GLYPHOSATE AND GLYPHOSATE-RESISTANT CROP INTERACTIONS WITH RHIZOSPHERE MICROORGANISMS

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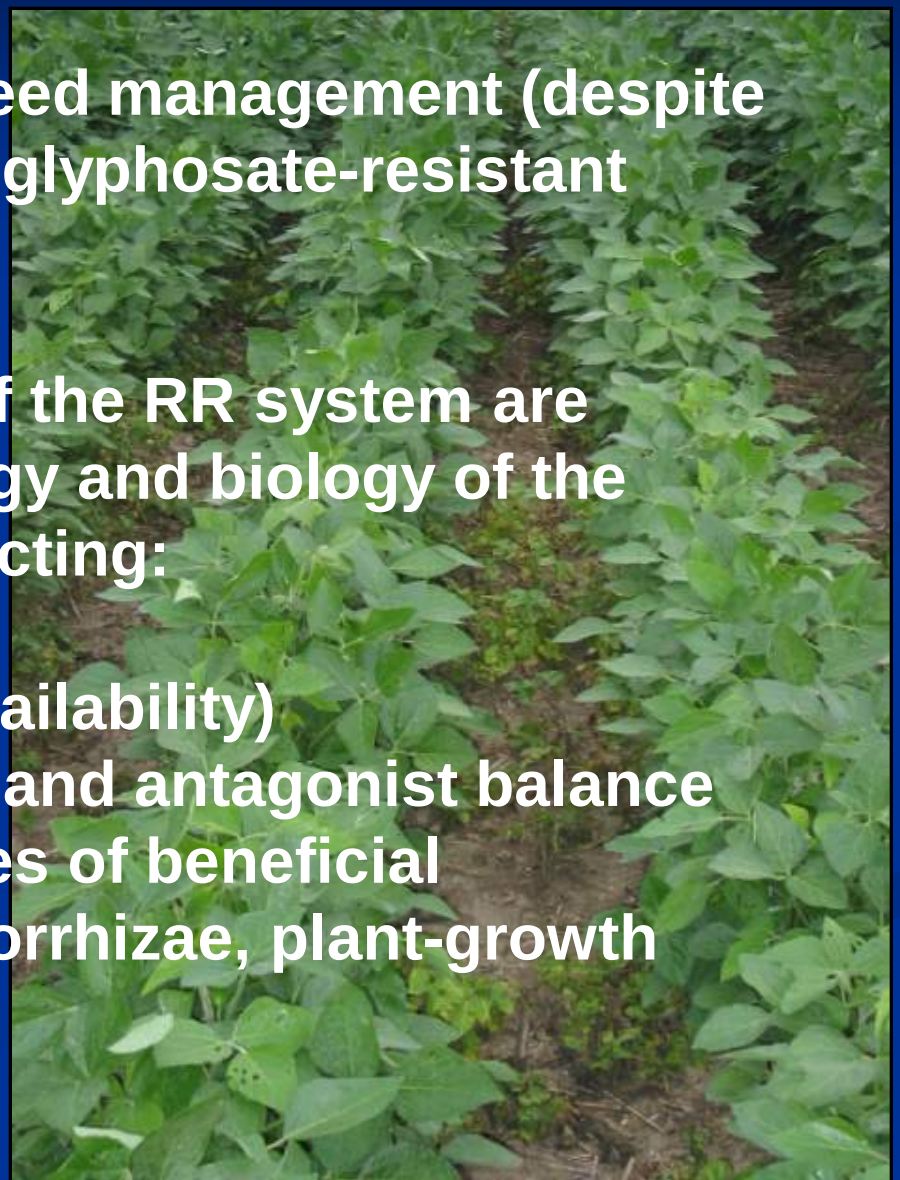
"Standard" field trials conducted with Roundup Ready soybean.
Field trial on Tiptonville silt loam, Pemiscot County, Missouri 1999

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)

TRANSGENIC CROPS AND SOIL BIOLOGY RESEARCH – USDA-ARS & UNIVERSITY OF MISSOURI

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**

Increased *Fusarium* colonization attributed to possible lower or altered synthesis of phytoalexins, based on previous work on glyphosate 'secondary mode of action' (Johal & Rahe. Phytopathology 74:950, 1984; Levesque & Rahe, Annu. Rev. Phytopathol. 30:579, 1992)
and altered root exudation patterns due to genetic modification or glyphosate interactions *in planta* (Kremer et al. Int. J. Environ. Anal. Chem. 85:1165, 2005)

These findings helped partially explain reported production problems with RR soybean experienced by many farmers in midwestern U.S.

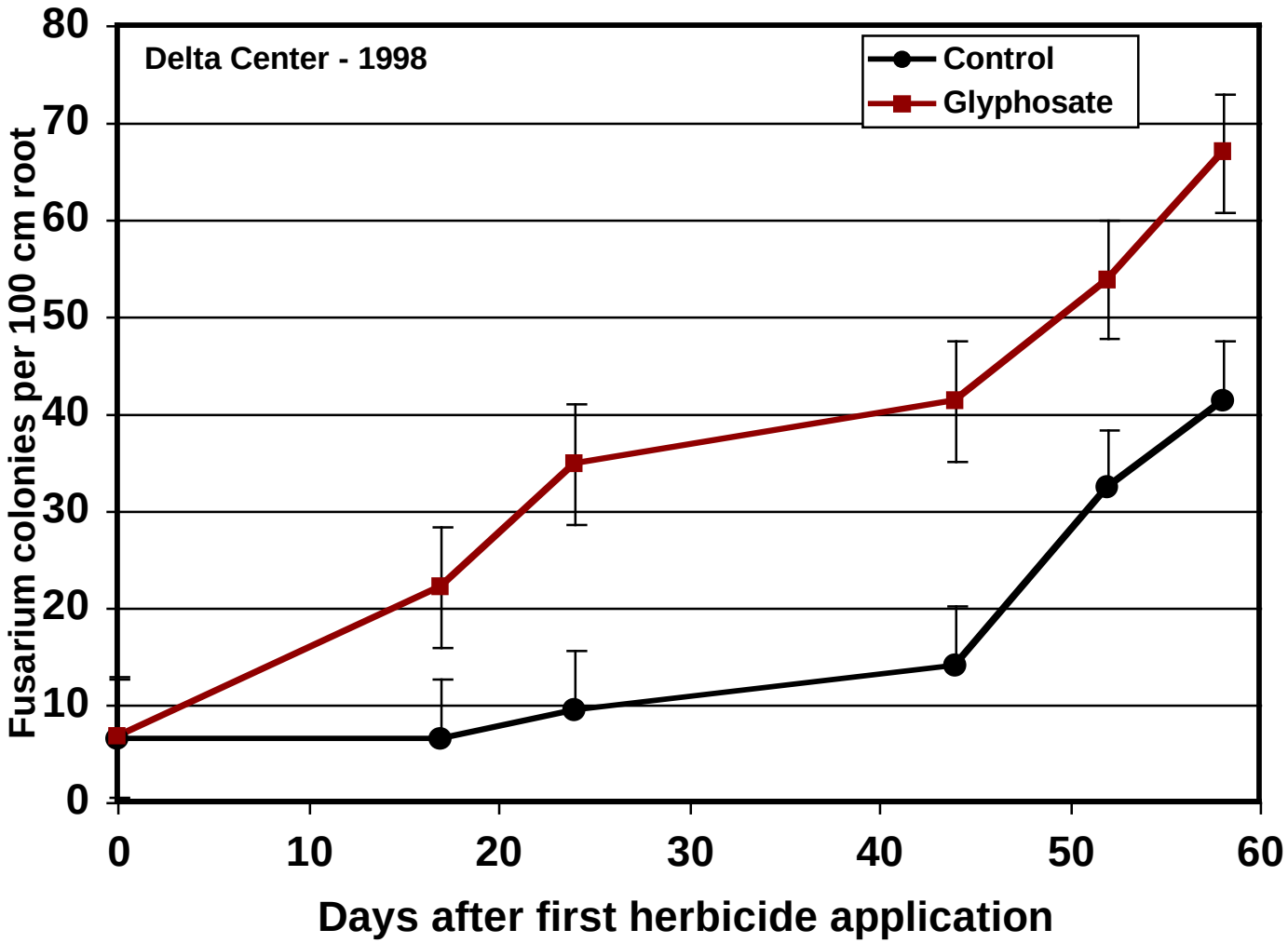
Typical soybean root colonization assay results observed consistently, 1997-2007

