GLYPHOSATE AND GLYPHOSATE-RESISTANT CROP INTERACTIONS WITH RHIZOSPHERE MICROORGANISMS

ROBERT J. KREMER
USDA-ARS & UNIVERSITY OF MISSOURI
COLUMBIA, MO

“Standard” field trials conducted with Roundup Ready soybean. Field trial on Tiptonville silt loam, Pemiscot County, Missouri 1999
Introductory Comments Regarding the Symposium

Development of genetically modified (GM) crops seeks to improve agricultural productivity. However, assessments of specific yet important processes and microorganisms have been largely neglected although these aspects contribute to a strong feedback that influence crop productivity and the ecological balance in our soil resource.

We must continue to emphasize our position in addressing the key issues concerning the impact of GM crops on soil systems and the sustainability of agriculture.
Glyphosate and Glyphosate-Resistant Crops (‘Roundup-Ready’ [RR] Production System)

- Advancement in effective weed management (despite the insidious development of glyphosate-resistant weed biotypes)

- Among the consequences of the RR system are alterations in microbial ecology and biology of the rhizosphere environment affecting:
  - Nutrient cycling (plant availability)
  - Potential phytopathogen and antagonist balance
  - Composition and activities of beneficial microorganisms (i.e., mycorrhizae, plant-growth promoting rhizobacteria)
OBJECTIVES:

1. UNDERSTAND IMPACTS OF TRANSGENIC CROP PRODUCTION ON
   a. SOIL BIOLOGICAL INTERACTIONS;
   b. DISCOVER MECHANISMS RESPONSIBLE FOR OBSERVED EFFECTS

2. DETERMINE APPROACHES TO OVERCOME PRODUCTION-LIMITING FACTORS EVOLVING FROM DETRIMENTAL TRANSGENIC CROP-SOIL BIOLOGICAL INTERACTIONS
BACKGROUND –

Evaluation of Genetically-Modified Crop X Soil/Rhizosphere Interactions since 1997

Original objectives –

Determine root-associated fungal pathogens on RR soybean (i.e., *Fusarium solani pv. glycines*) (what level of Sudden Death Syndrome develops in RR soybean?) What is impact of glyphosate use?

Determine root populations of soybean cyst nematode (SCN) (*Heterodera glycines*) on RR soybean

Evaluate *Fusarium* collected from roots for potential biological control of SCN (RR soybean promote populations of biocontrol fungi?)
Early Results –

- No consistent trends in population of SDS fungal pathogen
- No consistent trends in population development of SCN
- Few *Fusarium* cultures showed biological control activity against SCN
- Consistent increases in *Fusarium* spp. colonizing RR soybean roots with glyphosate applications at label rates


These findings helped partially explain reported production problems with RR soybean experienced by many farmers in midwestern U.S.
Typical soybean root colonization assay results observed consistently, 1997-2007

Fusarium colonization – 2007 study
+ Glyphosate  Check

+ Glyphosate  No herbicides
Figure 2. Incidence of rhizosphere *Fusarium* on RR soybean. Vertical bars denote LSD (p<0.05)
Summary - consistent trends over years

*Fusarium* Root Colonization on RR Soybean 1997-2007

Fusarium colonies per 100 cm root

Herbicide

- Roundup
- Check
- Tank Mix

Identification of Root Fusarium Isolates

1. Subculture, characterize based on cultural and microscopic morphology; key based on Nelson et al. (1983)

2. Verified via nuclear DNA translation elongation factor sequence primer and Penn State database (Skovgaard et al. (2001)*

Identifications Over All Treatments:

**Fusarium oxysporum** complex – 72%

**Fusarium solani** complex – 18%

**Fusarium equiseti** – 9%

*Analyses conducted at ARS Mycology Lab, Peoria, IL by Kerry O’Donnell, 2004*
*Fusarium* shown to be good indicator of potential impacts of GM crop on rhizosphere ecosystem – most can be opportunistic pathogens if not directly infectious, and respond to various factors and alterations in the rhizosphere.

However, the rhizosphere is a COMPLEX system – a more comprehensive examination of Structure and Function of microbial communities needed to provide a complete assessment of potential effects induced by GM cropping – This will yield better understanding of mechanisms contributing to impacts of GM on soil ecology/biology AND crop productivity.
Complexity of the rhizosphere / soil ecosystem presents formidable challenge in devising an appropriate Framework that effectively evaluates effects of GM crops.

