Glyphosate induced changes in plant resistance to diseases

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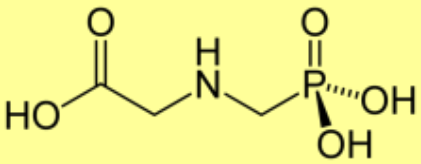
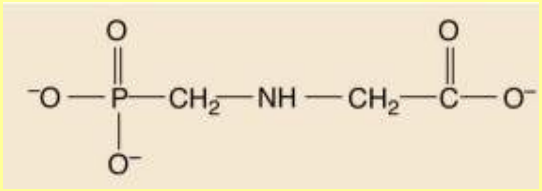
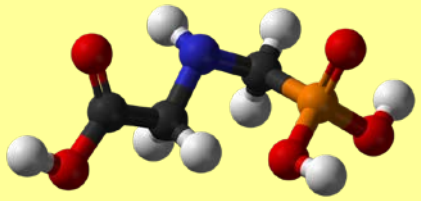






Glyphosate (N-Phosphonomethyl glycine)

Piracicaba, Sept. 21, 2007

The Message:

The effectiveness of glyphosate (Roundup) as a wonderful herbicide derives largely from its ability to compromise plants' ability to defend against pathogens.

The pathogens that are most important in this regard are those that live in the soil.

Two notable among them are:

- **Pythium (an oomycete/water mould) and,**
- **Fusarium (an ascomycete fungus)**

These pathogens are present ubiquitously in agricultural and other soils, thus they contribute significantly to the herbicidal efficacy of glyphosate on plants.

This talk has been divided into three parts:

1. **How was this discovery (about Glyphosate induced susceptibility) made? (M.S. research)**

2. **How does glyphosate make plants susceptible to pathogens? (Ph.D. research)**

3. **Are Roundup-Ready (RR) plants completely safe in this regard?**

Although I have not worked on glyphosate since 1988, I have been actively involved in disease resistance research. In fact, our group was the first to clone a disease resistance gene in plants.

The gene was the maize *Hm1*, which confers protection by inactivating a host-specific toxin produced and absolutely needed by a leaf blight and ear mold pathogen (*Cochliobolus carbonum*) to cause disease.

Johal and Briggs. 1992. **Science**. *258: 985-987.*

Part 1: How was the discovery about glyphosate induced susceptibility made?

• The year was 1982, and I had just landed in Jim Rahe's (pronounces Ray) lab at Simon Fraser University, Vancouver, Canada, to do M.S. in plant pathology.

• After realizing that I had expertise in genetics and strong interest in basic biology, Jim (Rahe) gave me a project on a disease on beans (*Phaseolus vulgaris*).

The bean anthracnose pathosystem

This was one of a few projects in his lab and our challenge was to figure out what causes bean plants to be resistant to *Colletotrichum lindemuthianum*, the pathogen that causes the disease 'bean anthracnose'.

Susceptible anthracnose lesions

A little bit about the pathogen, which is a fungus:

- **It is a true hemi-biotroph**
- **It belongs to the class Ascomycetes**
- **It can infect stems, leaf petioles and pods**
- **It causes brown oval lesions, a bit sunken, hence the name anthracnose**

This is what this disease looks like at least when hypocotyls are inoculated with the pathogen.

Bean anthracnose disease or pathosystem

However, if the interaction between the host (beans) and the pathogen (anthracnose fungus) is incompatible, it leads to a resistant reaction in the host, which appears as shown below:

Drop inoculation with fungal spores

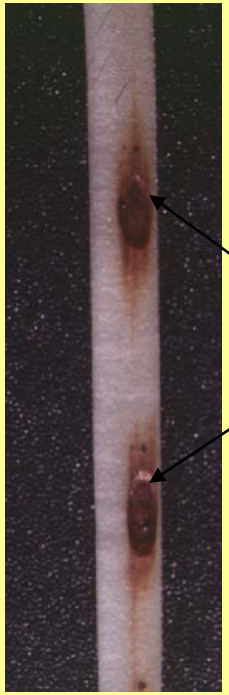
Spray inoculation with fungal spores

Bean anthracnose

- **Rahe had shown from previous work (in Joe Kuc' lab at Purdue and subsequent research at SFU) that *phytoalexins* were associated with fungal containment in both of these interaction types.**
- **These *phytoalexins* appeared responsible for the containment of the pathogen in both compatible and incompatible interactions.**

Drop inoculation with fungal spores

Spray inoculation with fungal spores



Susceptible anthracnose lesions

For those who may not know about phytoalexins

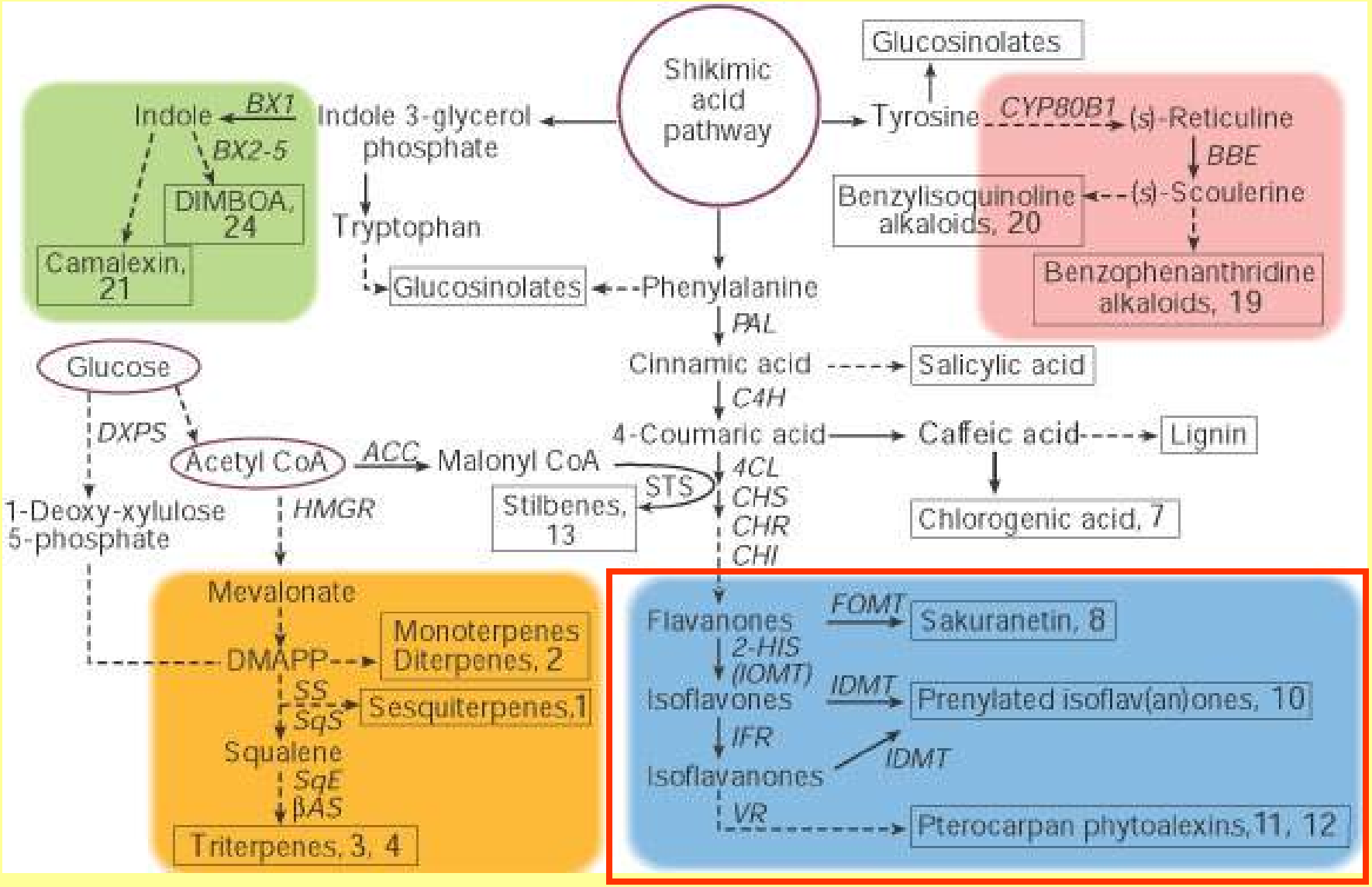
Phytoalexins are low-molecular weight, anti-microbial compounds that are both synthesized by and accumulate in plants after exposure to micro-organisms

• More than 300 chemicals with phytoalexin-like properties have been isolated from plants belonging to more than 30 families.

• They are mostly derived from the phenylpropanoid pathway and have a phenolic backbone.

- for example, in most legumes phytoalexins are isoflavonoids.

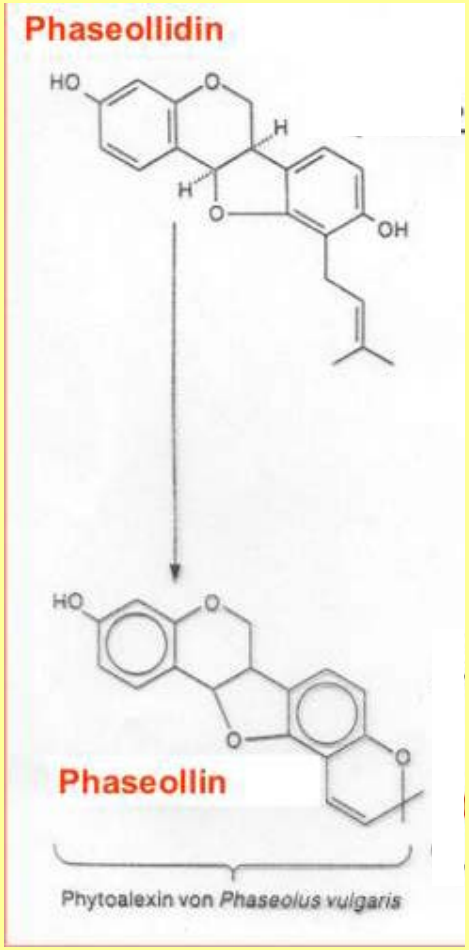
The general pathway leading to isoflavonoids in plants

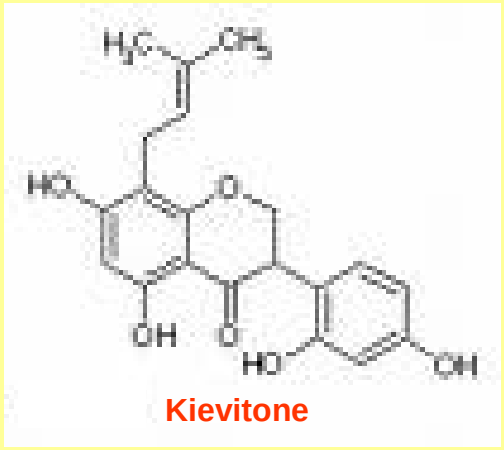


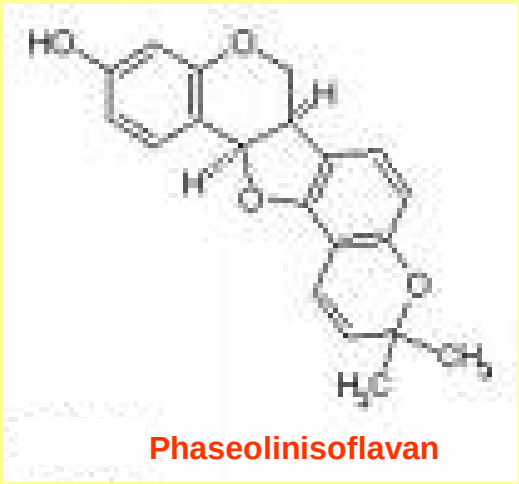
BBE, berberine bridge enzyme; PAL, L-phenylalanine ammonia-lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumarate CoA ligase; CHS, chalcone synthase; CHR, chalcone (polyketide) reductase; CHI, chalcone isomerase; STS, stilbene synthase; FOMT, flavanone 7-*O*-methyltransferase; 2-HIS, 2-hydroxyisoflavanone synthase ('isoflavone synthase'); IOMT, isoflavone 4'-*O*-methyltransferase; IDMT, isoflavone or isoflavanone dimethylallyl transferase; IFR, isoflavone reductase; VR, vestitone reductase; DXPS, 1-deoxy-xylulose 5-phosphate synthase; ACC, acetyl CoA carboxylase; HMGR, 3-hydroxy-3-methylglutaryl CoA reductase; SS, sesquiterpene synthase; SqS, squalene synthase; SqE, squalene epoxidase; AS, -amyrin synthase.

Bean plants (*Phaseolus vulgaris*) produce four different phytoalexins during interaction with the bean anthracnose fungus

Again, all isoflavonoids







Bean anthracnose disease system

As I mentioned earlier, Jim was almost certain that these phytoalexins were the reason why the pathogen was contained at individual infection sites.

But the question was: were these phytoalexins causally involved in providing protection against the fungus?

