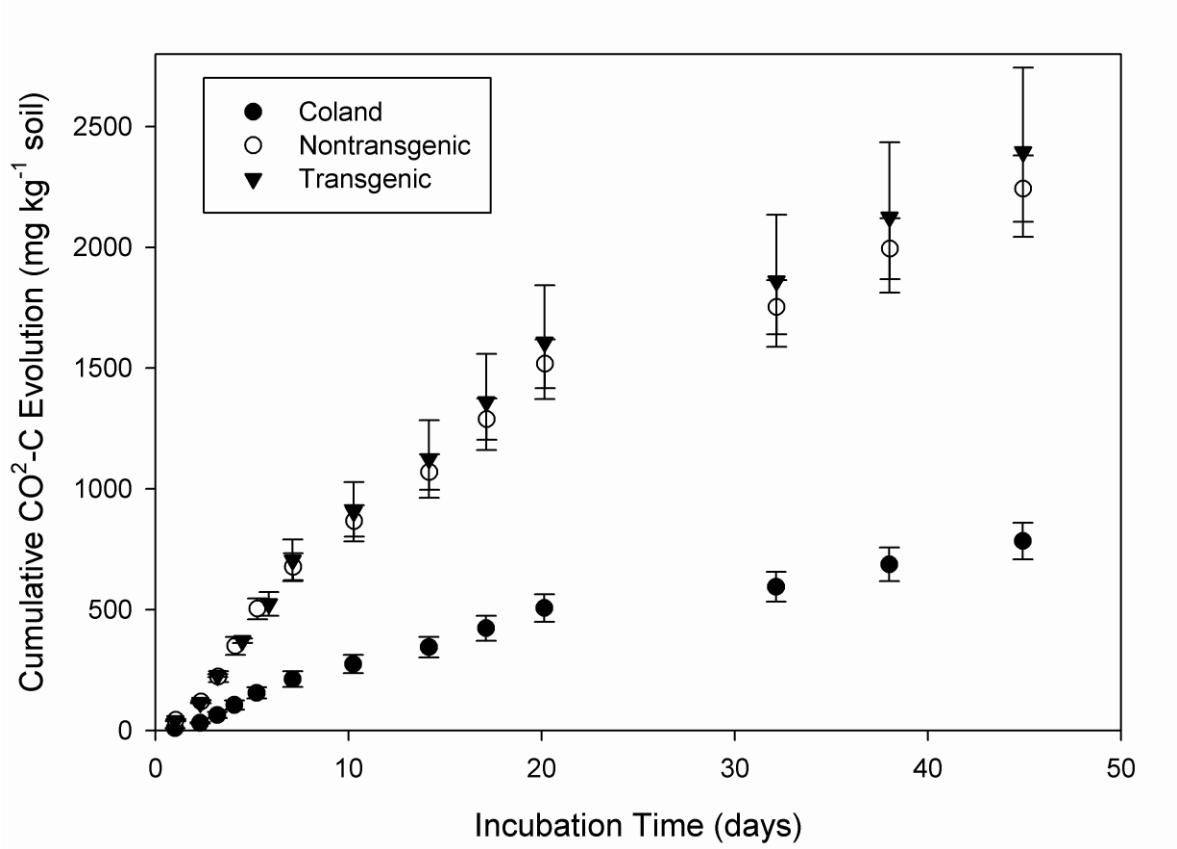

Fig 16. CO_2-C evolution from Spillville surface soils. Ten-gram soil samples were amended with 20 mg of tissue (equivalent to 3.58 Mg ha^{-1}) unless left unamended and CO_2-C evolution determined with a LI7000 infrared gas analyzer. There was no observed difference in CO_2-C release from Spillville soils amended with E1 maize and Wild-type maize. Each data point represents the average of triplicates ± 95% CI.