Fusarium colonization – 2007 study

+ Glyphosate Check

Typical trend in Fusarium colonization of RR roots

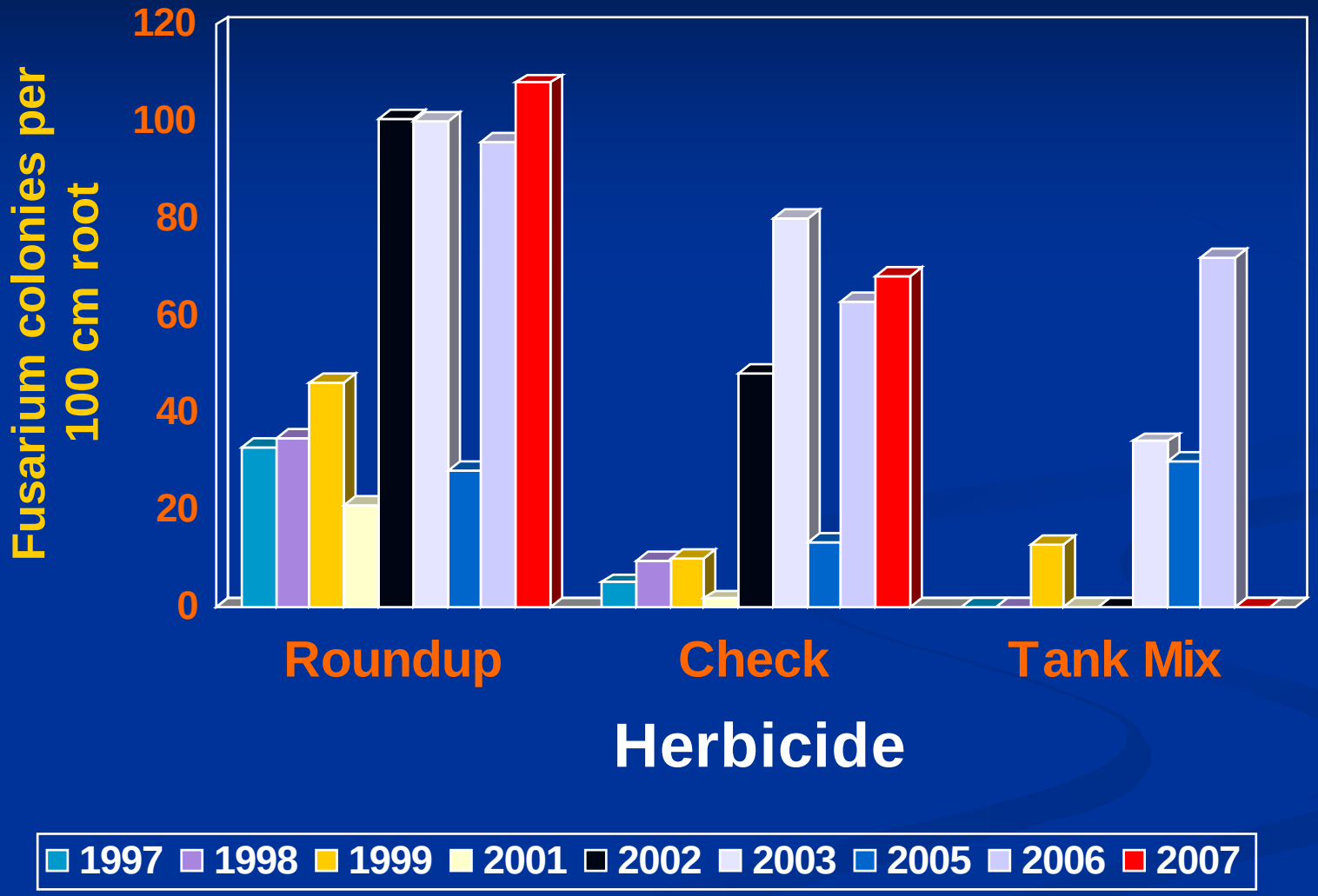
Figure 2. Incidence of rhizosphere *Fusarium* on RR soybean.
Vertical bars denote LSD (p<0.05)



Fusarium colonies per 100 cm root

Summary - consistent trends over years

Fusarium Root Colonization on RR Soybean 1997-2007



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

Photo: D. Sasseville

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

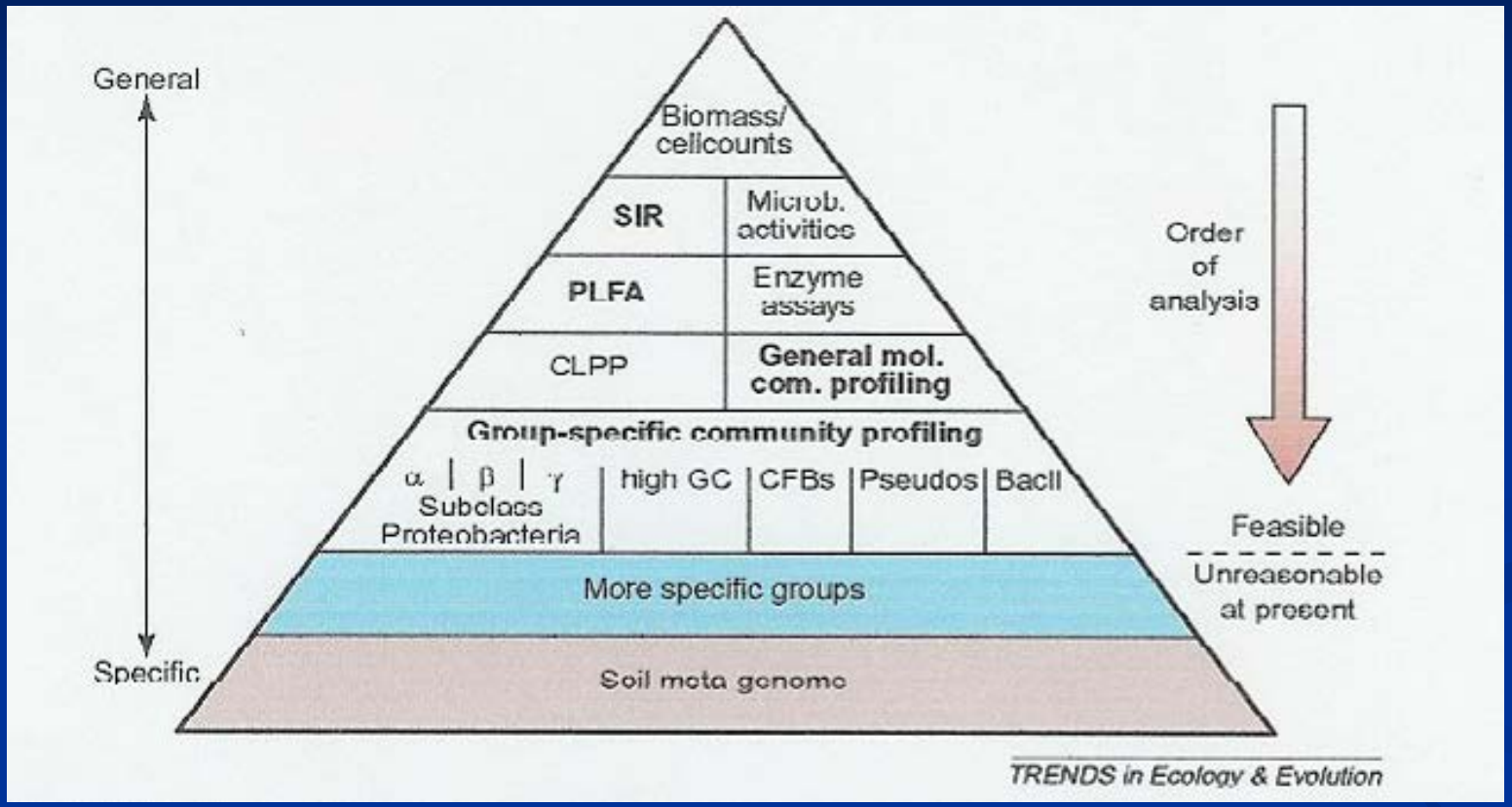
****Use of indicator groups and specific microbial activities that are sensitive to ecosystem changes and that are relevant to the particular GM system in place is proposed. A 'polyphasic' microbial analyses to target the broad impact on the total microbial community, thus a multiple analyses provides 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 –

Bruinsma et al. Biol. Fert. Soils 37:329, 2003;

Kowalchuk et al. Trends Ecol. Evolut. 18:403, 2003.

Proposed polyphasic microbial community analyses



Difficult to demonstrate impact

Likely most sensitive to ecosystem alteration by GM crops

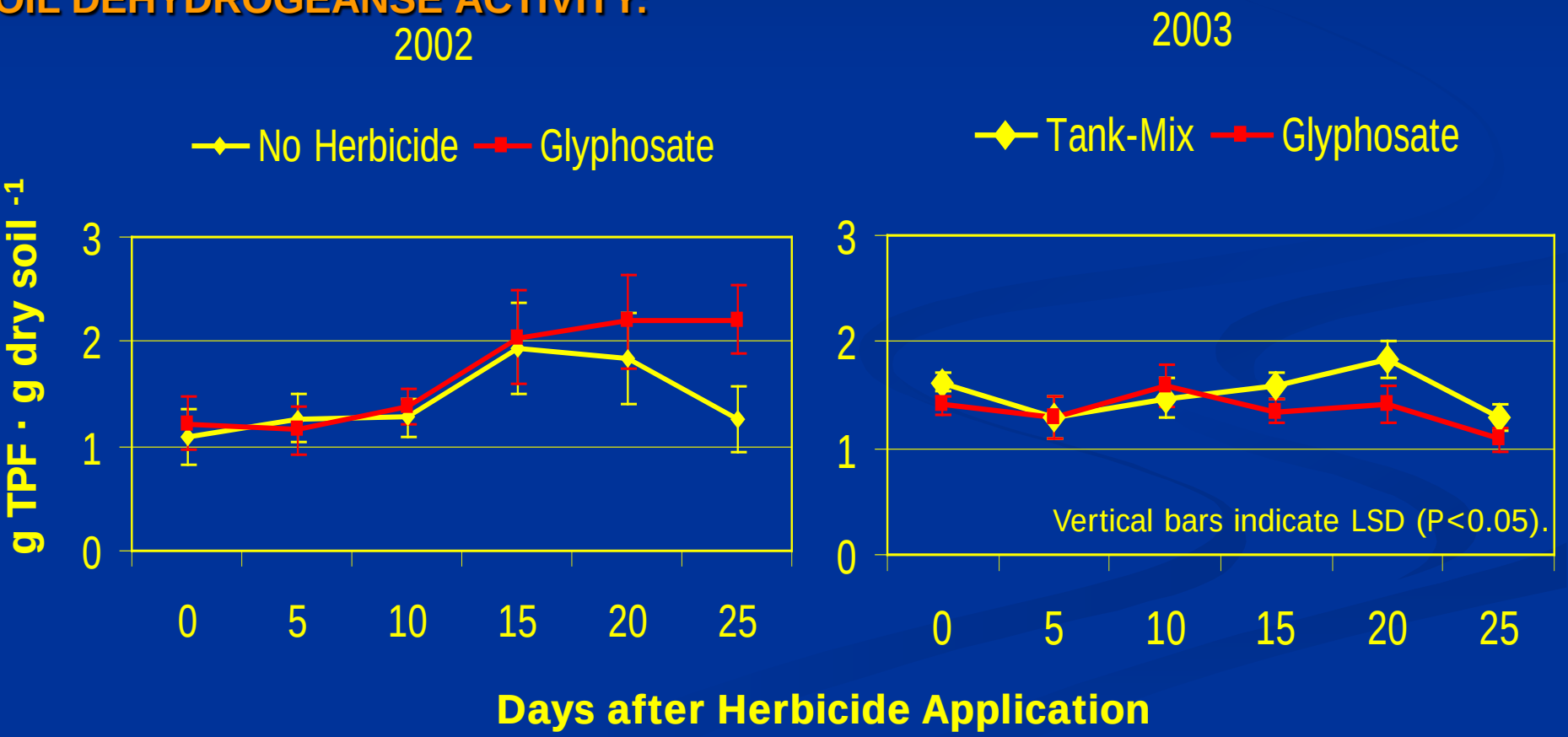
Source: Kowalchuk et al. 2003. Trends Ecol. Evolut. 18:403-410.