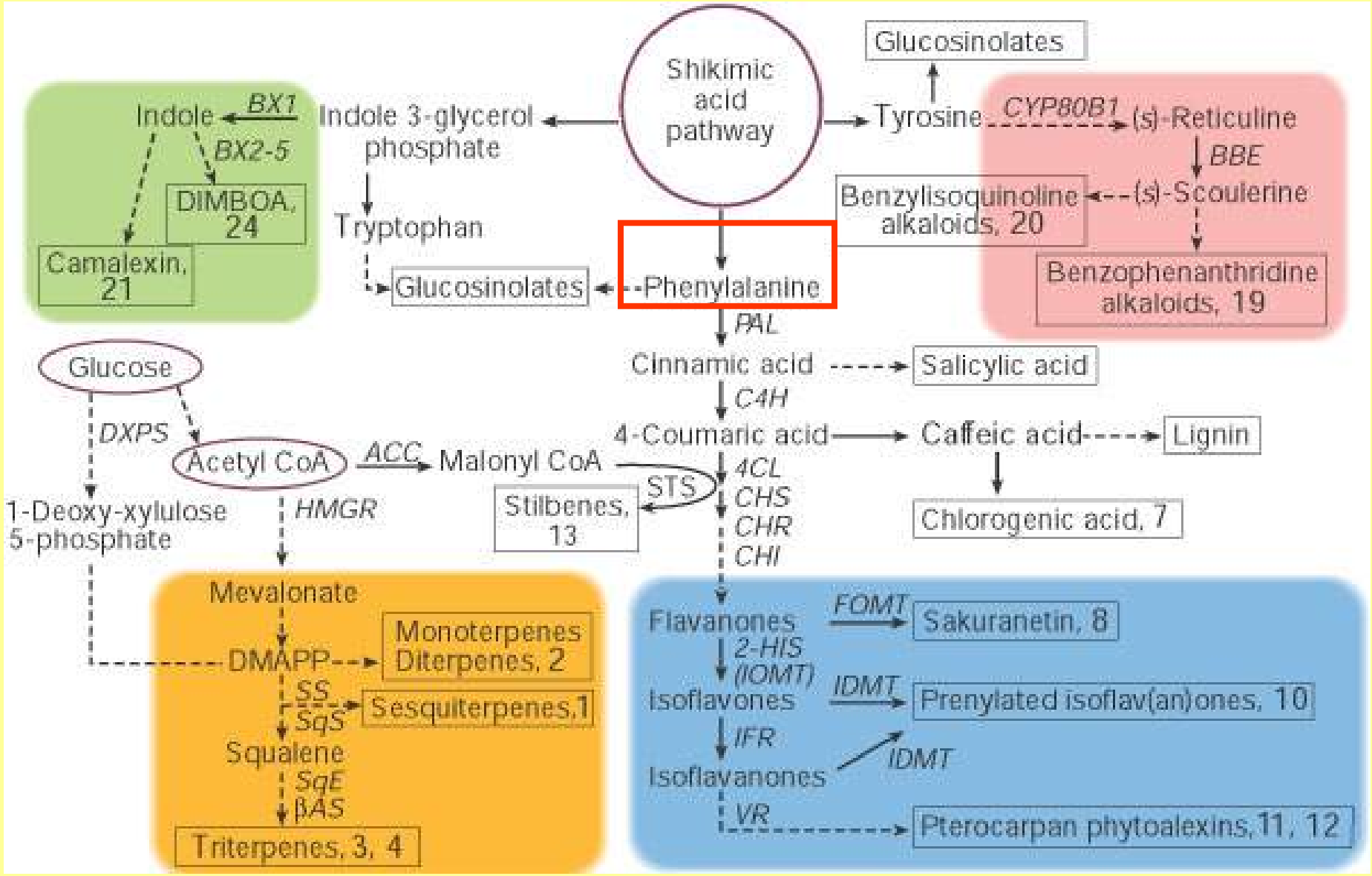
He asked me to think of ways and approaches to provide a definitive answer.

Bean anthracnose disease system

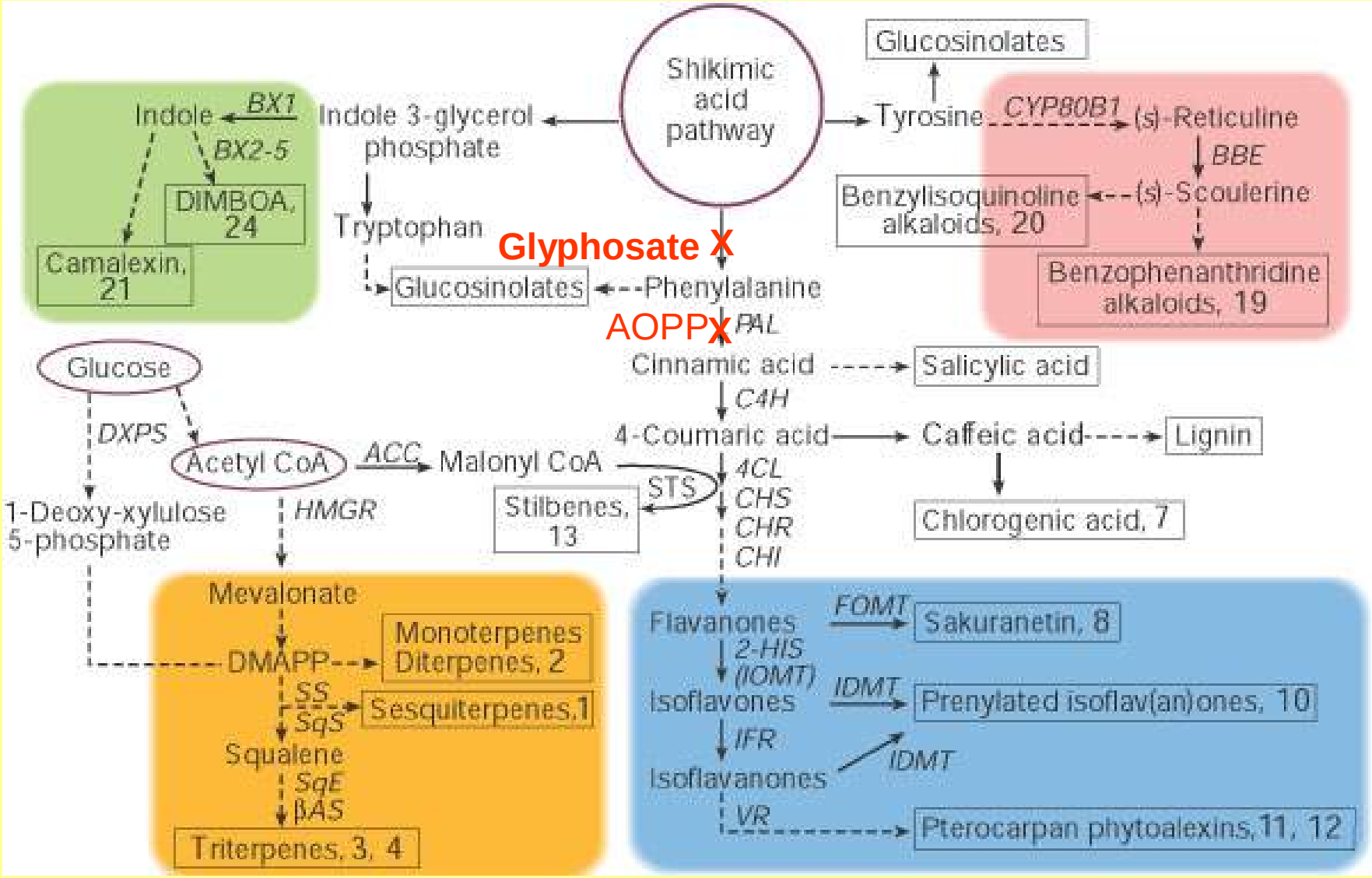
- **Having a training in genetics, my first idea was to use mutants defective in these phytoalexins to prove their role in disease resistance – however it was not - and still not - possible to do that kind of genetics with *Phaseolus vulgaris*.**

- **An alternative was to use a chemical approach – i.e., use some chemical inhibitors to block the pathway leading to phytoalexins.**

Target phenylalanine, the gateway to secondary metabolism



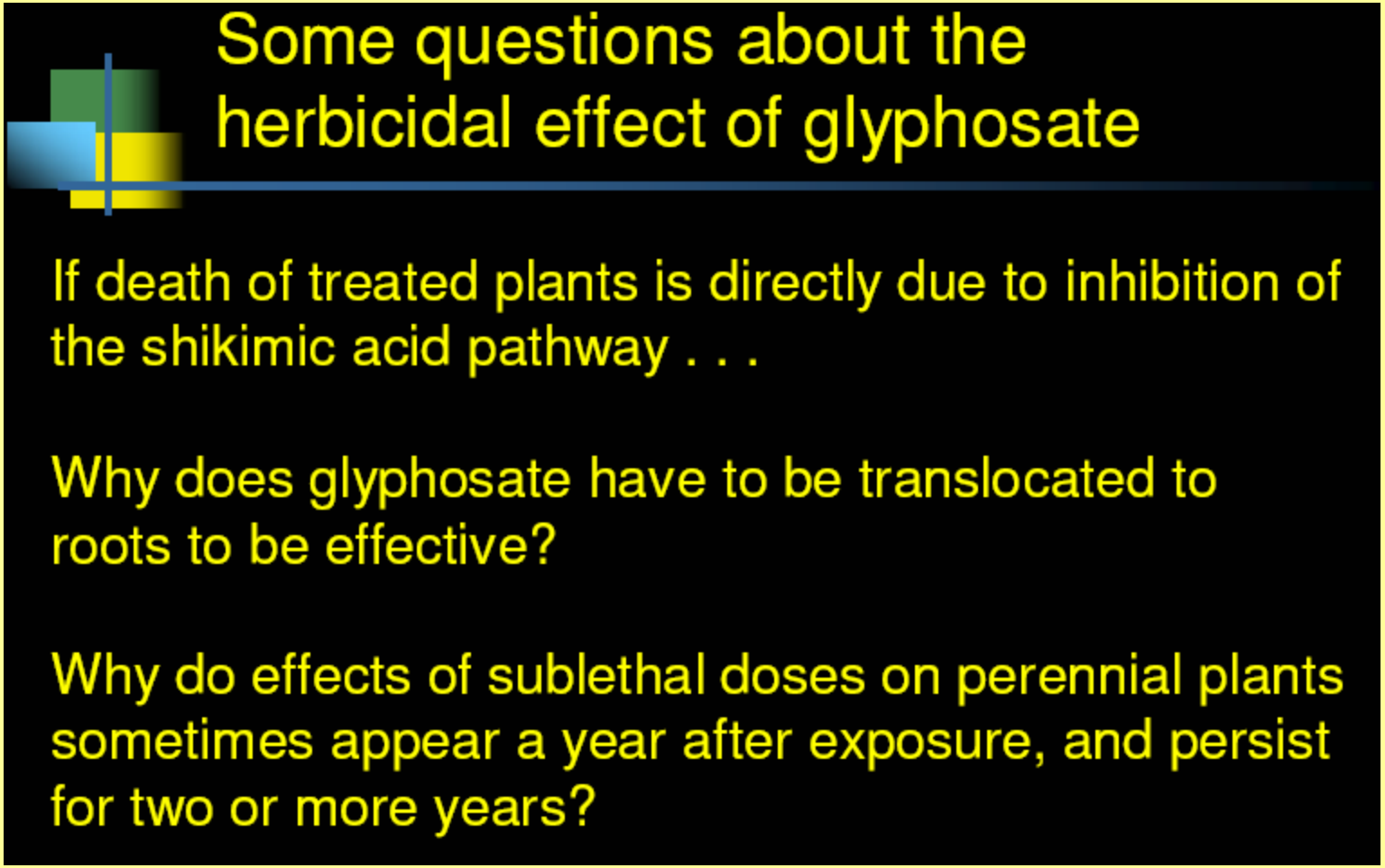
The general pathway leading to isoflavonoids in plants



Found two compounds in the literature that could block the production of isoflavonoids

We right away selected glyphosate, because as an apple orchard- and vegetable-grower, Rahe had experience working with this herbicide.

In fact, he was intrigued by it because of the following reasons:



Dose response tests:

So the first experiment to be done was to identify a dose of Roundup (the only commercial formulation of glyphosate at that time) that would be just below its lethal dose

Beans were grown in vermiculite, which is a soil-less synthetic medium that was routinely used in Rahe's lab for ease of handling and for its largely sterile nature (free of pathogens).

However, none of my plants died no matter how much Roundup I put on them. One of my dose was 10x higher than the recommended dose for broad leaf dicots.

Roundup probably had gone bad – was our first suspicion

So we repeated the experiment with a new batch of Roundup.