**Use of indicator groups and specific microbial activities that are sensitive to ecosystem changes and that are relevant to the particular GM system in place is proposed. A ‘polyphasic’ microbial analyses to target the broad impact on the total microbial community, thus a multiple analyses provides a more reliable view of GM effects than any single technique alone.**

Based on guidelines for assessing responses of soil microorganisms to GM plants by –


Proposed polyphasic microbial community analyses

Difficult to demonstrate impact

Likely most sensitive to ecosystem alteration by GM crops

In studies of effects of glyphosate on soil and rhizosphere microbial communities where only ‘general’ properties are measured, no effect due to glyphosate and/or RR soybean is concluded -


An example:

**SOIL DEHYDROGENASE ACTIVITY:**

2002

```
No Herbicide  | Glyphosate
```

2003

```
Tank-Mix  | Glyphosate
```

Vertical bars indicate LSD (P<0.05).

Source: Means et al., 2007
OUR ASSESSMENT OF POTENTIAL TRANSGENIC CROP EFFECTS IS AN “EVOLVING FRAMEWORK” BASED ON OBSERVATIONS OVER 10 YR

THUS, THE CURRENT APPROACH:
EXAMINE SPECIFIC RHIZOSPHERE MICROBIAL COMMUNITIES AND THE ASSOCIATED ACTIVITIES => DETERMINE ANY FUNCTIONAL CONSEQUENCES OF RR CROPPING SYSTEM

OUR SPECIFIC ANALYSES INCLUDE:
A. RHIZOSPHERE FUNGAL COMMUNITIES – *FUSARIUM*
B. Mn-TRANSFORMING MICROORGANISMS (OXIDIZERS & REDUCERS)
C. SYMBIOTIC NITROGEN FIXING RHIZOBIA
D. PSEUDOMONAD COMMUNITIES
E. ARBUSCULAR MYCORRHIZAL FUNGI (AMF)

THE ABOVE COMPONENTS ARE INTERACTIVE AND MAY EXPLAIN ANY LINKAGES OF SHIFTS IN MICROBIAL COMMUNITY STRUCTURE WITH FUNCTIONAL ALTERATIONS.
RECALL -

Glyphosate is *systemic* – the amount not adsorbed to the EPSPS enzyme is *transported throughout* the plant.

In RR soybean, glyphosate has been found *localized* in meristematic tissues of growing points, leaves and roots; pod and seed; nodules.

Also glyphosate is *actively released through roots* into rhizosphere soil (root zone) likely with high amounts of carbohydrates & N-compounds in RR soybean.

Interacting factors that determine rhizosphere community - overlaid with Management, may have additive effect on microorganisms with root exudation patterns of plants (see de Boer et al. New Phytol. 170:3, 2006)

Representation of components and their interactions involved in plant growth
Methodology –

* Protocols to define the sample under test must be followed: what constitutes ‘rhizosphere soil’ vs ‘bulk soil’ or rhizosphere vs rhizoplane?

Considerably more activity and alterations occur in the rhizosphere micro-environment than in ‘bulk soil’

Remove bulk soil from roots with vigorous shaking; meticulously collect tightly adhering rhizosphere soil with intense brushing of root surface
Variety and Herbicide Effects on Soybean Nodule Mass
2005 Field Study, R1 Growth Stage – Mid-Missouri USA

Roundup Ready*

Nodule mass (mg/plant)

Herbicide Treatment

*DeKalb DKB38-52
RU=Roundup; TM=Tankmix conventional herbicide;
HW=Handweeded check

Nodulation generally lower on RR regardless of treatment
Does glyphosate interfere with flavonoid signals required for rhizobia attachment to root?
Impact on PGR substances?