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 -

See Liphadzi et al. Weed Sci. 53:536, 2005; Weaver et al. Pest Manage. Sci. 63:388, 2007; Means et al. J. Environ. Sci. Health B 42:125, 2007. (Note: most studies deal with soil, not rhizosphere communities)

An example:

SOIL DEHYDROGEANSE ACTIVITY:



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.

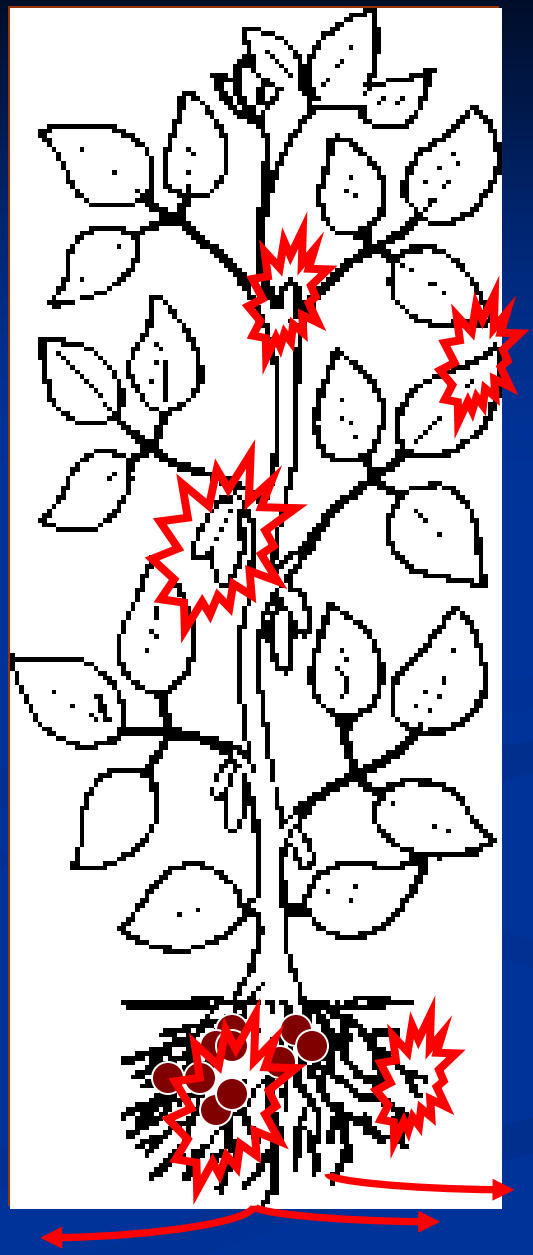
RECALL -

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

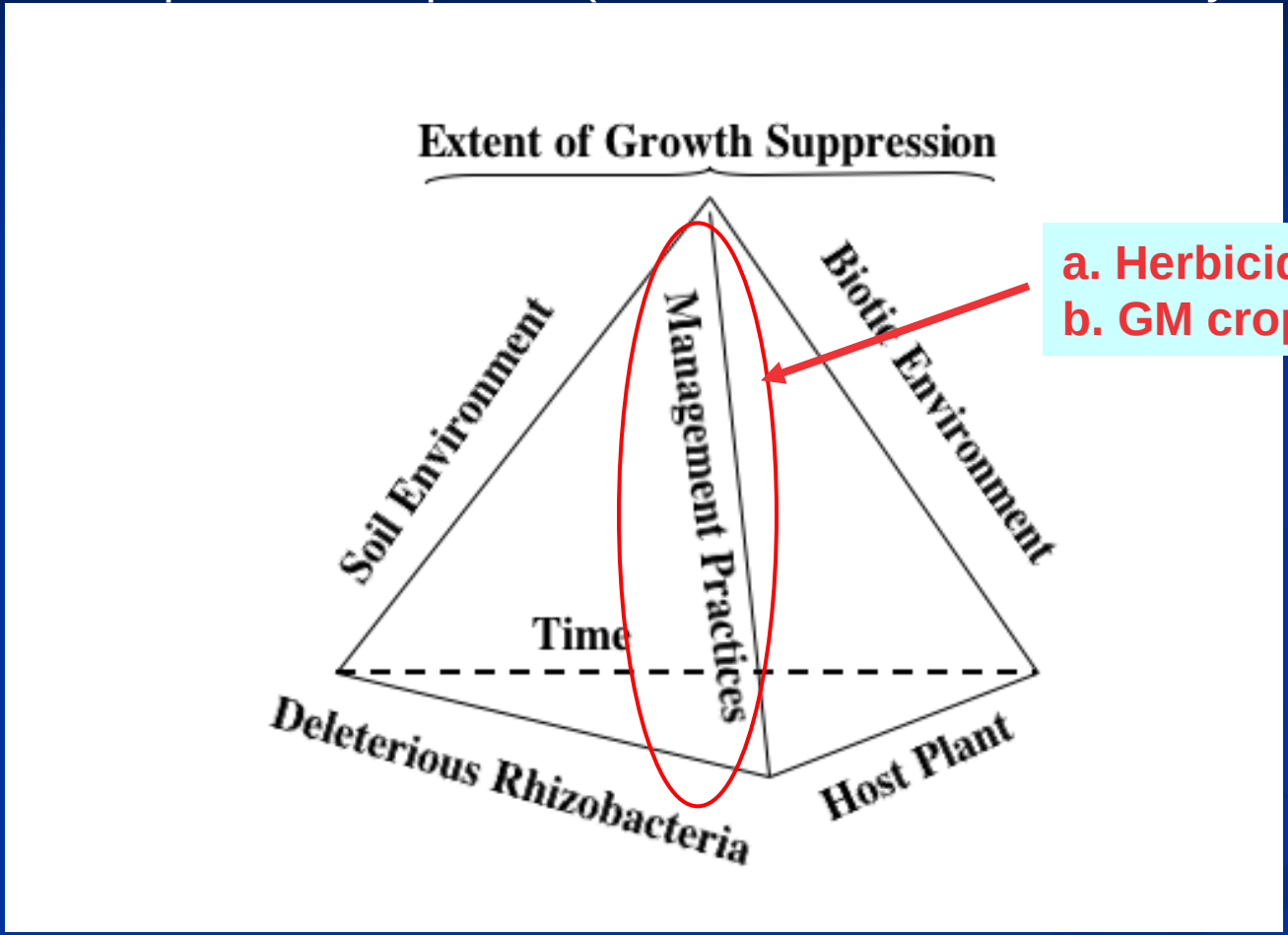
In RR soybean, glyphosate has been found *localized* in meristematic tissues of growing points, leaves and roots; pod and seed; nodules.

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

(Kremer et al. Int. J. Environ. Anal. Chem. 85:1165, 2005**)**



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 Kremer, 2006 (modified from Agrios, 1988: Huber & McCay-Buis, 1993).