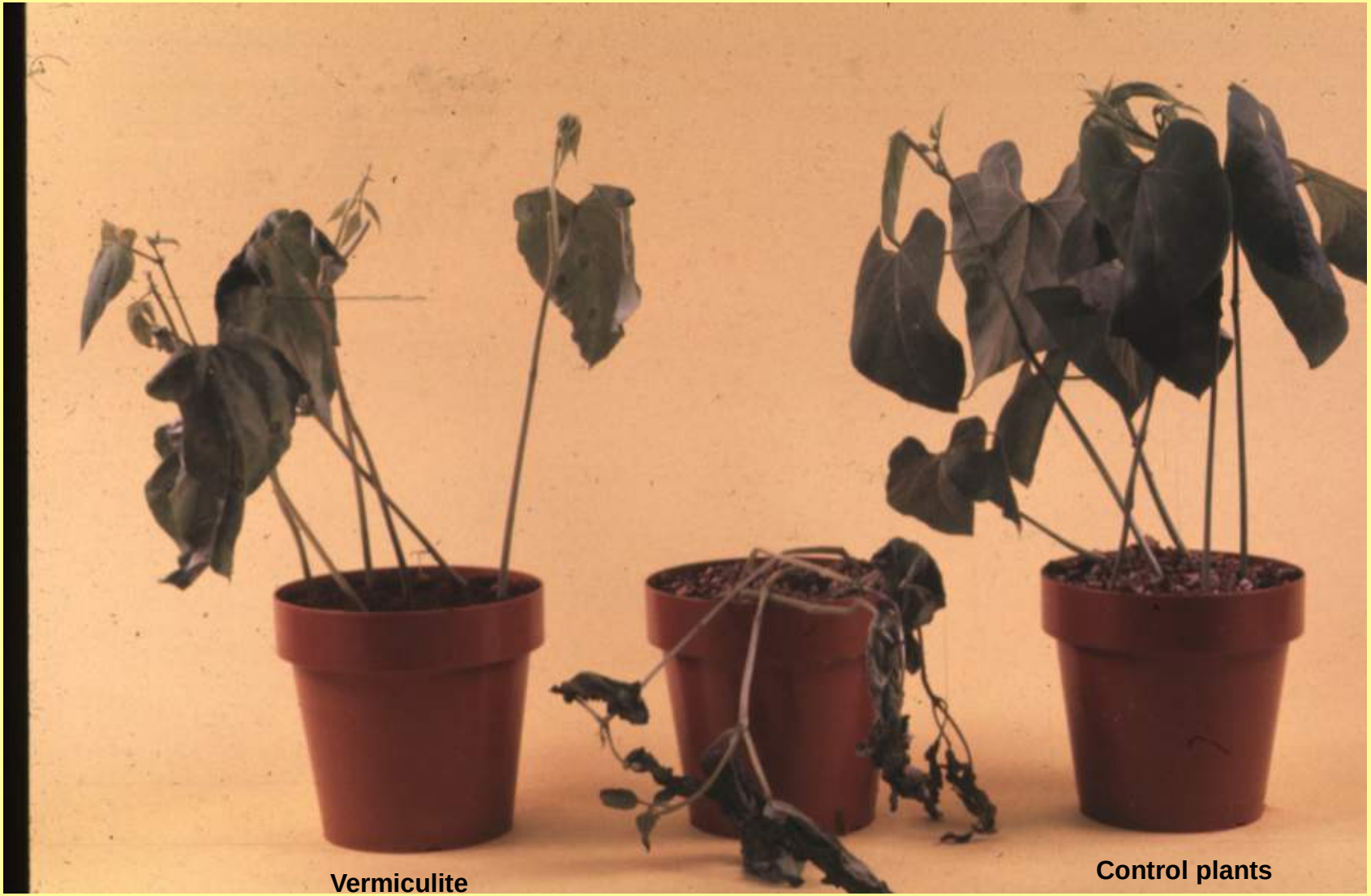
But same results again.

None of the plants died, although their growth was inhibited.

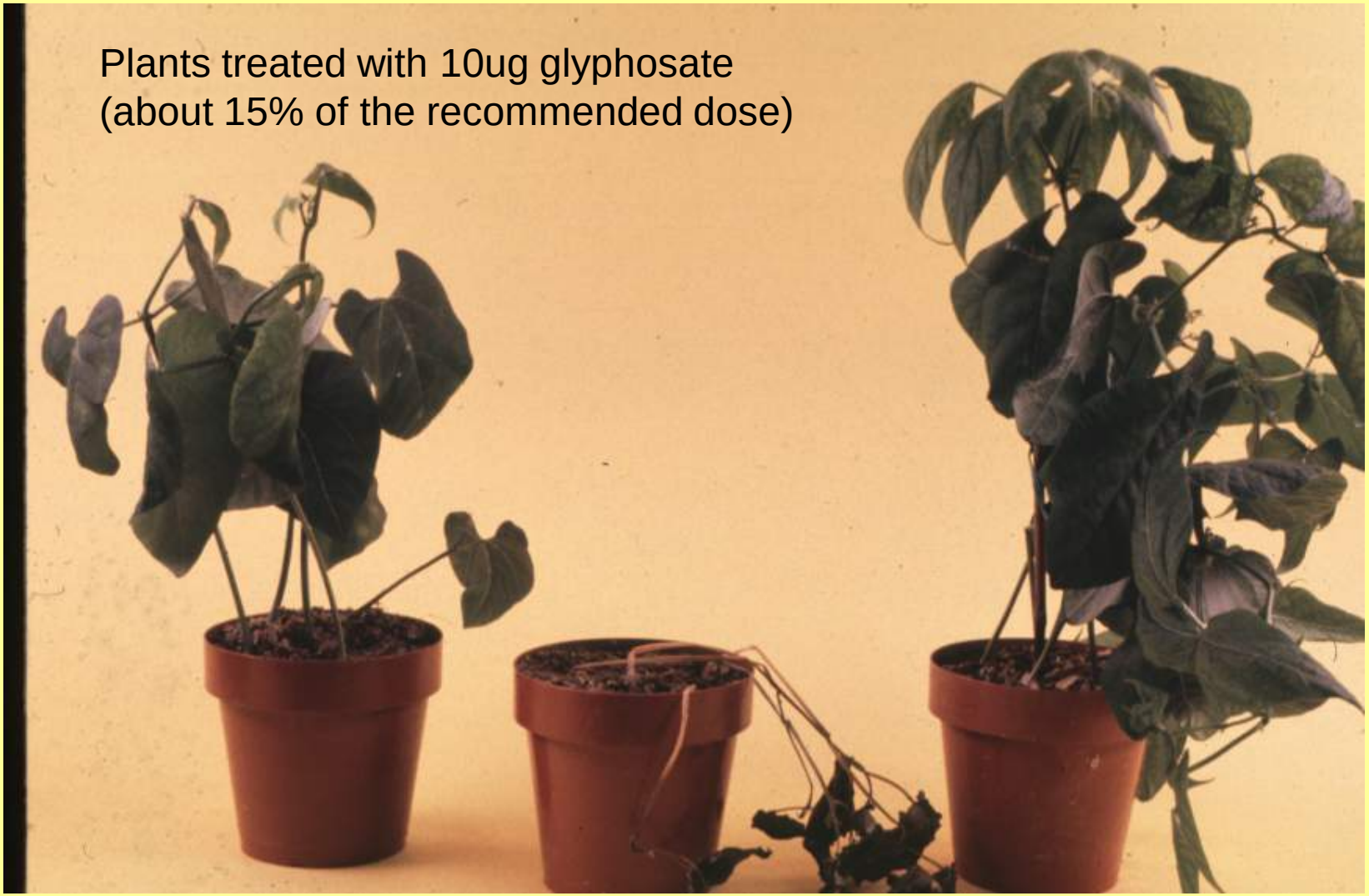
Right away Rahe knew there was something about the growth medium (vermiculite) that abolished the lethal bite of Roundup.

So in the next experiment, bean plants were grown side-by-side in vermiculite and field soil (unammended) and treated with Roundup.

Eureka!!!



Plants growing in the field soil died, but those growing in vermiculite did not.



At the 10ug per plant dose, plants growing in vermiculite not only survived, they started recovering (from growth inhibition) after a few weeks and grew normally to produce viable seed.

They eventually appeared more bushier and vigorous than the control plants.

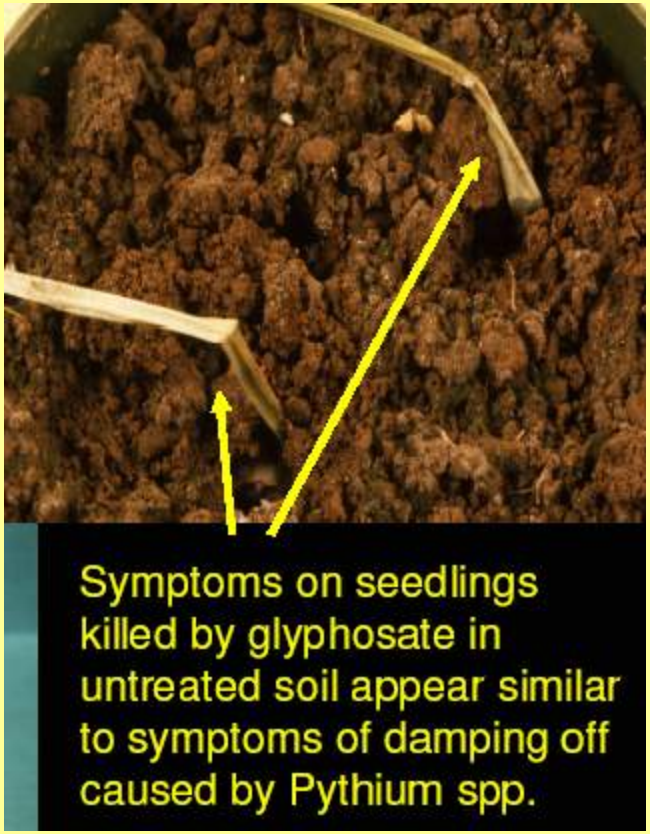
So what kills glyphosate treated plants in natural soils?

% Mortality in Bean Seedlings Grown in Autoclaved Soil Amended with Extracts from Untreated Soil

Extract:	Raw	Filtered	Autoclaved
Glyphosate	100	0	0
Control	0	0	0

So the agent(s) that killed our plants was of particulate nature (could be be filtered out by a 0.2 uM sieve) and was heat sensitive.

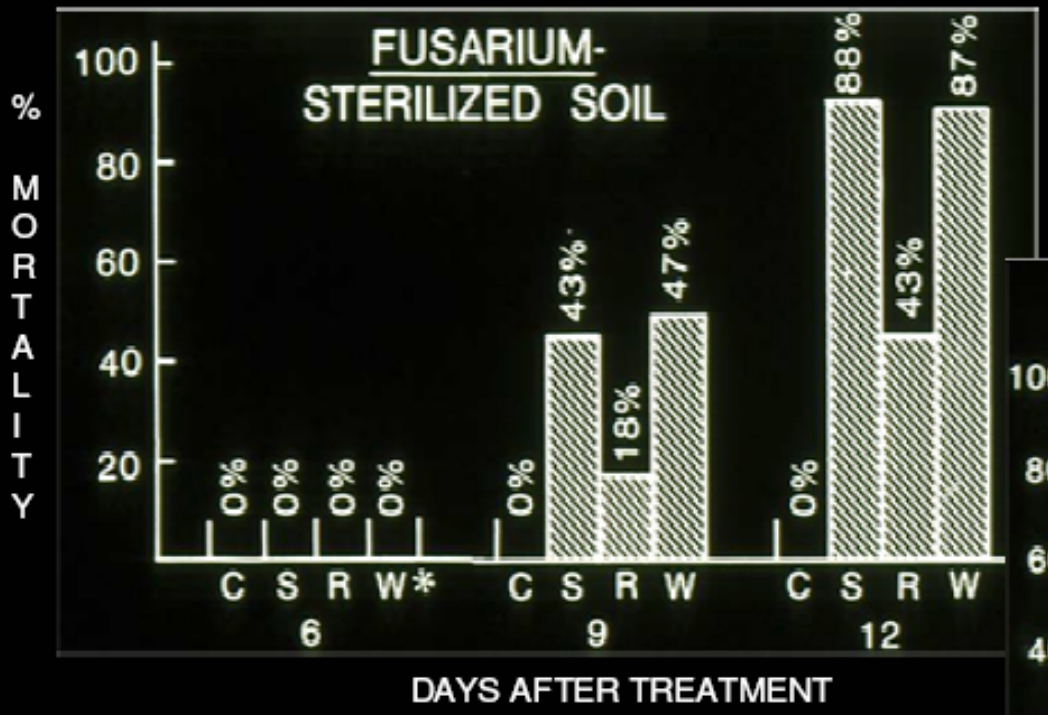
So what kills glyphosate treated plants in natural soils?