Glyphosate Effects on Soybean Nodule and Root Mass
2007 Field Study, R2 Growth Stage – Mid-Missouri USA
(similar to 2006 results)

RR variety:
Pioneer 93M92

Root mass can be decreased by Fusarium infection (Agrios, 1988)
Potential Mn-oxidizing (black) & Mn-reducing bacteria (white, clear) and fungi on roots, in rhizosphere soils

Key Indicator - Mn transformations primarily microbially-mediated, thus have major impact on plant nutrient availability
Ratio of potential Mn-reducing and Mn-oxidizing bacteria in rhizosphere of soybean-herbicide treatments - Stage V4, 2006

Higher ratio of Mn-reducers:Mn oxidizers implies higher plant availability of Mn (Rengel. Plant Soil 196:255, 1997)

- W82 = ‘Williams 82’
- RR 2006 = DK-838-52
- TM = Conventional herbicide tankmix
- RU = Roundup

Soybean var. X Herbicide

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mn Red</th>
<th>Mn Ox</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>W82</td>
<td>0.66</td>
<td></td>
<td>0.66</td>
</tr>
<tr>
<td>W82+TM</td>
<td>0.51</td>
<td></td>
<td>0.51</td>
</tr>
<tr>
<td>RR</td>
<td>0.33</td>
<td></td>
<td>0.33</td>
</tr>
<tr>
<td>RR+TM</td>
<td></td>
<td>0.33</td>
<td>0.33</td>
</tr>
<tr>
<td>RR+RU</td>
<td>0.17</td>
<td></td>
<td>0.17</td>
</tr>
</tbody>
</table>
Ratio of potential Mn-reducing and Mn-oxidizing bacteria in rhizosphere of soybean-herbicide treatments - Stage R2, 2006

W82 = ‘Williams 82’; RR 2006=DK-838-52; TM= Conventional herbicide tankmix; RU= Roundup
Ratio of potential Mn-reducing and Mn-oxidizing bacteria in rhizosphere of soybean-herbicide treatments - Stage R1, 2007

Rhizosphere bacteria (cfu x 10^5 /g soil)

RR variety: Pioneer 93M92
Secondary Observation: majority of bacterial colonies with Mn-oxidizing activity also produce copious amount of exopolysaccharides (EPS); very frequently observed with RR+RU; most colonies classified as *Agrobacterium* spp.; Suggests formation of ‘biofilms’ on roots -- What is relationship between *Agrobacterium*, biofilms, and glyphosate?
**Biofilm** - microbes attached to surface (rhizoplane), aggregated in polymeric matrix (EPS); concentrates extracellular enzymes; mediates many functions (Jass et al., 2002),

**Including Mn oxidation** - in which Mn oxides are precipitated and retained (Toner et al. Appl. Environ. Microbiol. 71:1300, 2005)

Is biogenic Mn oxidation within biofilms enhanced on RR soybean with or without glyphosate amendment??

Demonstration of EPS production by Agrobacterium cultures
Rhizosphere bacteria on plant root surfaces as BIOFILMS

Role of rhizosphere bacteria on GM crop growth?

Bacterial aggregates in layer of EPS forming **biofilm**
KEY GROUP: Rhizosphere Pseudomonads


Many fluorescent pseudomonads associated with antagonism of fungal pathogens (Schroth & Hancock. Science 216:1376, 1982) and Mn transformations (Rengel, 1997)
Relative proportion or composition of Pseudomonas spp. altered in RR or RR+RU treatments. Does glyphosate alter this component in rhizosphere; does genetically-altered RR plant affect composition?

Fluorescent pseudomonad colonies visualized under UV light.
Populations of fluorescent pseudomonads in rhizospheres of soybean-herbicide treatments - 2006

Rhizosphere bacteria (cfu x 10^5 /g soil)

Pseudomonad populations greater in conventional system than RR across growth stages

W82= ‘Williams 82’; RR 2006=DK-838-52; TM= Conventional herbicide tankmix; RU= Roundup
Populations of fluorescent pseudomonads in rhizospheres of Roundup Ready soybean (R1) - 2007

Glyphosate seemed to be associated with greater suppression of fluorescent pseudomonads in 2007 – variety effect?
Relationship between colonization of soybean roots by fluorescent *Pseudomonas* spp. and by *Fusarium* spp.

\[ y = -0.8303x + 94.951 \]

\[ R^2 = 0.761 \]
In vitro assays for interactions of rhizosphere bacteria with fungi: I. Antagonism of Fusarium by bacteria