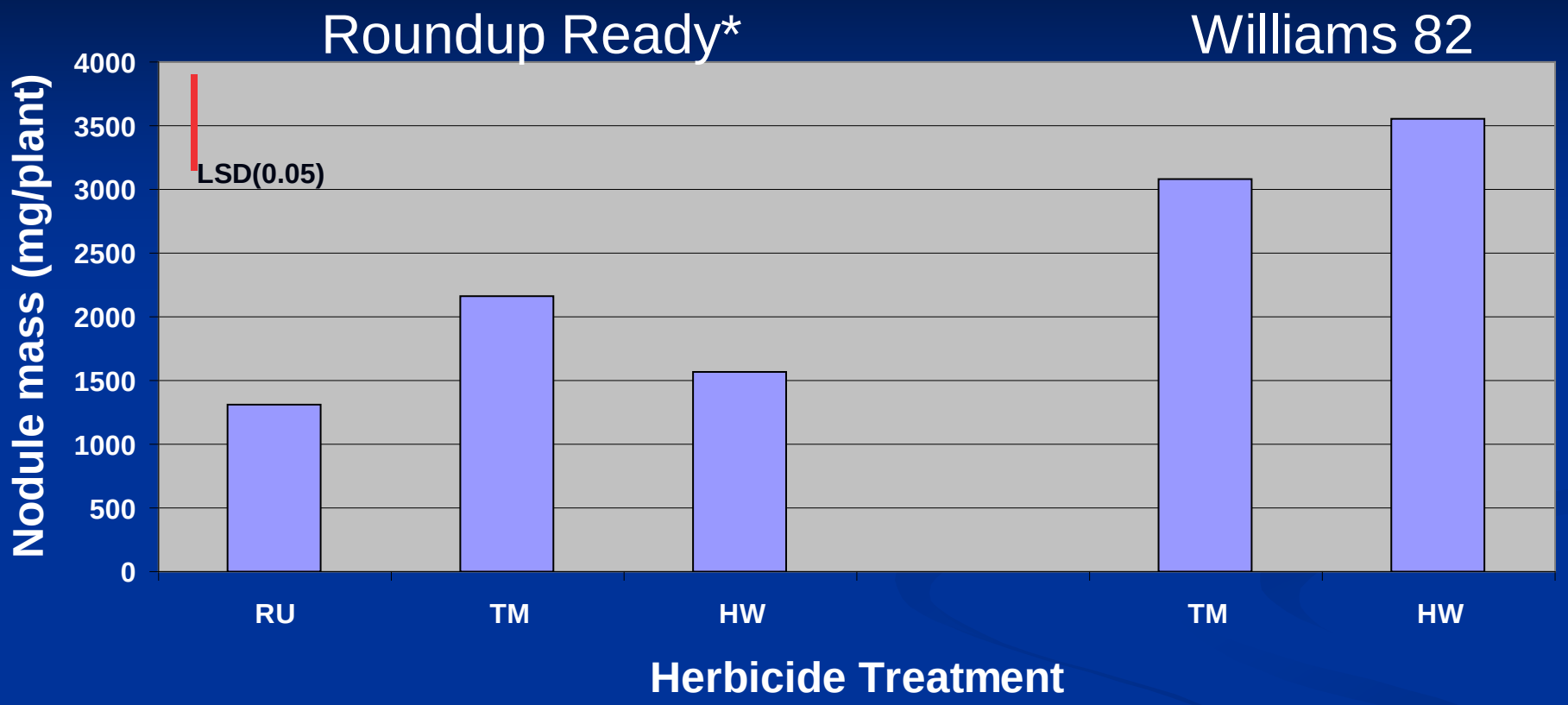
Methodology –

***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

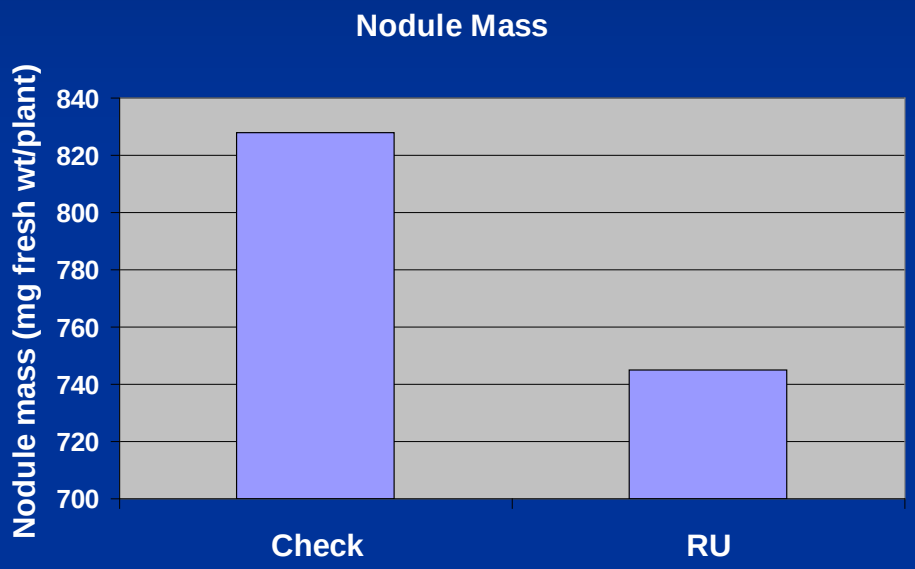


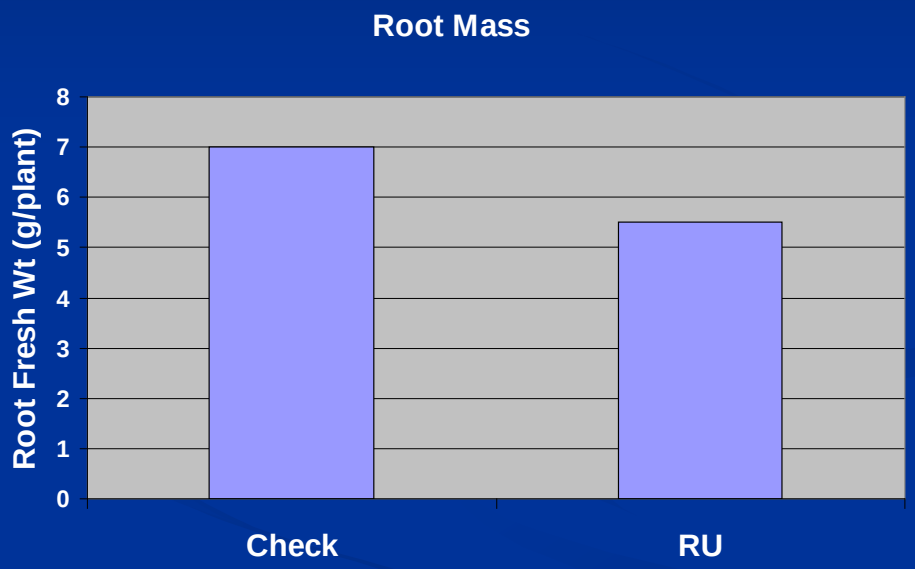
*DeKalb DKB38-52
RU=Roundup; TM=Tankmix conventional herbicide;
HW=Handweeded check

Recent report of alteration of rhizobial cell structure by glyphosate in de Maria et al. Appl. Environ. Microbiol. 73:5075, 2007

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)





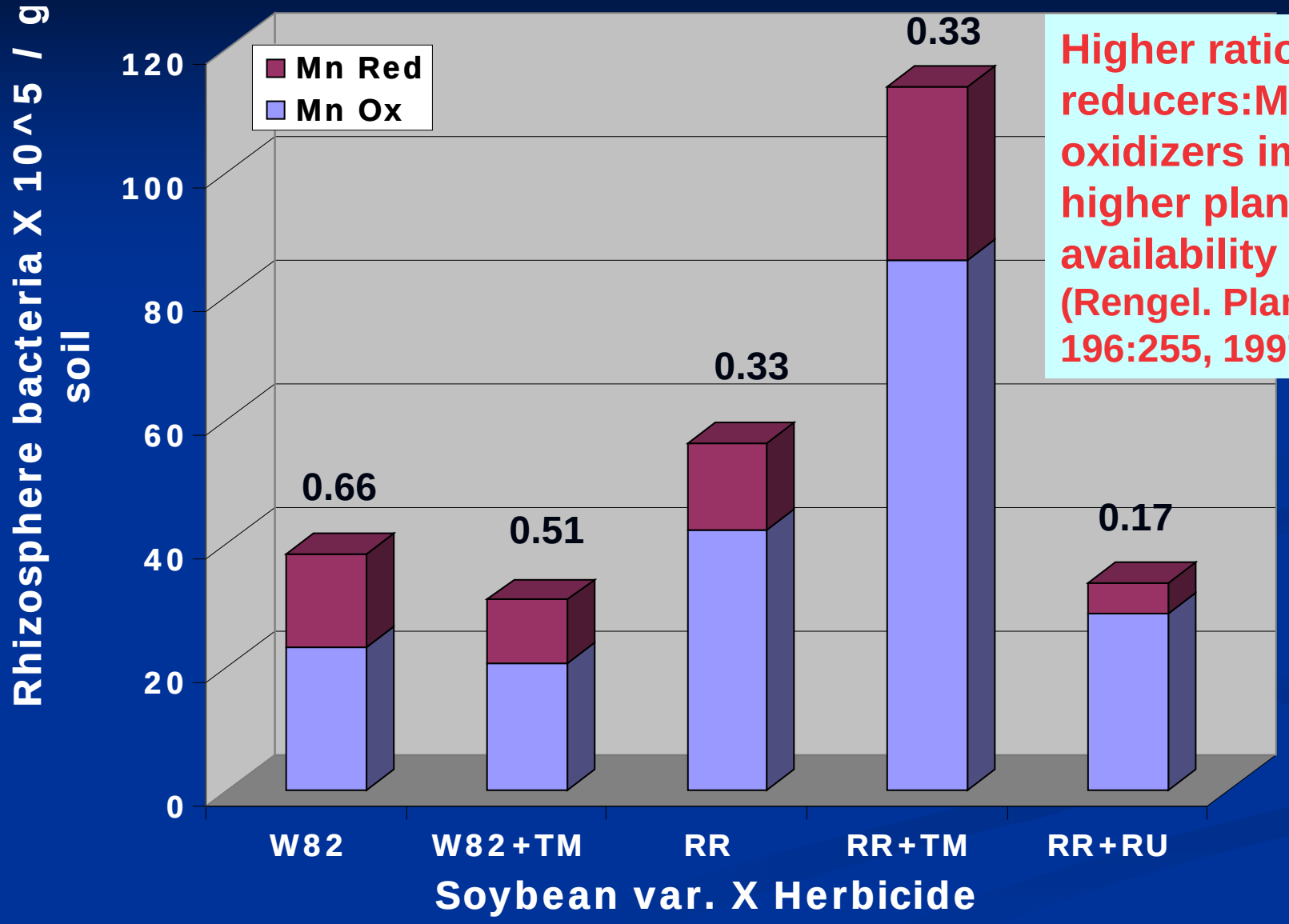
RR variety:
Pioneer 93M92

Root mass can be decreased by Fusarium infection (Agrios, 1988)

Key Indicator -
Mn transformations primarily microbially-mediated, thus have major impact on plant nutrient availability

Potential Mn-oxidizing (black) & Mn-reducing bacteria (white, clear) and fungi on roots, in rhizosphere soils

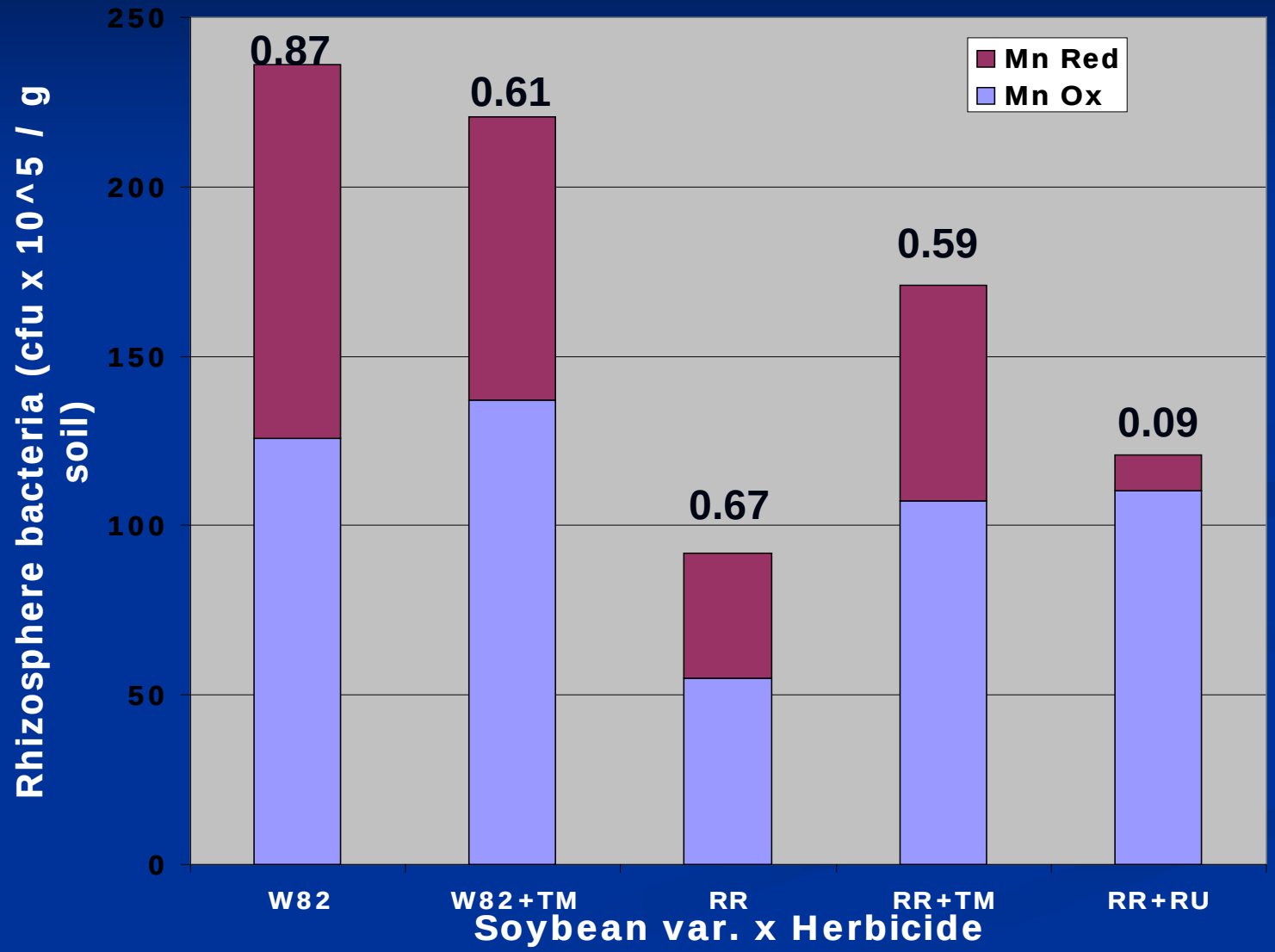
Ratio of potential Mn-reducing and Mn-oxidizing bacteria in rhizosphere of soybean-herbicide treatments - Stage V4, 2006