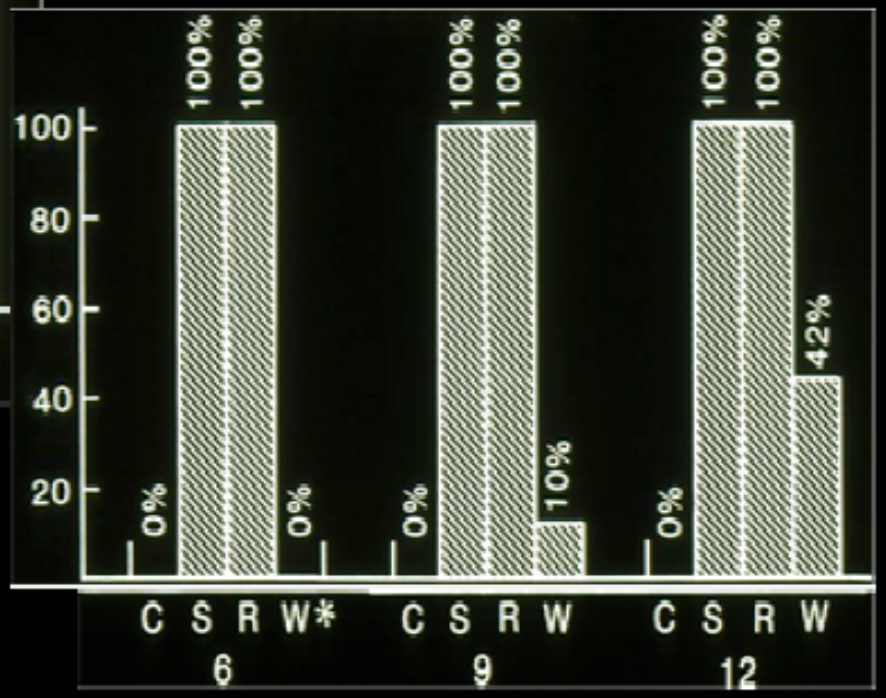


We were able to isolate two different pathogens from the roots of dead plants. These were:

Pythium and *Fusarium*

Fusarium and Pythium restore herbicidal activity of glyphosate to bean seedlings growing in sterilized soil





* FUNGUS APPLIED BY
C = CONTROL
S = SEED TREATMENT
R = ROOT DIP
W = WOUNDING

Effect of Ridomil on % Mortality on Bean Seedlings Growing in Different Media, 12 Days After Treatment with Glyphosate

To summarize:

- Herbicidal activity is reduced in sterilized soils, and in soilless plant growth media
- Pythium and Fusarium spp. rapidly colonize roots of glyphosate treated plants growing in non sterile soil
- Adding Pythium or Fusarium restores herbicidal activity of glyphosate to seedlings growing in sterilized soil or soilless media
- Metalaxyl blocks restoration of activity when Pythium is used to amend sterilized soil

These results clearly demonstrate that the herbicidal efficacy of glyphosate is largely due to the action of pathogens like *Pythium* and *Fusarium* which are present in most soils

Johal, G. S. and J. E. Rahe. (1984). Effect of soilborne plant pathogenic fungi on the herbicidal action of glyphosate on bean seedlings. *Phytopathology* 74: 950-955.

Andre Levesque, another graduate student in the lab, extended these studies to the field.

He demonstrated that:

***Pythium* – an oomycete or water mould (not considered a fungus anymore) – was the main culprit if the soil was heavy and wet.**

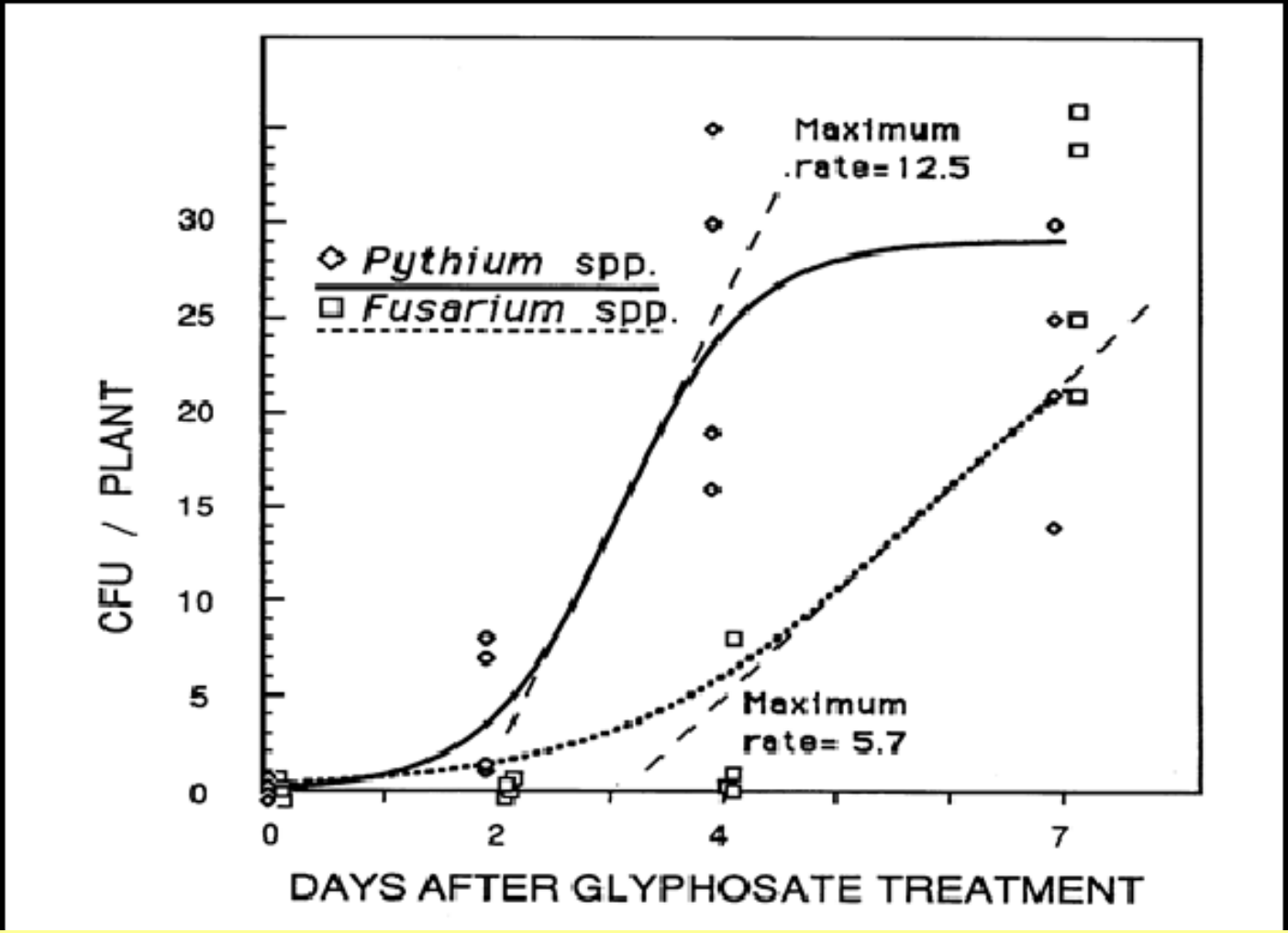
It was *Fusarium* – a soil-borne fungal pathogen - if the soil was light (sandy loam e.g.) and dry.

Andre' also devised a whole root plating technique to show that these soil borne pathogens start colonizing the roots of glyphosate treated plants within 2-3 days after treatment.

Wheat seedling roots

Levesque et al. 1987. Can. J. Micribiology. 33: 354-360.

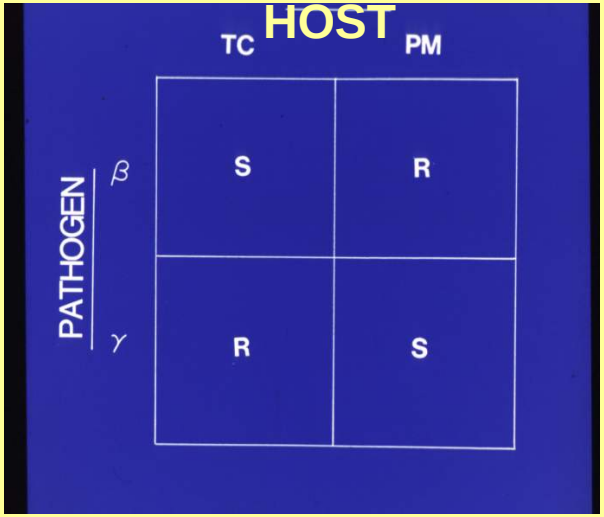
Rates of Colonization of Roots of Bean Seedlings Growing In Untreated Soil by Pythium and Fusarium Following Foliar Treatment With Glyphosate

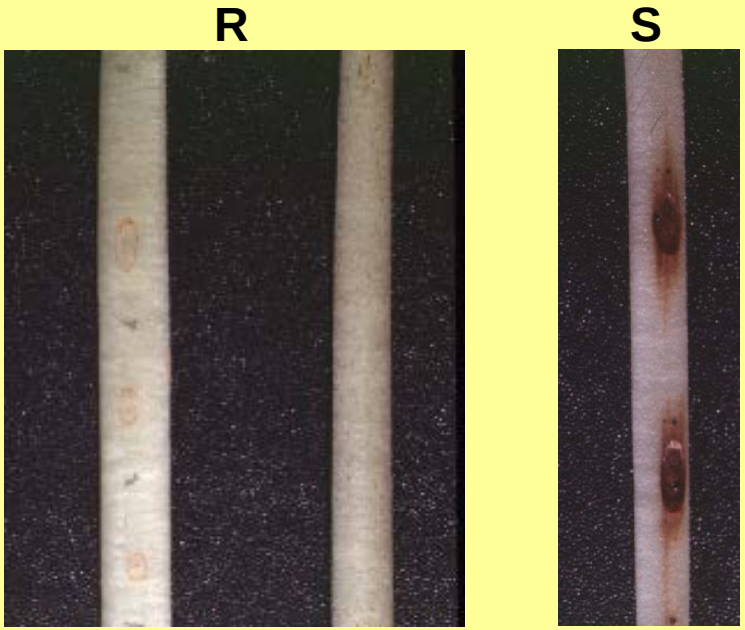


II. How does glyphosate make plants susceptible to pathogens?

After this little distraction, I went back to the bean anthracnose system to address this question.

In the BA pathosystem, the host and the pathogen follow a gene-for-gene relationship to determine the outcome of the interaction, which is either compatible (susceptible reaction) or incompatible (resistant reaction)





Bean anthracnose (*Phaseolus vulgaris-Colletotrichum lindemuthianum)*

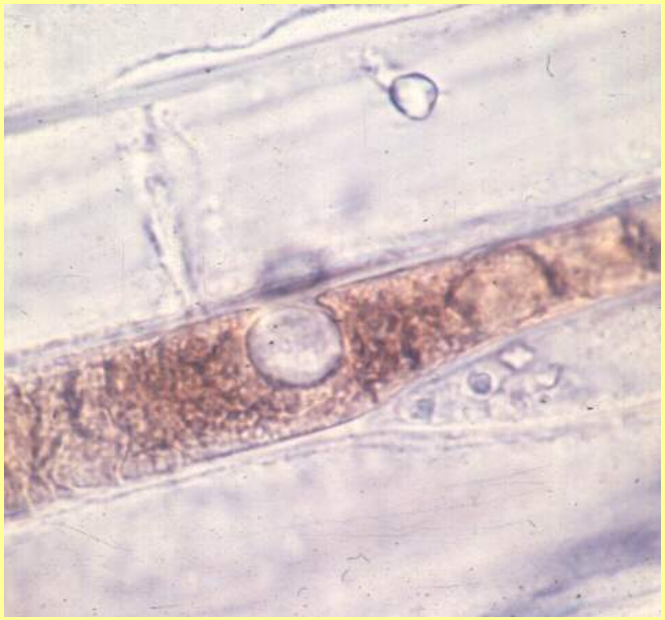
To take a look at how these interactions develop;

Fungal spores (conidia) germinate to produce appressoria by 24-36 h after inoculation (hai).

And the infection process is similar in both compatible and incompatible interactions until direct penetration of the host around 48 hai.

In an incompatible interaction

The very first cell that the pathogen penetrates, commits suicide. This reaction is called a hypersensitive reaction (HR). It is triggered by recognition of a pathogen avirulence/effector gene by a corresponding disease resistance gene (termed an R receptor).

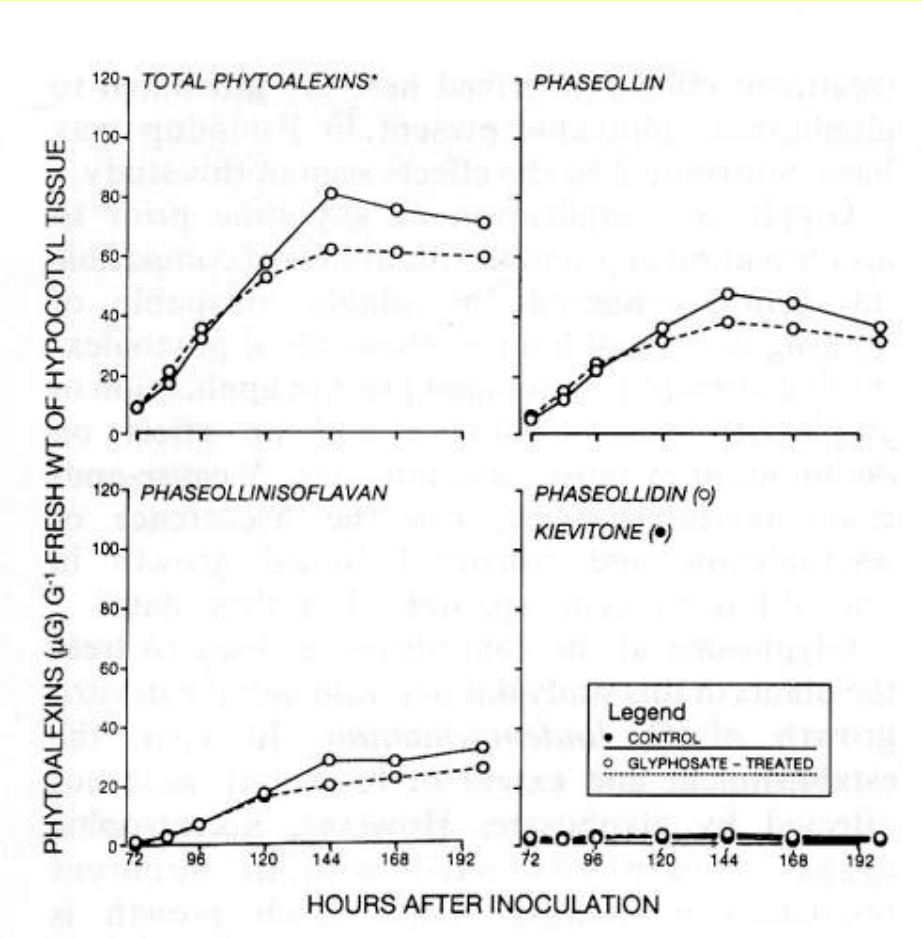


Over 8-10 hours, the HR cell turns brown, and the pathogen stays trapped in it.