Fungal growth suppression or conidial germination stimulation by rhizobacterial cultures
II. Protease assay of selected rhizosphere bacteria on milk protein agar [positive test indicated by clearing around bacterial growth] => *possible antagonism mechanism*
### Characteristics of Some Rhizosphere Bacteria Cultured from Soybean, 2006-2007

<table>
<thead>
<tr>
<th>Bacterium</th>
<th>Source</th>
<th>Fluor</th>
<th>EPS</th>
<th>Mn</th>
<th>Fusarium Suppression</th>
<th>Protease</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Pseudomonas</em></td>
<td>W82+HW</td>
<td>+</td>
<td>-</td>
<td>Red</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>Pseudomonas</em></td>
<td>W82+HW</td>
<td>+</td>
<td>-</td>
<td>Red</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td><em>Pseudomonas</em></td>
<td>RR+TM</td>
<td>+</td>
<td>+</td>
<td>Red</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td><em>Pseudomonas</em></td>
<td>W82+TM</td>
<td>+</td>
<td>-</td>
<td>Red</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td><em>Pseudomonas</em></td>
<td>RR+RU</td>
<td>+</td>
<td>++</td>
<td>Ox</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Pseudomonas</em></td>
<td>RR+RU</td>
<td>-</td>
<td>-</td>
<td>Red</td>
<td>±</td>
<td>-</td>
</tr>
<tr>
<td><em>Pseudomonas</em></td>
<td>RR+HW</td>
<td>+</td>
<td>-</td>
<td>Red</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td><em>Chryseobacterium</em></td>
<td>RR+HW</td>
<td>-</td>
<td>-</td>
<td>Red</td>
<td>++</td>
<td>±</td>
</tr>
<tr>
<td><em>Agrobacterium</em></td>
<td>RR+RU</td>
<td>-</td>
<td>++</td>
<td>Ox</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Agrobacterium</em></td>
<td>RR+RU</td>
<td>-</td>
<td>+</td>
<td>Ox</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Agrobacterium</em></td>
<td>RR+RU</td>
<td>-</td>
<td>+</td>
<td>Ox</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Agrobacterium</em></td>
<td>RR+RU</td>
<td>-</td>
<td>++</td>
<td>Red</td>
<td>-</td>
<td>±</td>
</tr>
<tr>
<td><em>Agrobacterium</em></td>
<td>W82+HW</td>
<td>-</td>
<td>+</td>
<td>Red</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

Fluor=Fluorescence; EPS=Exopolysaccharide

Mn reduction greater factor in fungal suppression than enzymatic process or antibiosis in conventional system? Suggested by Huber & McCay-Buis, 1993
Linking Function to Structure – Molecular Fingerprint of Microbial Communities

PCR-DGGE of bacterial DNA extracted from soybean rhizosphere and bulk soils

- Agrobacterium marker
- Pseudomonas marker

Soil - 2006
W82 R3 2006
RR 2006
W82 V4 2006
RR 2007
RR 2006
Bacterial diversity (no. of DNA bands, Shannon index) in soils and soybean rhizospheres affected by variety, herbicide, growth stage.