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

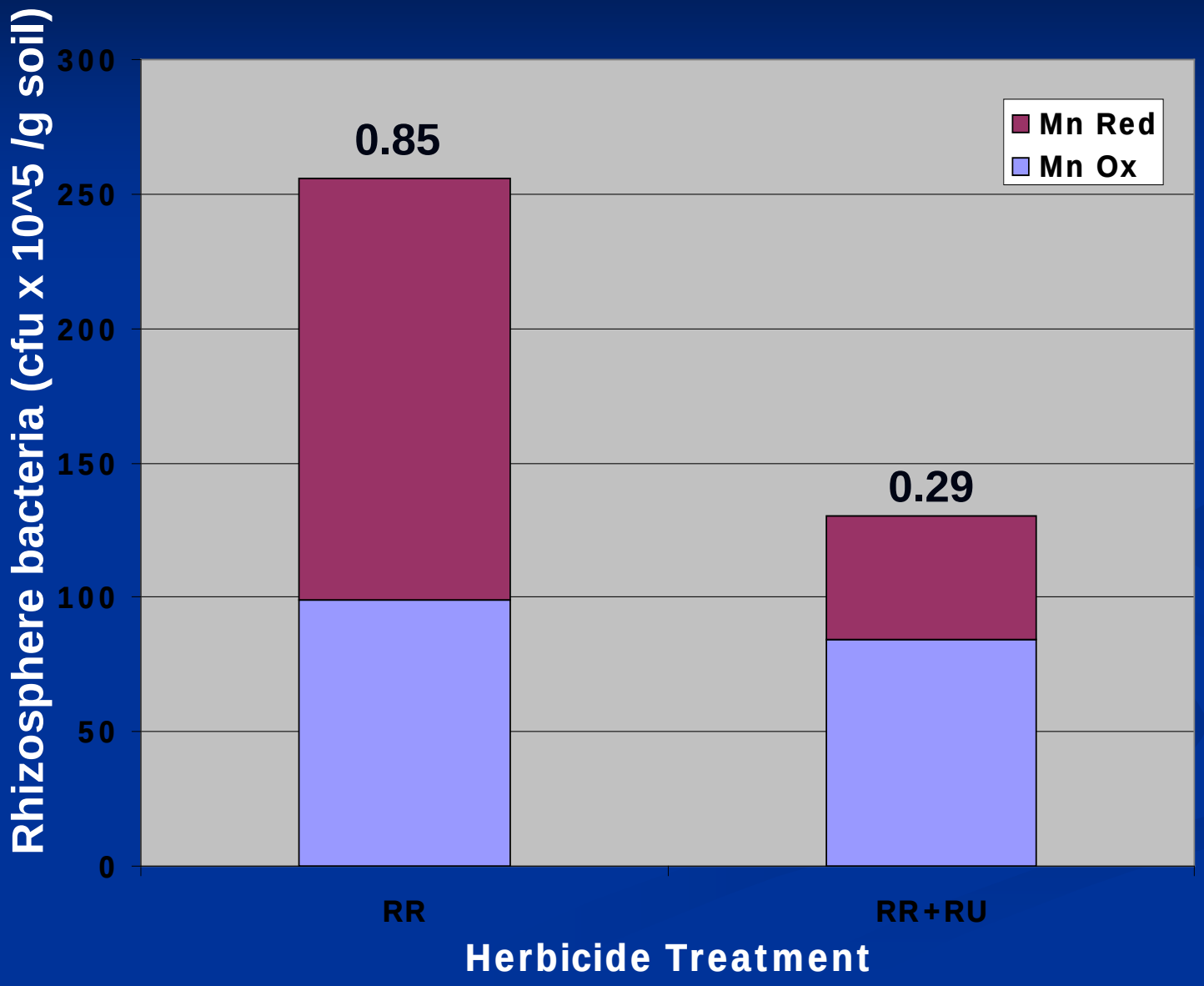
W82= 'Williams 82'; RR 2006=DK-838-52; TM= Conventional herbicide tankmix; RU= Roundup

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



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?

Agrobacterium sp. colony with presumed Mn-oxidizing ability showing excess EPS

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)

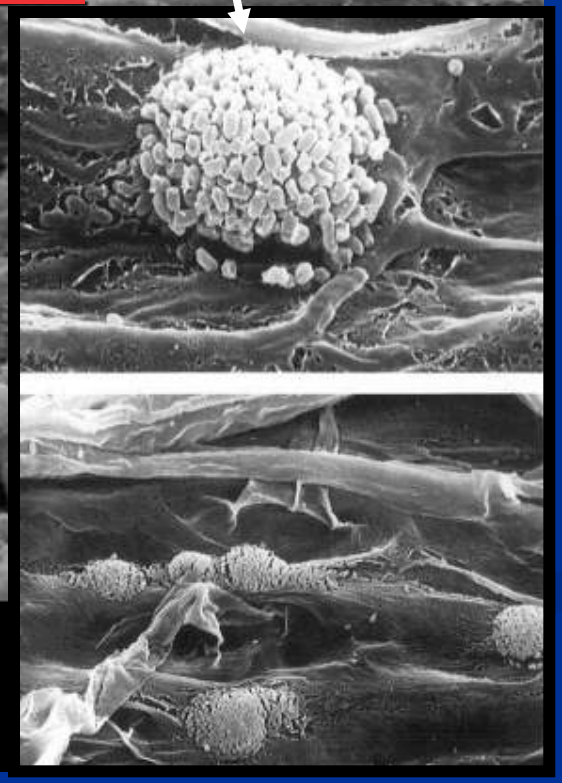
Is biogenic Mn oxidation within biofilms enhanced on RR soybean with or without glyphosate amendment??

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



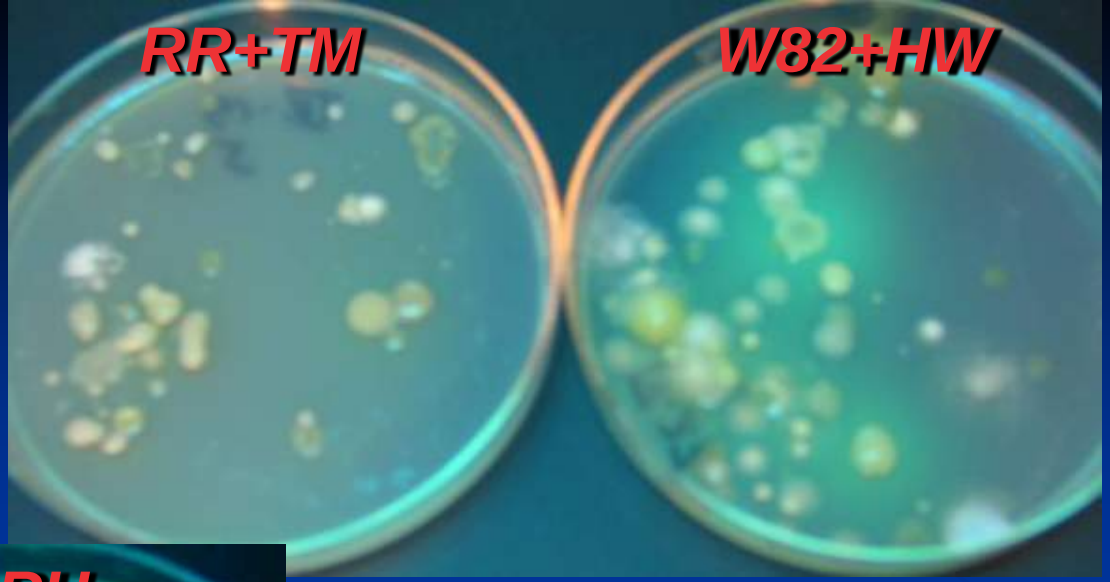
10KV 10U

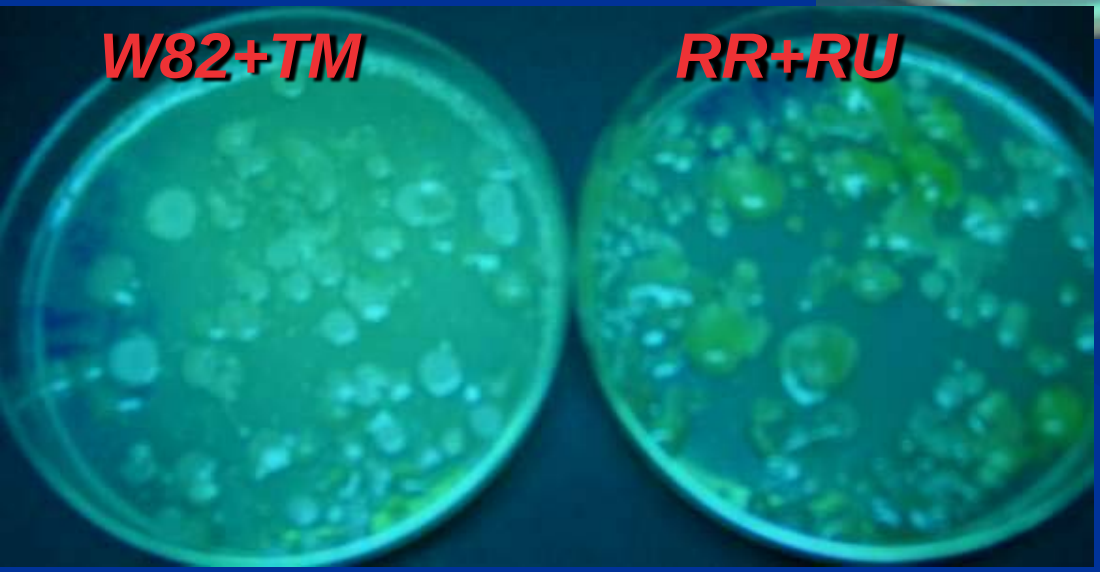
KEY GROUP: Rhizosphere Pseudomonads

Important multi-functional bacterial group in rhizosphere community (Schroth et al. Prokaryotes 6:714, 2006)