Resistant or incompatible interaction

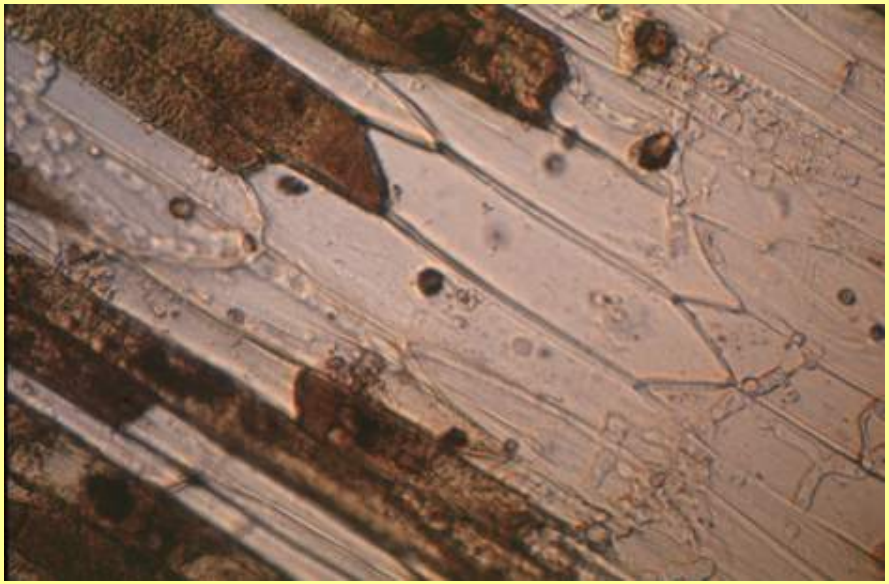
Roundup does not suppress the HR reaction, and the fungus stays contained inside the HR cells.

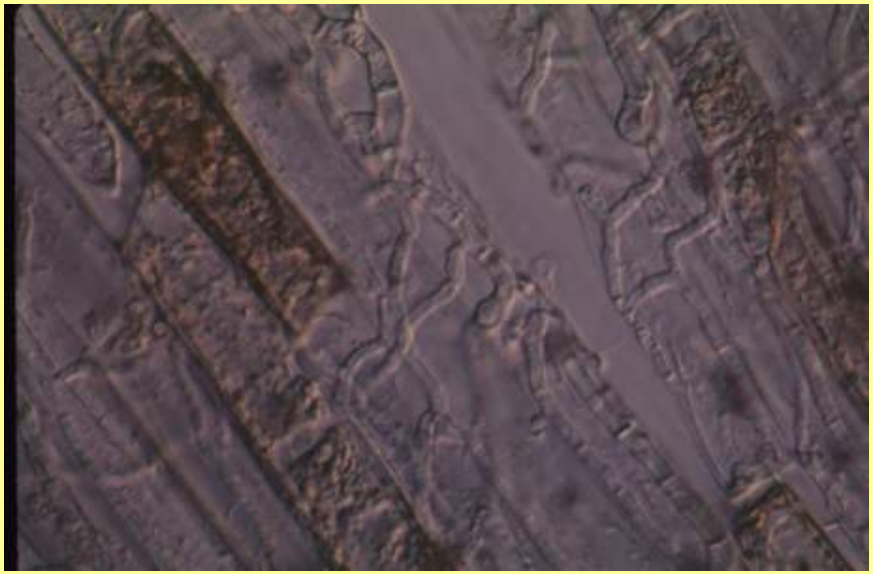
BA - Incompatible interaction



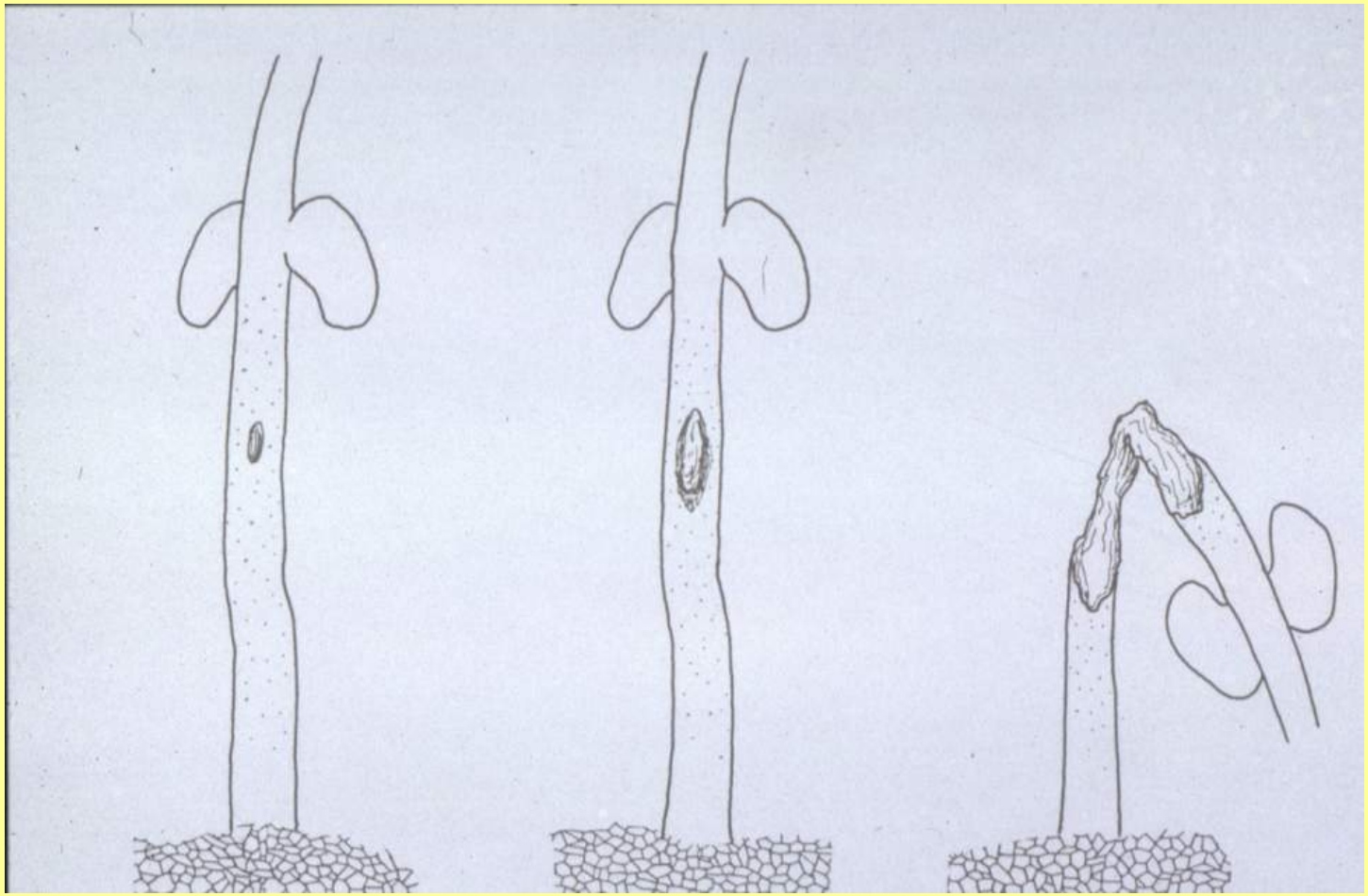
The total amount of phytoalexins produced are slightly lower in glyphosate treated vs. untreated plants.

However, once in a while –especially near the glyphosate treatment site – the fungus escapes an HR cell and starts growing in adjacent cells.





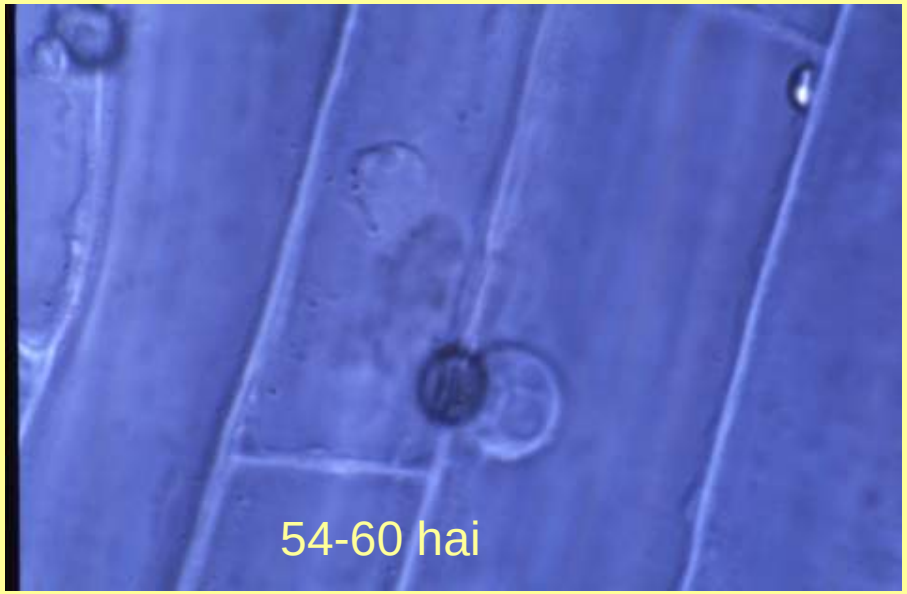
This results in a spreading lesion that continues to grow and engulfs much of the stem tissue, causing it to collapse.

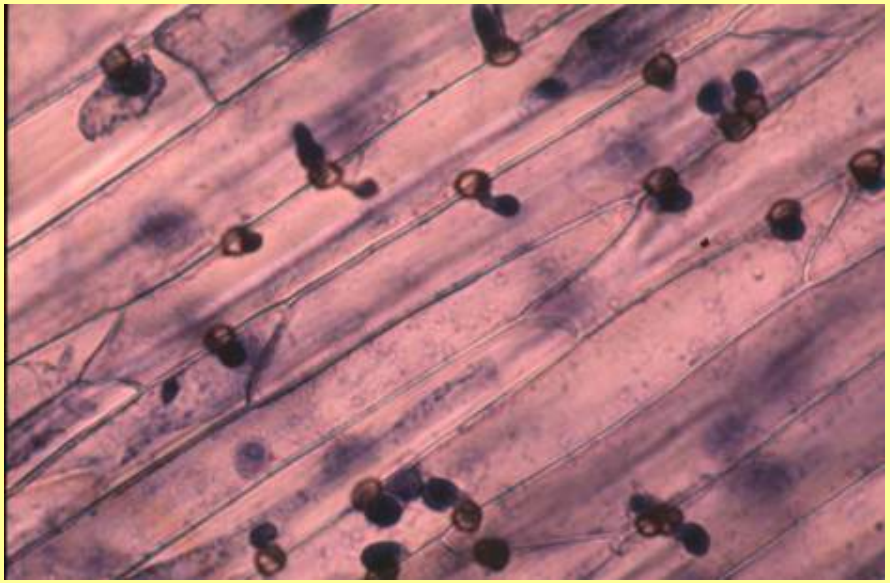


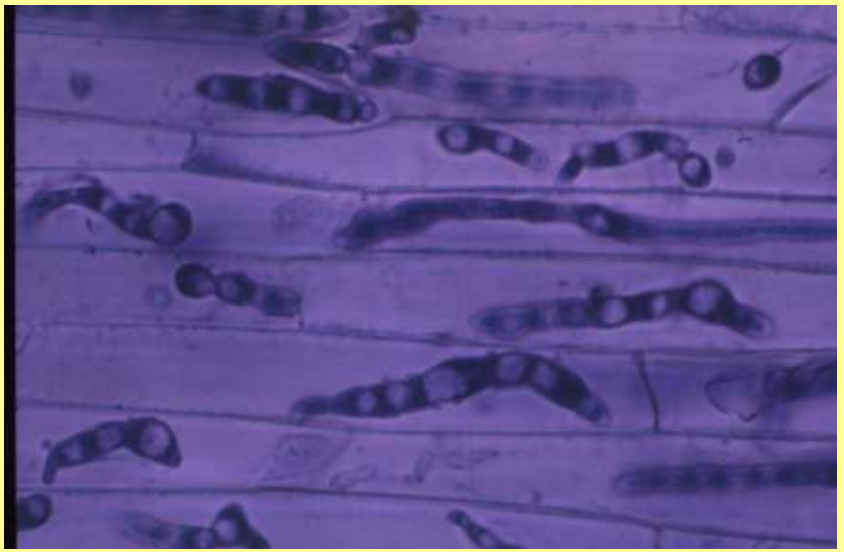
No phytoalexins are produced at these spreading lesion sites

Johal, G. S. and J. E. Rahe. (1990). *Physiological and Molecular Plant Pathology* 32: 267-281.