<table>
<thead>
<tr>
<th>Variety X Herbicide</th>
<th>Soybean Growth Stage</th>
<th>Season</th>
<th>No. of DNA Bands</th>
<th>Diversity Index</th>
</tr>
</thead>
<tbody>
<tr>
<td>W82</td>
<td>V4</td>
<td>2006</td>
<td>20</td>
<td>3.50</td>
</tr>
<tr>
<td>W82 + Herb</td>
<td>V4</td>
<td>2006</td>
<td>18</td>
<td>3.40</td>
</tr>
<tr>
<td>W82</td>
<td>R3</td>
<td>2006</td>
<td>22</td>
<td>3.89</td>
</tr>
<tr>
<td>W82 + Herb</td>
<td>R3</td>
<td>2006</td>
<td>22</td>
<td>3.25</td>
</tr>
<tr>
<td>RR</td>
<td>V4</td>
<td>2006</td>
<td>21</td>
<td>2.80</td>
</tr>
<tr>
<td>RR + Herb</td>
<td>V4</td>
<td>2006</td>
<td>27</td>
<td>3.00</td>
</tr>
<tr>
<td>RR + RU</td>
<td>V4</td>
<td>2006</td>
<td>21</td>
<td>2.20</td>
</tr>
<tr>
<td>RR</td>
<td>R3</td>
<td>2006</td>
<td>20</td>
<td>2.85</td>
</tr>
<tr>
<td>RR + Herb</td>
<td>R3</td>
<td>2006</td>
<td>26</td>
<td>3.20</td>
</tr>
<tr>
<td>RR + RU</td>
<td>R3</td>
<td>2006</td>
<td>18</td>
<td>2.10</td>
</tr>
<tr>
<td>‘Bulk’ Soil</td>
<td>2006</td>
<td></td>
<td>16</td>
<td>0.45</td>
</tr>
<tr>
<td>RR</td>
<td>V8</td>
<td>2007</td>
<td>24</td>
<td>3.20</td>
</tr>
<tr>
<td>RR + RU</td>
<td>V8</td>
<td>2007</td>
<td>18</td>
<td>2.00</td>
</tr>
<tr>
<td>‘Bulk’ Soil</td>
<td>2007</td>
<td></td>
<td>15</td>
<td>0.52</td>
</tr>
</tbody>
</table>
High carbohydrate contents

Glyphosate

Fusarium spp.

Enzymes, Toxins

PGPRs (auxins)

Soybean root

Roundup Application (systemic movement of glyphosate to roots)

Factors affecting interaction:
- Soil moisture/temperature
- Clay mineralogy/soil type
- Soil organic matter content
- RR soybean variety
- Soil nutrient status - i.e., Mn
- Management - crop rotation, tillage

Beneficial (i.e., biocontrol) or Pathogenic (i.e., SDS agent)?

Rhizosphere bacteria interactions with fungi

Complexity of the soil & rhizosphere ecosystem confounded by RR technology

Mycorrhizal Interactions

Microbial Biofilms
- Bradyrhizobia, nodulation interactions?
- Mn, Fe oxidation/reduction

Mycotoxins

antagonisms

Complexity of the soil & rhizosphere ecosystem confounded by RR technology
Observations on Assessment of Glyphosate and Glyphosate-Resistant Crops for Soil-Microbial-Plant Interactions (“More questions, some answers”)

Examine Key Indicators -

* Specific microbial groups and functions (nutrient transformations, specific fungi/ bacteria, mycorrhizae); specific plant parameters (root growth, nodulation) may be most sensitive to potential impacts of GM plants.

Decreased diversity in RR soybean (molecular fingerprint) is of concern because high diversity is essential to maintain stable ecosystem productivity. (Dunfield & Germida. J. Environ. Qual. 33:806, 2004)
Use of ‘conventional cropping system’ illustrated a more balanced rhizosphere community relative RR system demonstrated by proportion of Mn transforming bacteria; pseudomonad population size relative to Fusarium colonization; and apparent nitrogen fixation represented by root nodulation.

Polyphasic microbial analyses (integrated approach combining specific assays with structural characterization) shows impacts not limited to *Fusarium*; several key microbial groups/functions involved in rhizosphere interactions are affected.

*General analyses will not likely yield measurable or differential responses (soil respiration; microbial biomass C/N; general soil enzyme activities)

See Liphadzi et al., 2005; Means et al., 2007; Weaver et al., 2007)
IMPACT OF RESEARCH - “in a word” - MANAGEMENT

An understanding of factors in soils and rhizospheres (root exudation, glyphosate release, microbial characteristics) that interact with root-associated microorganisms is essential for developing ways to manipulate the rhizosphere environment to reduce/eliminate adverse effects (i.e., root infection, nutrient availability) -- especially critical in context of transgenic crops as part of a management system.
ACKNOWLEDGMENTS

- USDA Special Grant – SCN
- Technicians: Jenan Nichols; Jim Ortbals
- Field crew: Tim Reinbott; Steve Troesser
- Graduate students: Nathan Means; Su-jung Kim
- Undergrad student assistants: Heidi, Atim, Michael, Sarah, Ashley, Luke

Thanks to Yamada for organizing and the invitation to participate in this important symposium
MAIZE ROOT FUSARIAUM

CFU per 100cm root

Days + Herbicide Application

 Glyphosate

 tank-mix