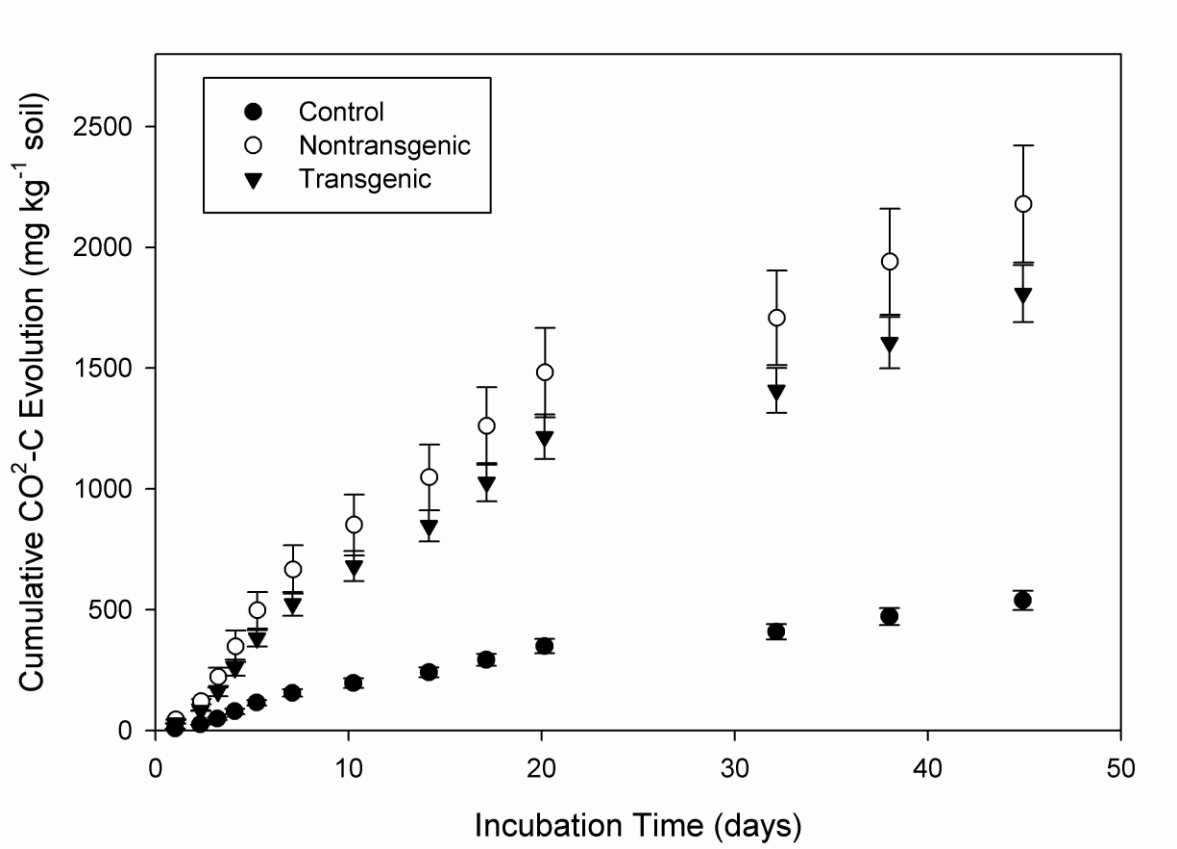

Fig 17. CO_2-C evolution from Coland surface soils. Ten-gram soil samples were amended with 20 mg of tissue (equivalent to 3.58 Mg ha^{-1}) unless left unamended and CO_2-C evolution determined with a LI7000 infrared gas analyzer. There was no observed difference in CO_2-C release from Coland soils amended with E1 maize and Wild-type maize. Each data point represents the average of triplicates ± 95% CI.