Infection process during the compatible interaction

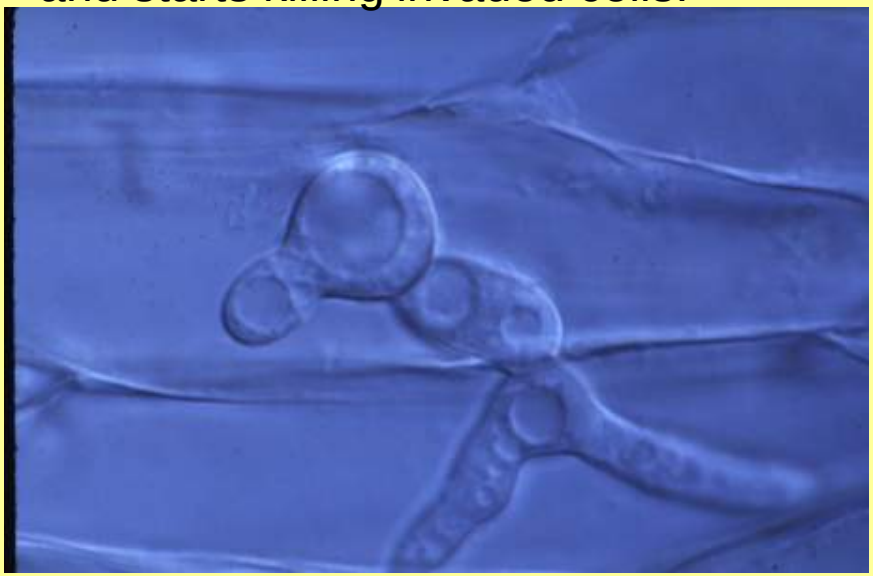


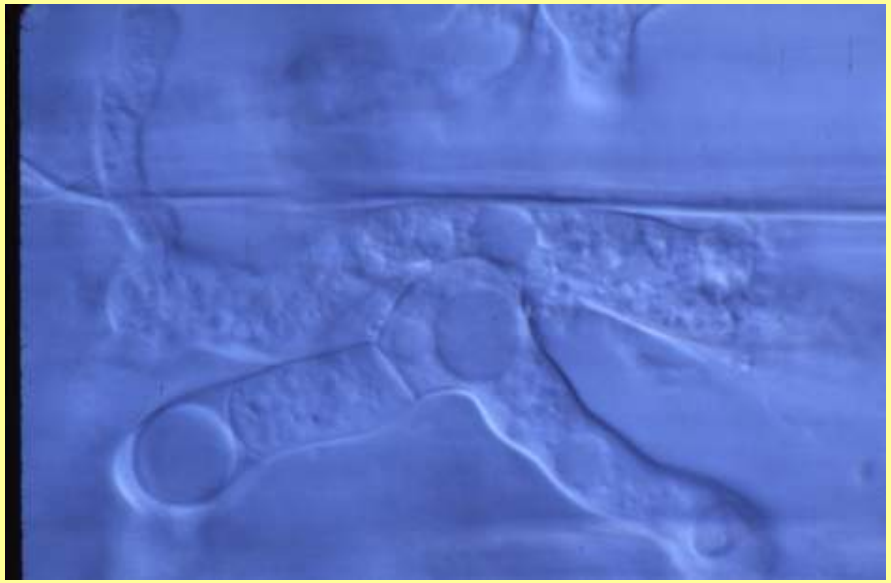


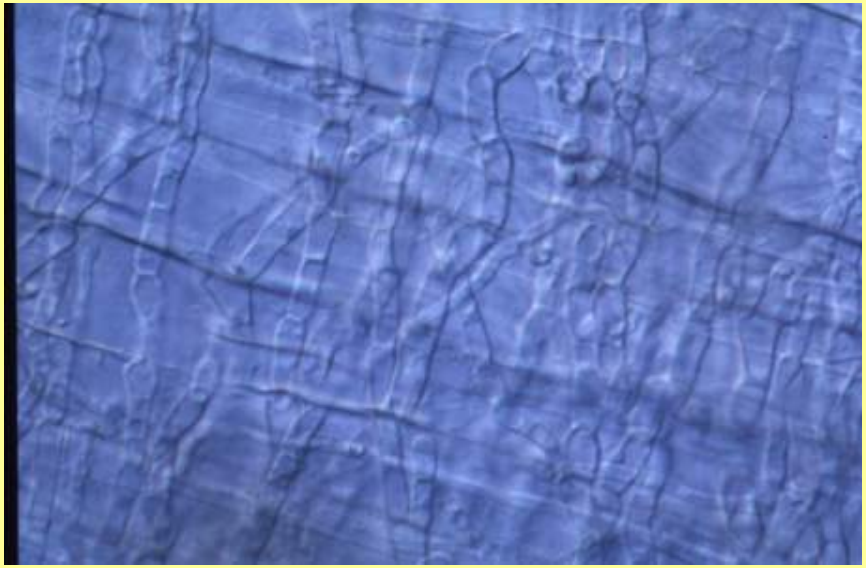


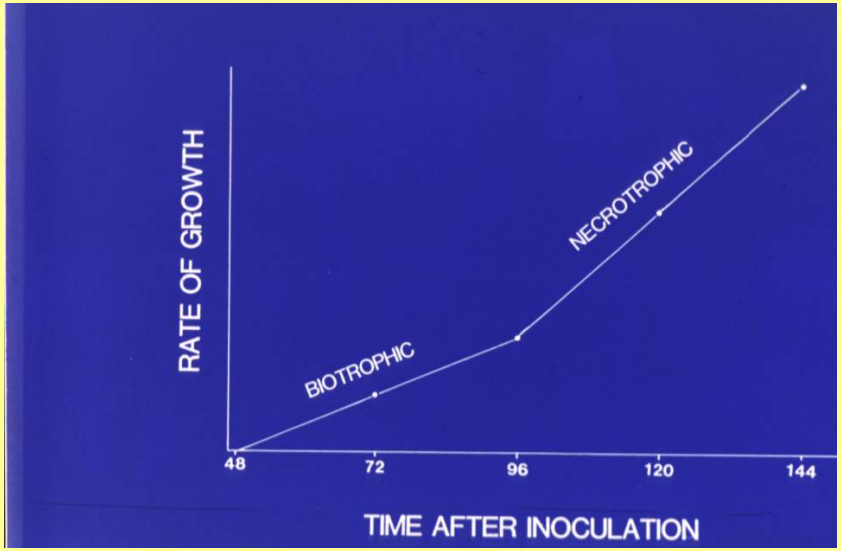
The fungus starts growing inside the bean epidermal cells largely unnoticed

Initially the fungal mode of colonization is biotrophic (does not kill invaded host cells), and the fungus moves from epidermal cells to underlying cortical cells. This continues till 90-96 hai. After that the fungus transforms into a necrotroph and starts killing invaded cells.

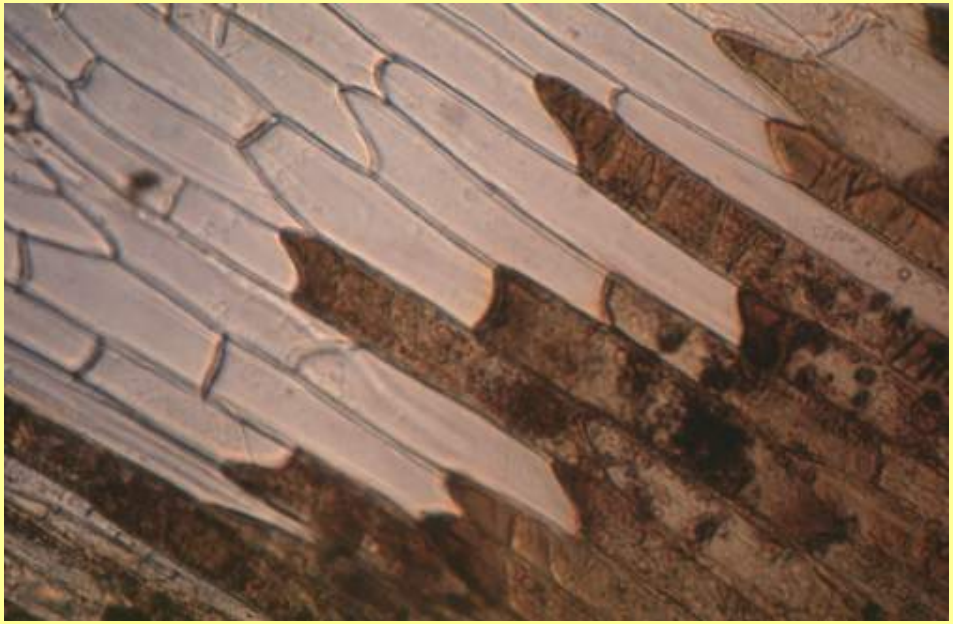






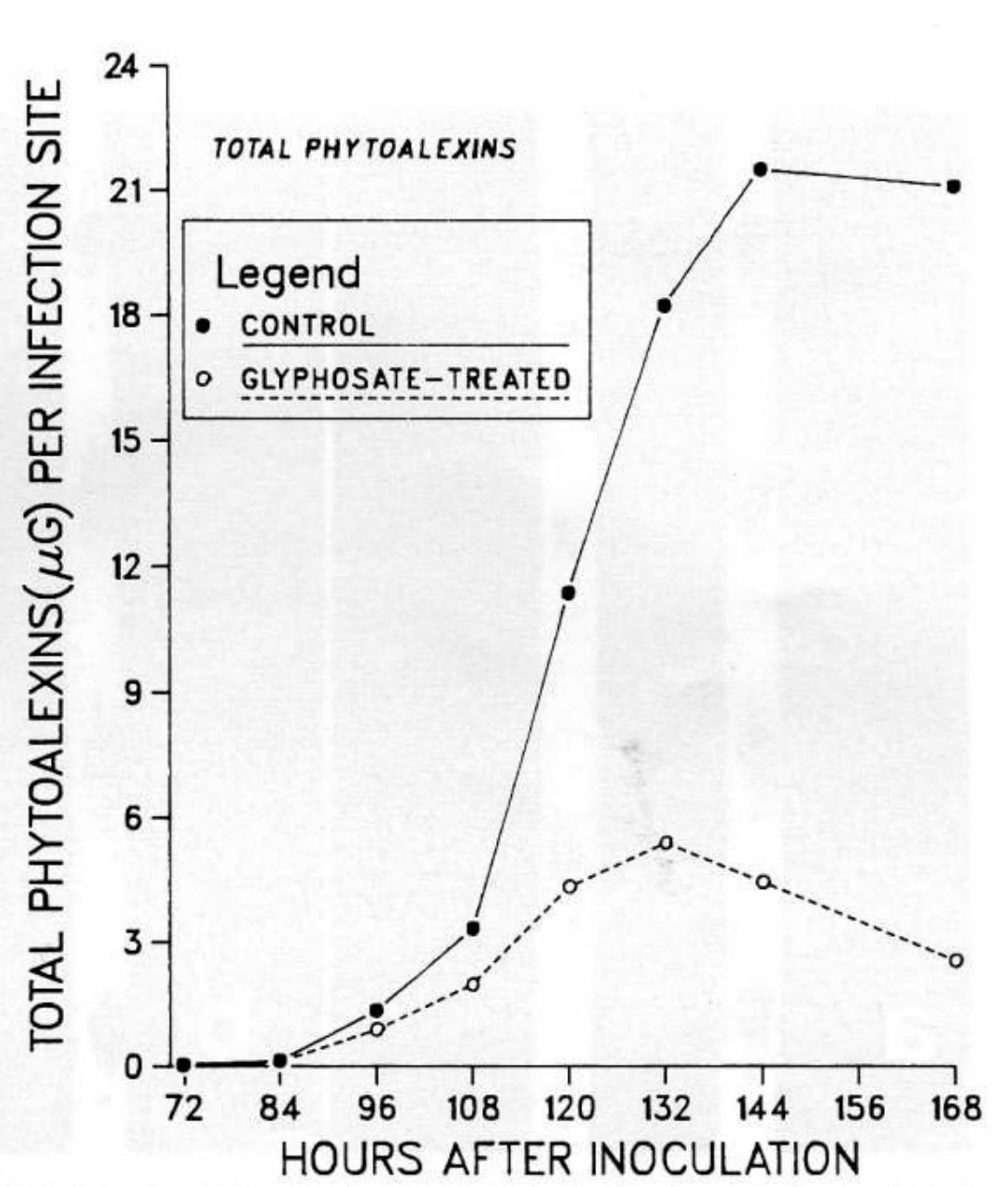


The infected tissue collapses and becomes dark brown to form typical anthracnose lesions