Many fluorescent pseudomonads associated with antagonism of fungal pathogens (Schroth & Hancock. Science 216:1376, 1982) and Mn transformations (Rengel, 1997)

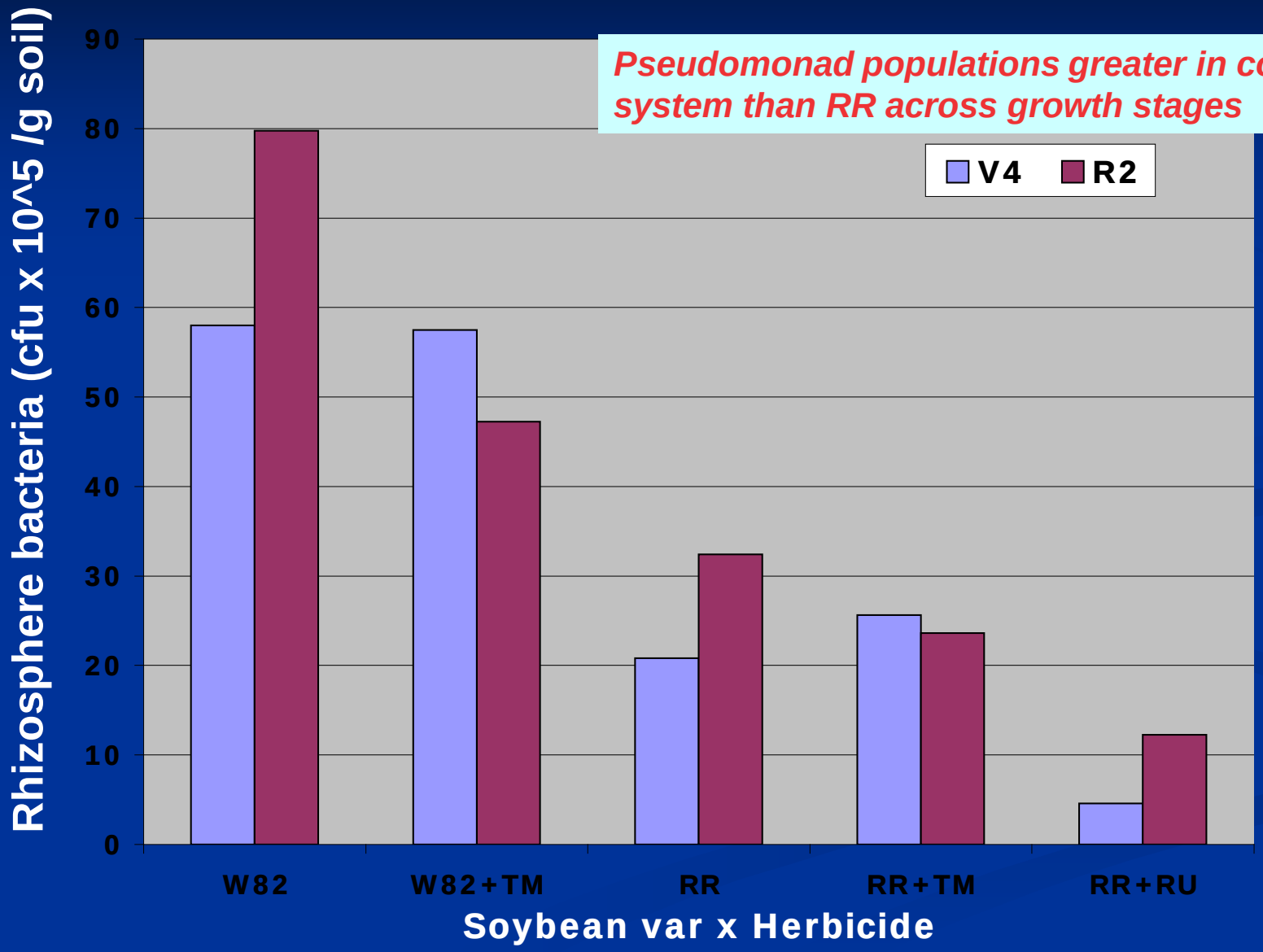
Fluorescent pseudomonad colonies visualized under UV light.





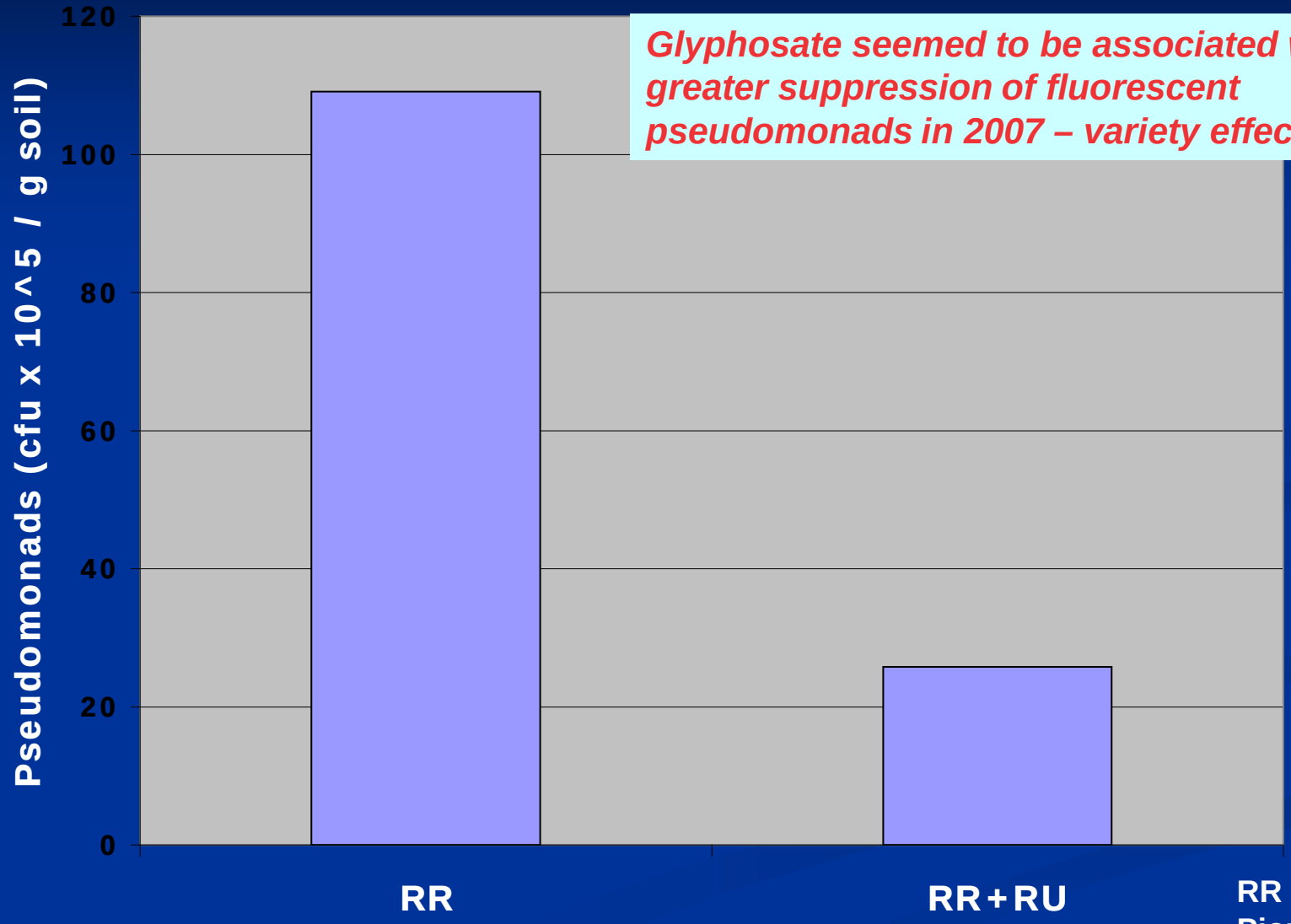
Relative proportion or composition of Pseudomonas spp. altered in RR or RR+RU treatments. Does glyphosate alter this component in rhizosphere; does genetically-altered RR plant affect composition?