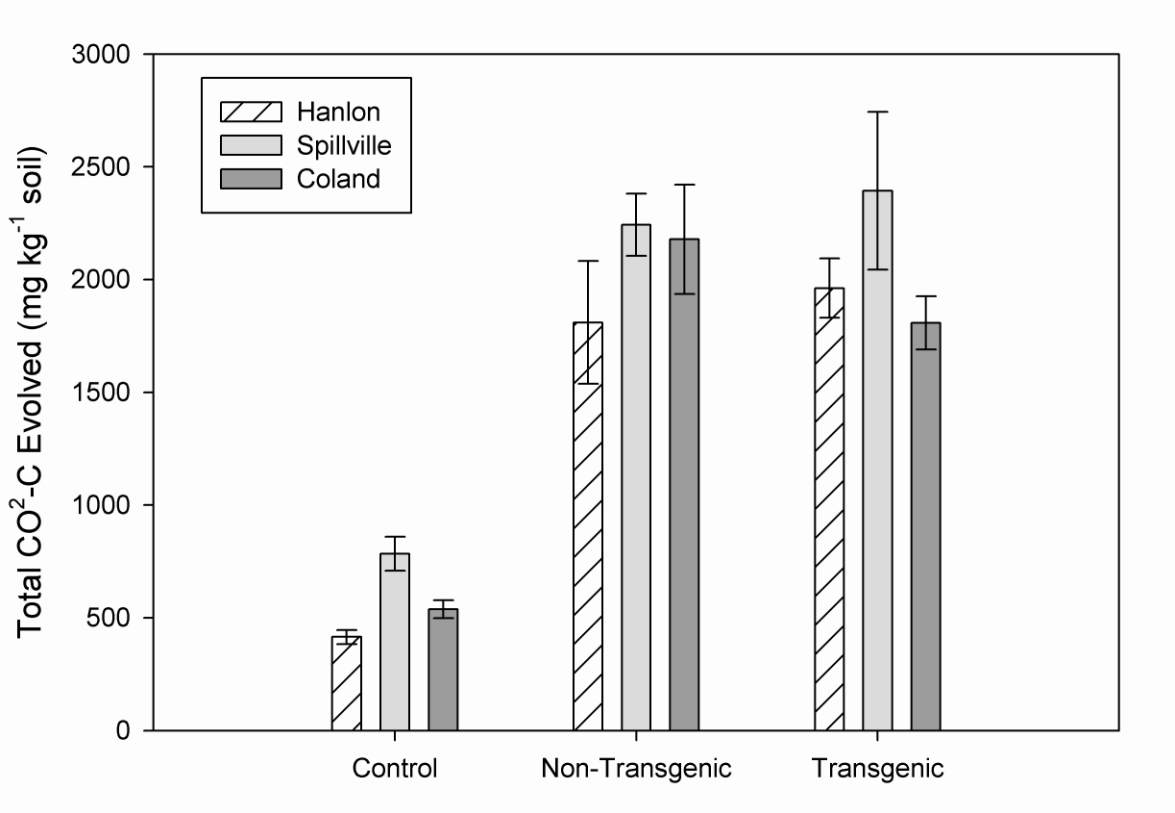

Fig 18. Total CO_2-C evolution for all treatments and soils. Ten-gram soil samples were amended with 20 mg of tissue (equivalent to 3.58 Mg ha^{-1}) unless left unamended and CO_2-C evolution determined with a LI7000 infrared gas analyzer. CO_2-C measurements from 45-day incubation were summed to give cumulative evolution. Differences within each treatment reflect a significant effect from soil type whereas there was no effect from treatment on CO_2-C evolution. Each data point represents the average of triplicates ± 95% CI.

APPENDIX A. EXPOSURE ASSESSMENT

Assumptions

- Average corn yield = 182 bushels/acre [1]
- Grain to stover ratio 1:1 [2]
- 1/3 of stover left on field post-harvest for erosion control and nutrient sustainability [3]
- Soil mass for 1 hectare x 15 cm depth = 2 x 10^3 Mg ha-1 [4]

Given

b = 3.58 Mg ha^{-1} (stover mass left on field per area) – obtained from assumptions

m = 2 x 10^3 Mg ha^{-1} (mass of applicable soil per area)

a = 17-33 g Mg^{-1} (E1 expression in mitochondria – maize stover) [5]

$\left(\frac{b}{m}\right)$ = tissue loading estimate, 2 mg/g soil

x = E1 estimated environmental concentration -EEC- (ng E1/g soil)

$x = a\left(\frac{b}{m}\right)$ x = 30-60 ng E1/g soil)

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APPENDIX B. STATISTICAL DATA

Table 1. Effect Tests. Reduced model effects initially determined from full factorial method of variables describing CO_2 evolution from soils.

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
TIME	1	1	0.00001593	636.6999	< 0.0001
TREATMENT	2	2	0.00001455	290.7248	< 0.0001
TIME*TREATMENT	2	2	0.00000505	101.0348	< 0.0001
SOIL	2	2	0.00000145	29.0252	< 0.0001
TIME*SOIL	2	2	0.00000018	3.6322	0.0276
TREATMENT*SOIL	4	4	0.00000045	4.5265	0.0014

Table 2. Treatment*Soil. Tukey's Honestly Significant Difference (HSD) test from CO_2-C evolution sampling points. (Treatment, Soil) No difference in CO_2 release between T and NT treatment within a given soil, however, there are marked differences in CO_2 evolution between soils. [(S) Spillville, (C) Coland, (H) Hanlon] for a given treatment (T)Transgenic E1 maize, (NT) Non-transgenic Wild-type maize, (C) Control – no amendment.

Level							Least Sq Mean
T, S	A						0.00078388
NT, S	A	B					0.00068663
NT, C		B	C				0.00065200
T, H		B	C	D			0.00058727
T, C			C	D			0.00054990
NT, H				D			0.00053009
C, S					E		0.00026136
C, C					E	F	0.00017905
C, H						F	0.00014228

ACKNOWLEDGEMENTS

I would like to thank my major professor Dr Jeff Wolt for his exceptional scientific mentoring and ardent support throughout my graduate education and research. I also appreciate the guidance and encouragement received from those closest to me, especially my family.