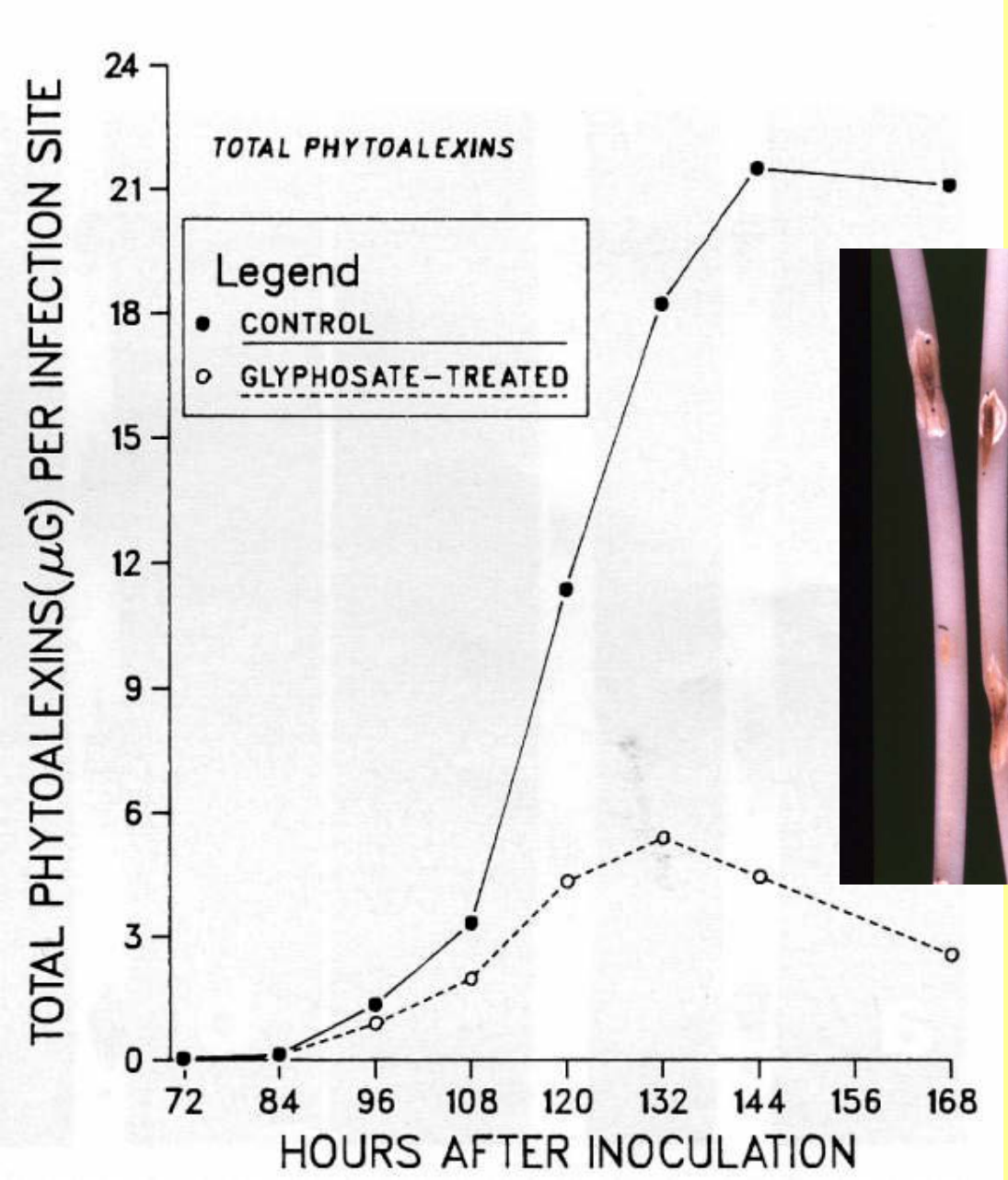


Cells at the boundary of anthracnose lesions

Phytoalexins that are produced at lesion sites are markedly suppressed by glyphosate



Roundup effectively suppresses phytoalexins and eliminates delimitation of the compatible bean anthracnose lesions



Exogenous application of phenylalanine helps contains bean anthracnose lesions on susceptible plants treated with glyphosate

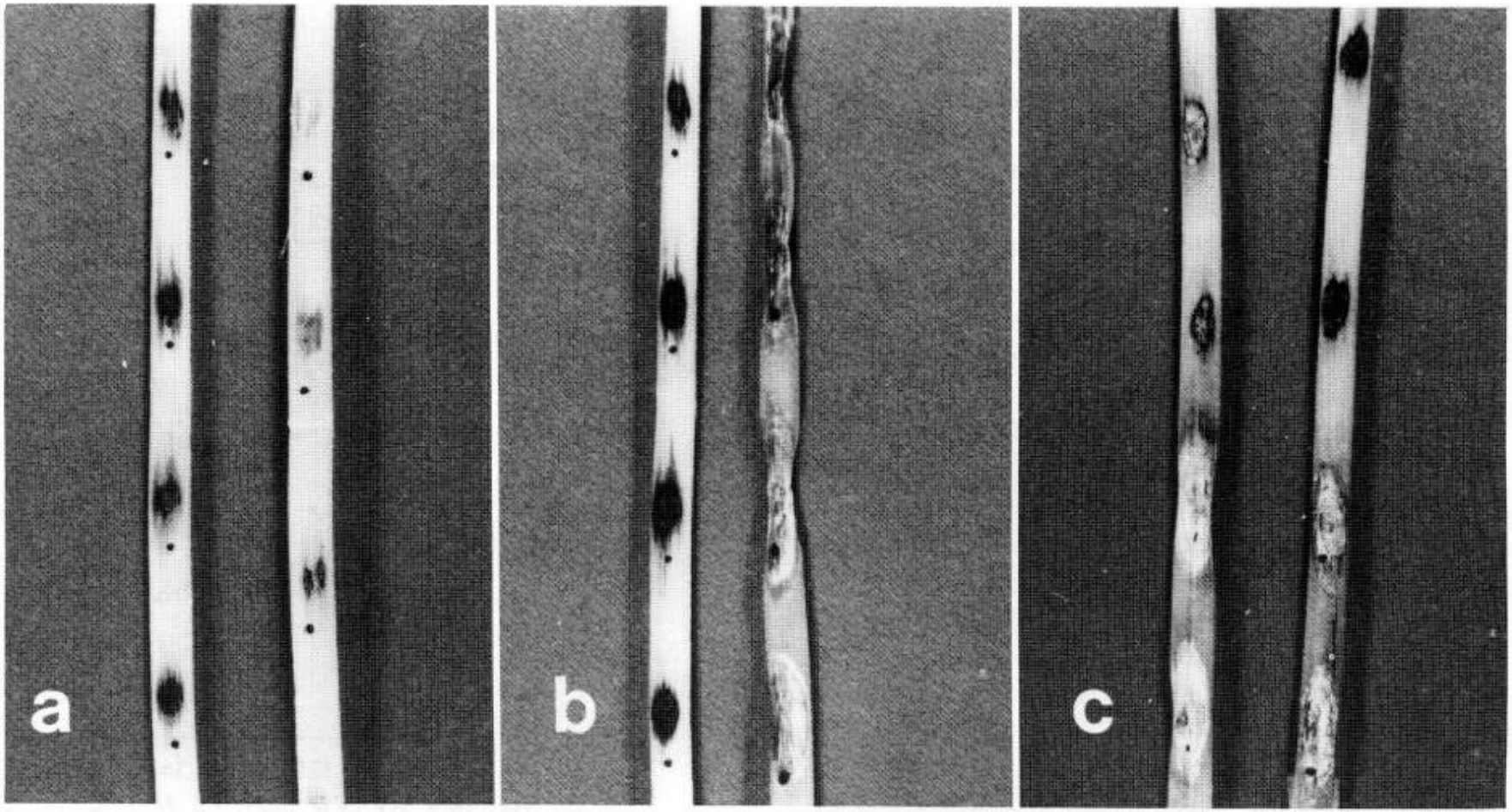


Figure 1. Segments of etiolated bean hypocotyls showing the effects of glyphosate and glyphosate plus phenylalanine on anthracnose lesion development. **a and b)** Typical appearance of lesions on control (left segment) and glyphosate treated (right segment) plants at 120 h (Fig. 1a) and 190 h (Fig. 1b) after inoculation. **c)** Appearance of lesions 144 h after inoculation of glyphosate treated plants that received, at 24 h post-inoculation, topical application of phenylalanine (top two sites on each plant) or water (bottom two sites on each plant) at the sites of inoculation.

However, if treatment with glyphosate is delayed, lesions do not become spreading

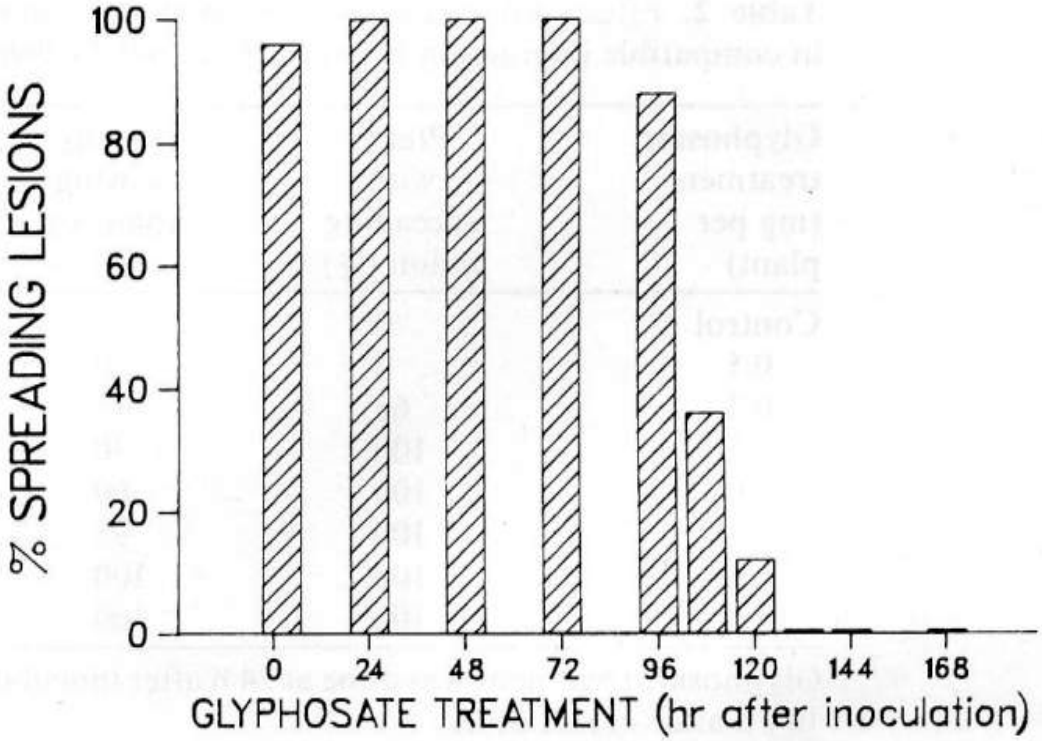
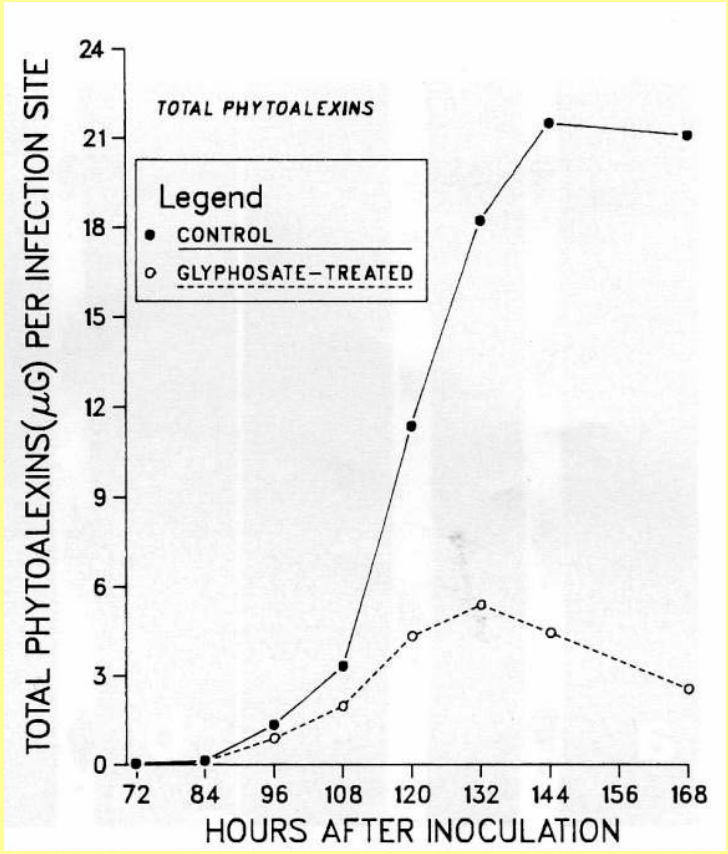


Figure 4. Ability of glyphosate to suppress delimitation of lesions in relation to the time of glyphosate treatment in beans infected with a compatible race of *Colletotrichum lindemuthianum*.