Populations of fluorescent pseudomonads in rhizospheres of soybean-herbicide treatments - 2006



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

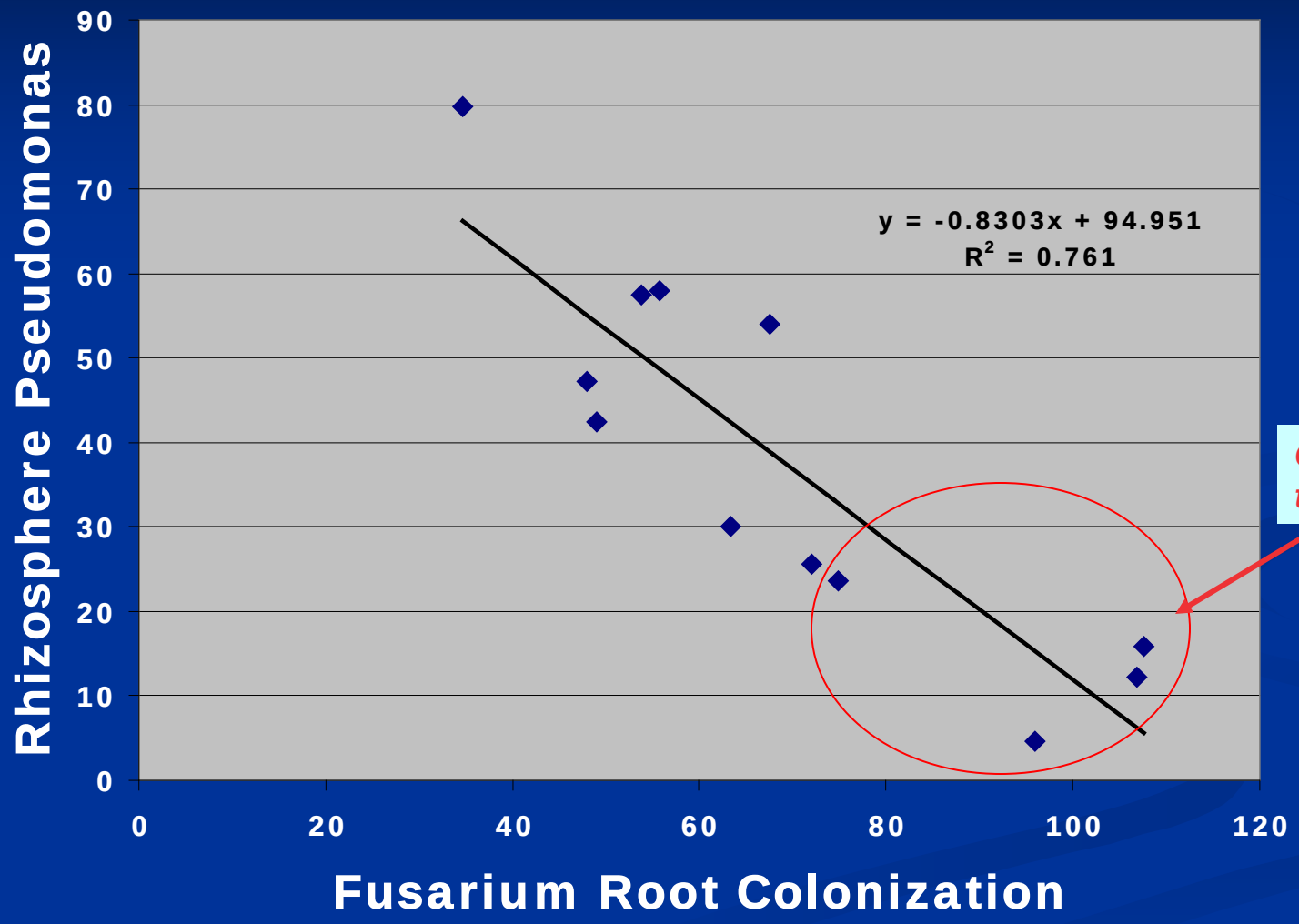
Populations of fluorescent pseudomonads in rhizospheres of Roundup Ready soybean (R1) - 2007



Glyphosate seemed to be associated with greater suppression of fluorescent pseudomonads in 2007 – variety effect?

RR variety:
Pioneer 93M92

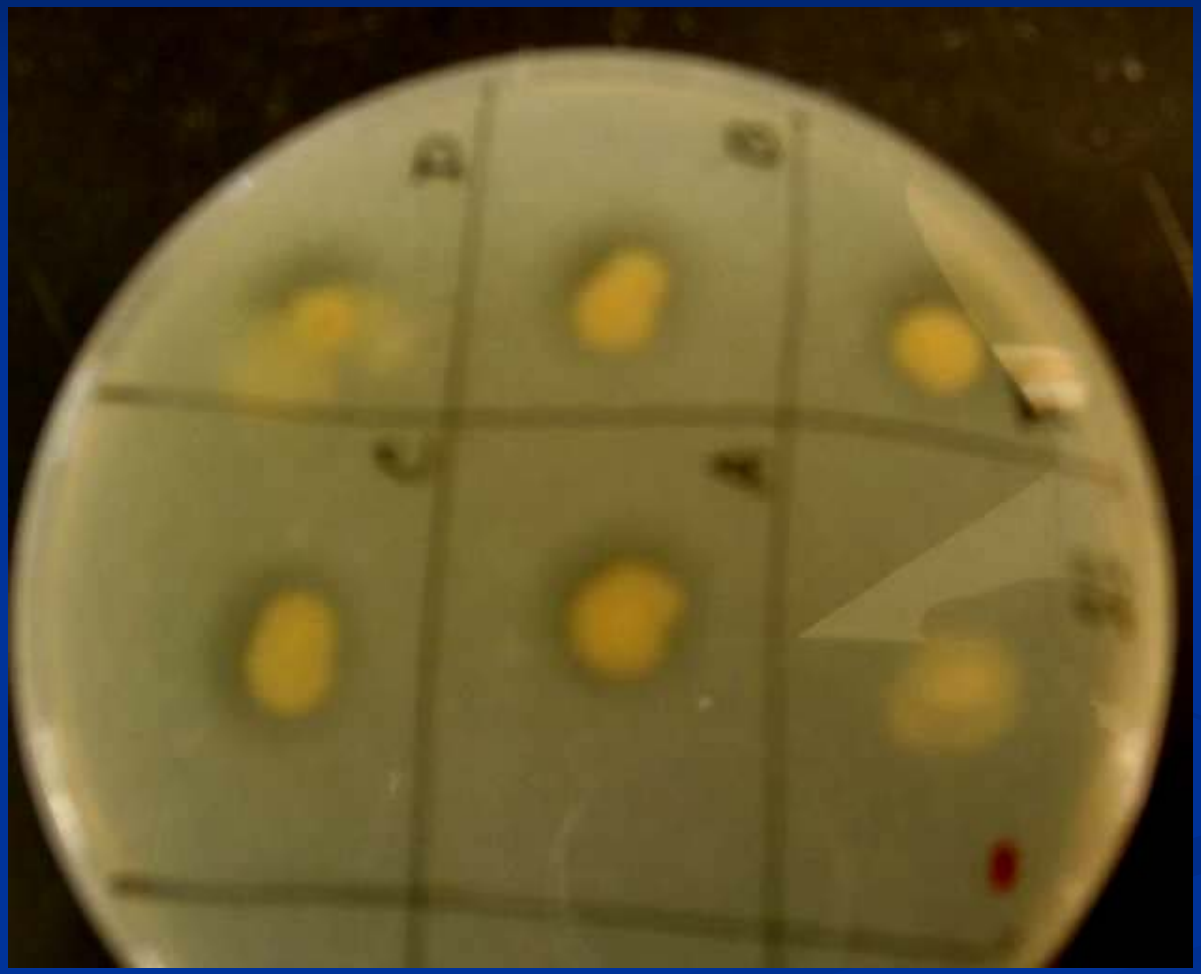
Relationship between colonization of soybean roots by fluorescent *Pseudomonas* spp. and by *Fusarium* spp.



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

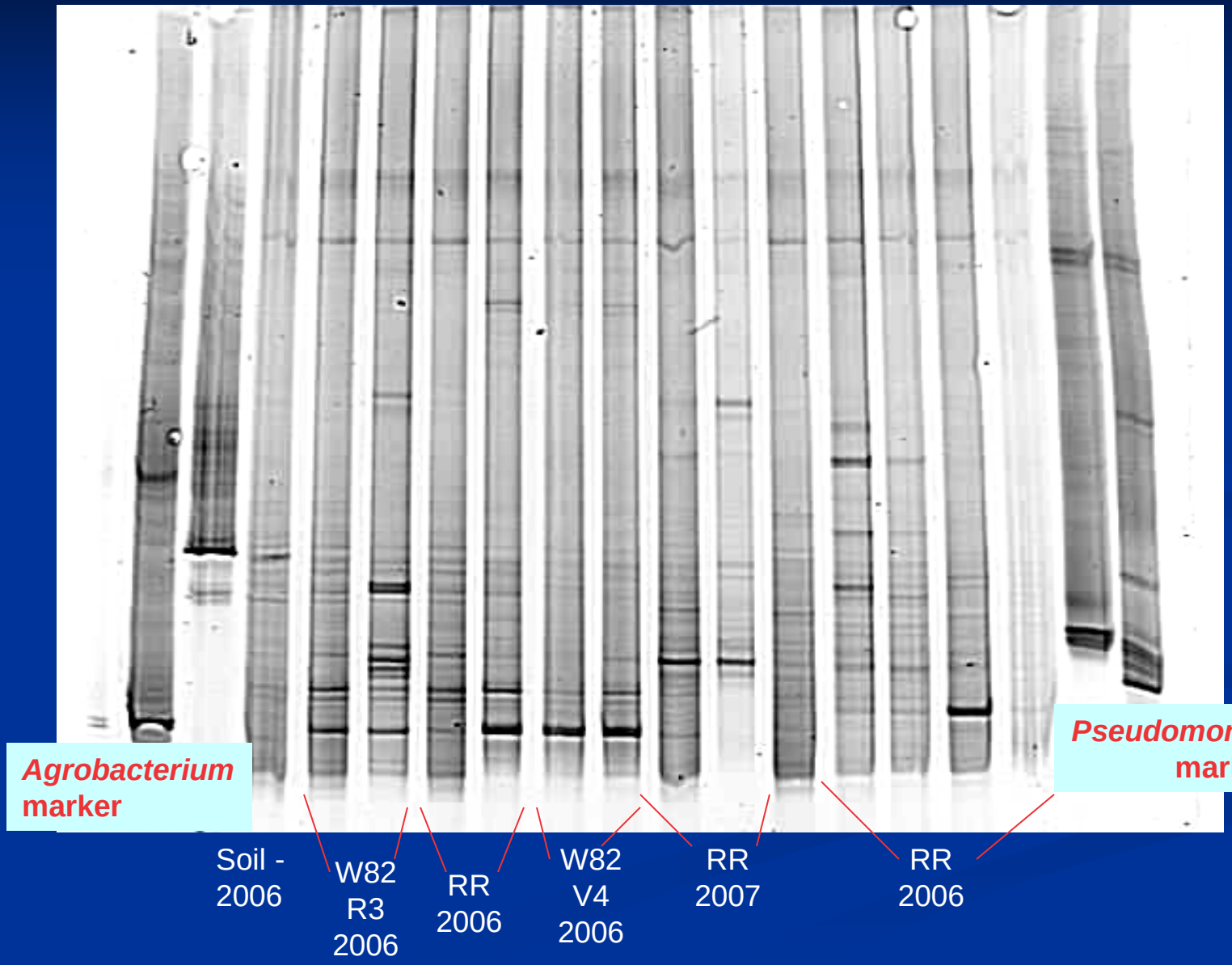
Bacterium	Source	Fluor	EPS	Mn	Fusarium Suppression	Protease
Pseudomonas	W82+HW	+	-	Red	+	+
Pseudomonas	W82+HW	+	-	Red	+	-
Pseudomonas	RR+TM	+	+	Red	+	++
Pseudomonas	W82+TM	+	-	Red	++	++
Pseudomonas	RR+RU	+	++	Ox	-	-
Pseudomonas	RR+RU	-	-	Red	±	-
Pseudomonas	RR+HW	+	-	Red	+	-
Chryseobacterium	RR+HW	-	-	Red	++	±
Agrobacterium	RR+RU	-	++	Ox	-	-
Agrobacterium	RR+RU	-	+	Ox	-	-
Agrobacterium	RR+RU	-	+	Ox	-	-
Agrobacterium	RR+RU	-	+	Ox	-	-
Agrobacterium	RR+RU	-	++	Red	-	±
Agrobacterium	W82+HW	-	+	Red	+	-

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

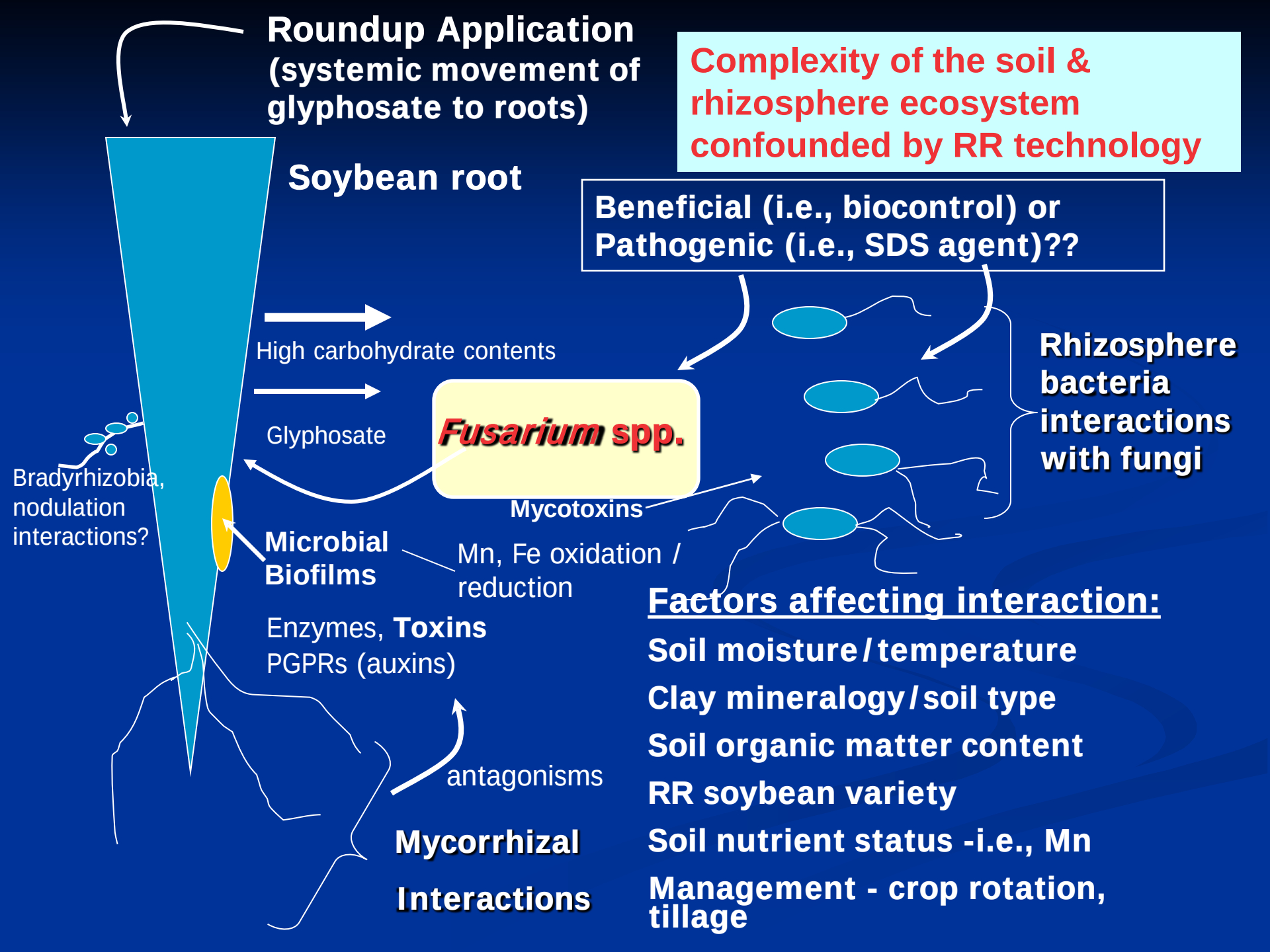
Linking Function to Structure – Molecular Fingerprint of Microbial Communities

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



Bacterial diversity (no. of DNA bands, Shannon index) in soils and soybean rhizospheres affected by variety, herbicide, growth stage.

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


Roundup Application
(systemic movement of glyphosate to roots)
Soybean root
Complexity of the soil & rhizosphere ecosystem confounded by RR technology
Beneficial (i.e., biocontrol) or Pathogenic (i.e., SDS agent)??
High carbohydrate contents
Glyphosate
Fusarium spp.
Rhizosphere bacteria interactions with fungi
Bradyrhizobia, nodulation interactions?
Mycotoxins
Microbial Biofilms
Mn, Fe oxidation / reduction
Enzymes, Toxins
PGPRs (auxins)
antagonisms
Mycorrhizal Interactions
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

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

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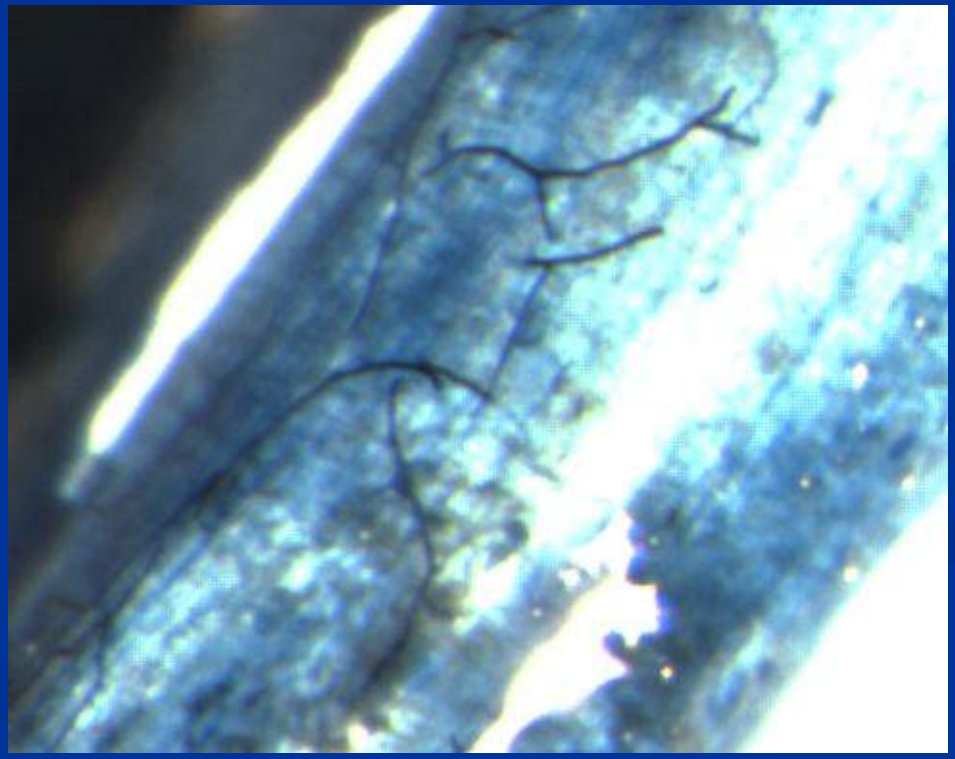
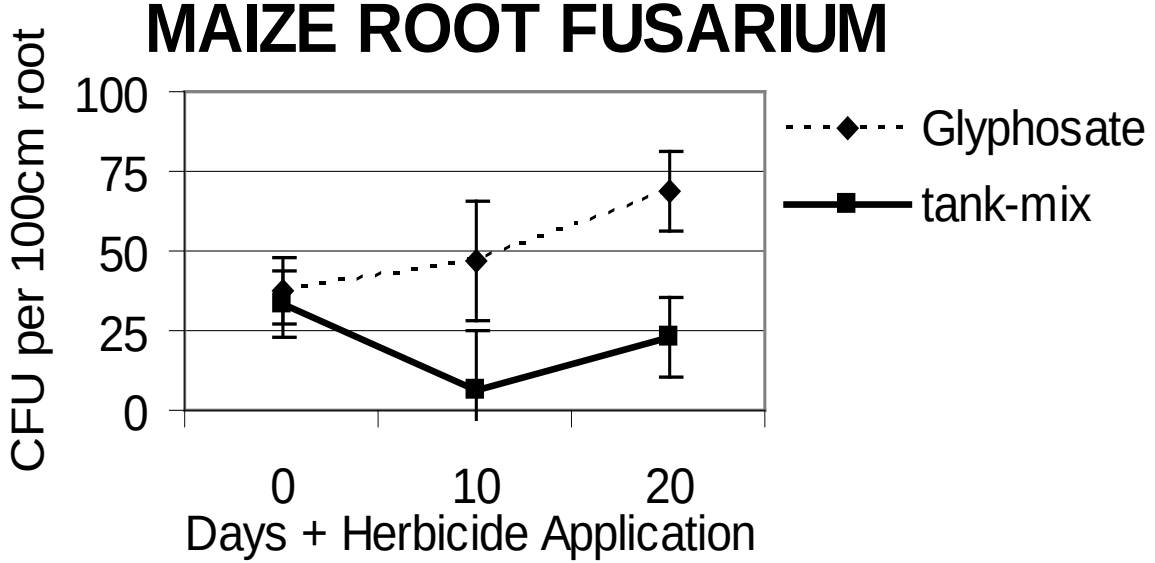
IPNI International Plant Nutrition Institute

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




MAIZE ROOT FUSARIUM
CFU per 100cm root
100
75
50
25
0
0
10
20
Days + Herbicide Application
Glyphosate
tank-mix