As little as 0.2 ug per plant can increase the severity of BA lesions

Table 2. Effect of different doses of glyphosate on symptom expression and accumulation of phytoalexins in compatible interaction between bean and *Colletotrichum lindemuthianum*

Glyphosate treatment (ug per plant)	Plants with spreading lesion (%)	Lesions showing expansion (%)	Lesion color[a]	Lesion size[a] (mm)	Total phytoalexins[b] (μg per lesion)
Control	0	0	dark brown	5.0	21.35
0.1	0	0	dark brown	5.0	NE[d]
0.2	60	15	mid brown	7.0	NE
0.5	100	30	light brown	8.5	16.50
1.0	100	60	pale brown	12.0	10.85
2.5	100	95	colorless	> 15[c]	NE
5.0	100	100	colorless	> 15[c]	NE
10.0	100	100	colorless	> 15[c]	4.60

Glyphosate treatment was done at 24 h after inoculation. Symptoms were rated and phytoalexins extracted 164 h after inoculation.

[a]For those lesions which showed expansion in response to glyphosate treatment.

[b]Sum of phaseollin, phaseollinisoflavan, phaseollidin, and kievitone.

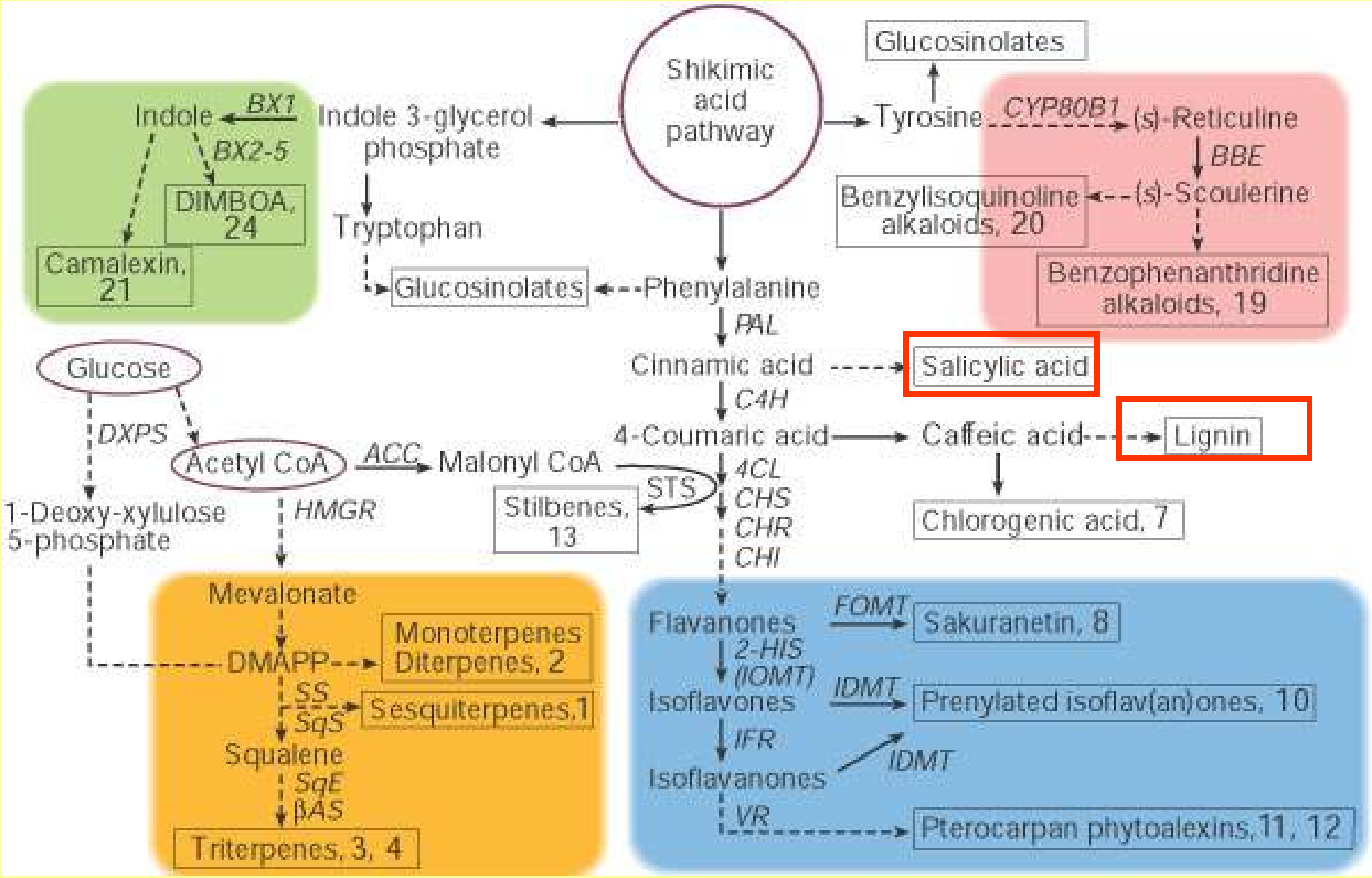
[c]Coalescing lesions

[d]NE = not estimated

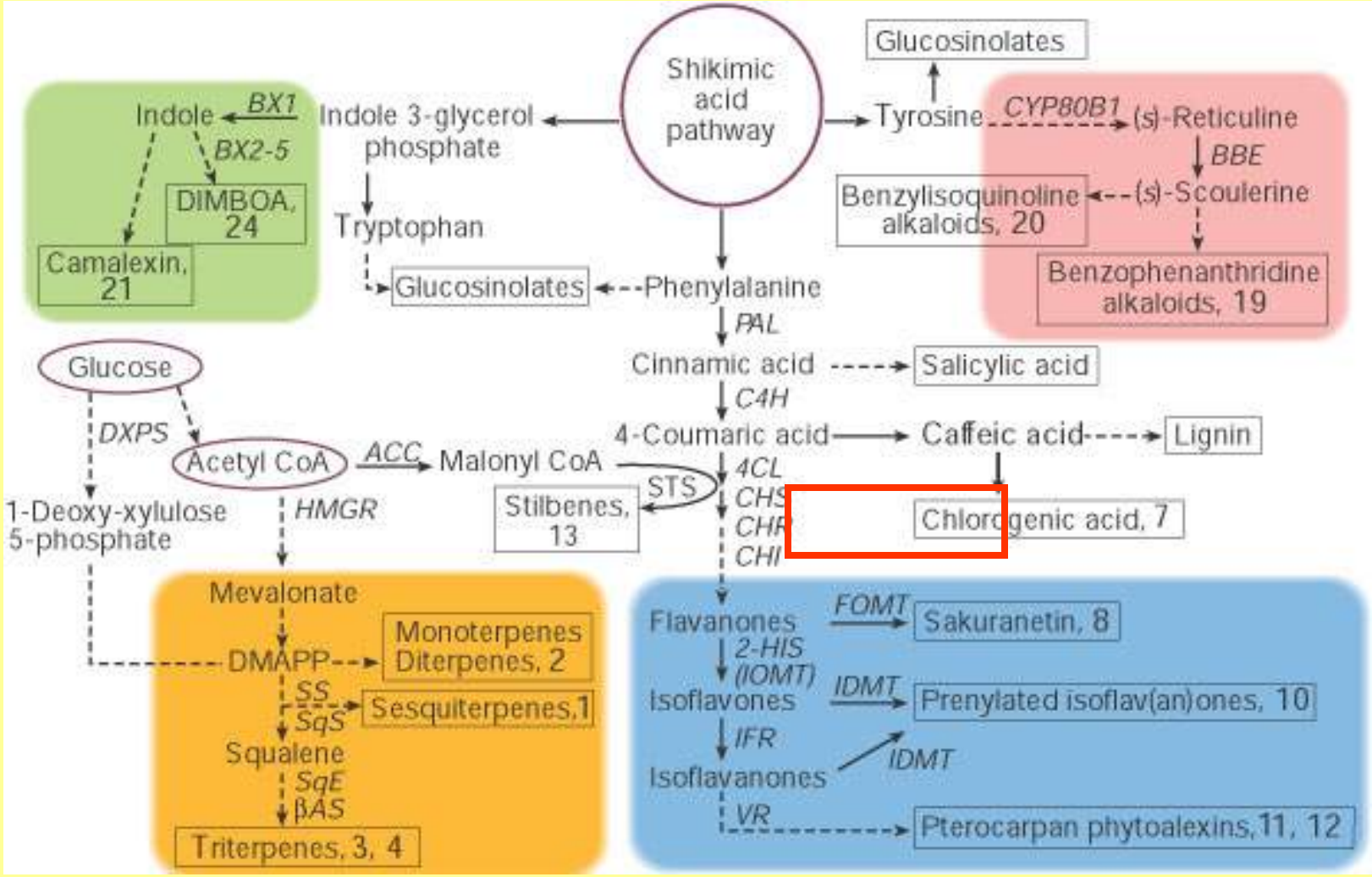
Taken together, these results are consistent with the idea that glyphosate makes plants susceptible to pathogens by inhibiting the production of phytoalexins

Johal, G. S. and J. E. Rahe. (1990). *Canadian Journal of Plant Pathology* 12: 225-235.

However, it remains open that other components derived from the shikimate pathway are also important in this regard.



In addition, the aroma of Coffee beans and their strength also derives from phenolics



Phenolics also sequester, inactivate, or facilitate transport of some phytohormones, especially auxins.

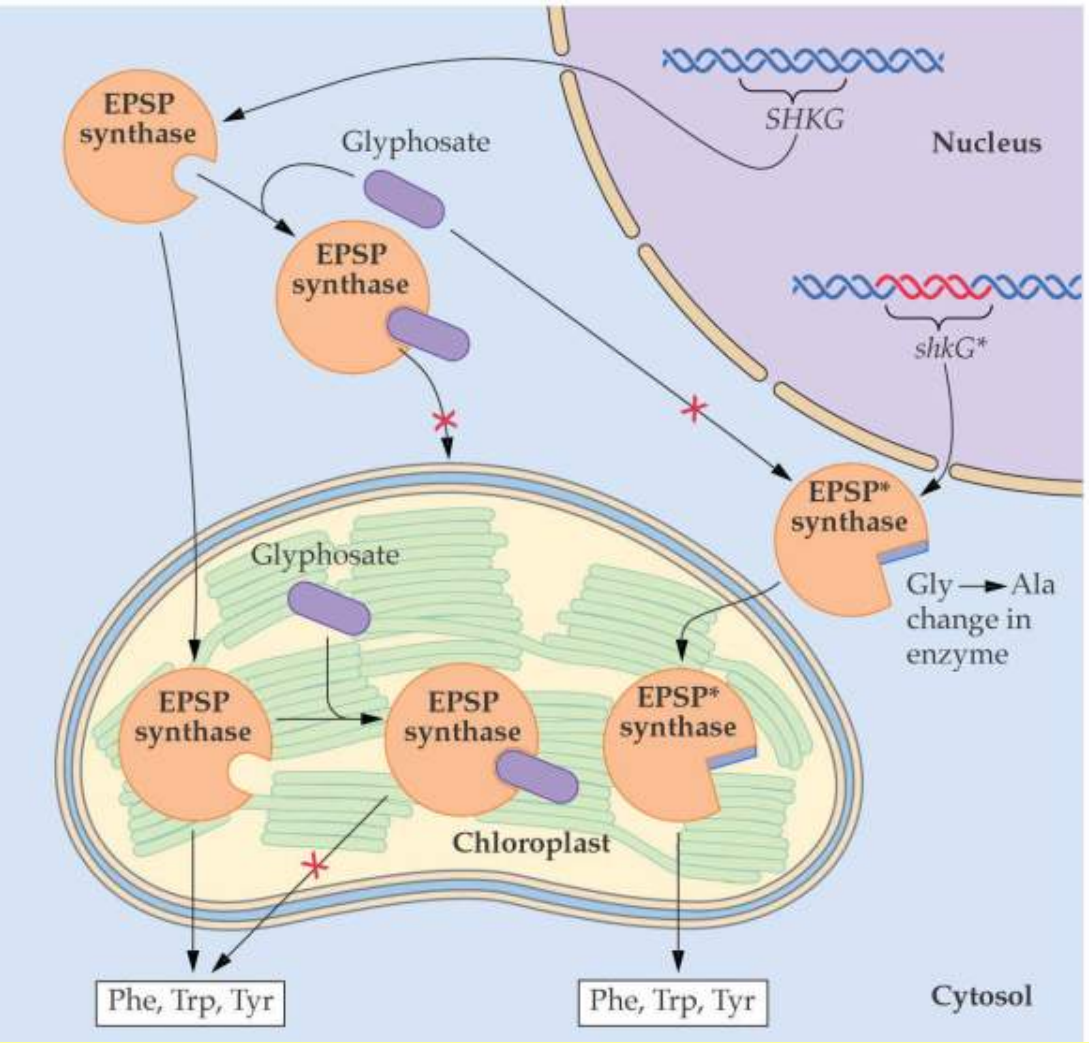
Part 3: Are ‘Roundup Ready’ (RR) plants completely resistant to glyphosate induced changes?

- In my opinion, there are reasons to be concerned about it.

First, the RR transgene may not be fully effective:

Because:

- **It being a bacterial gene, it may suffer from some ‘codon bias’**

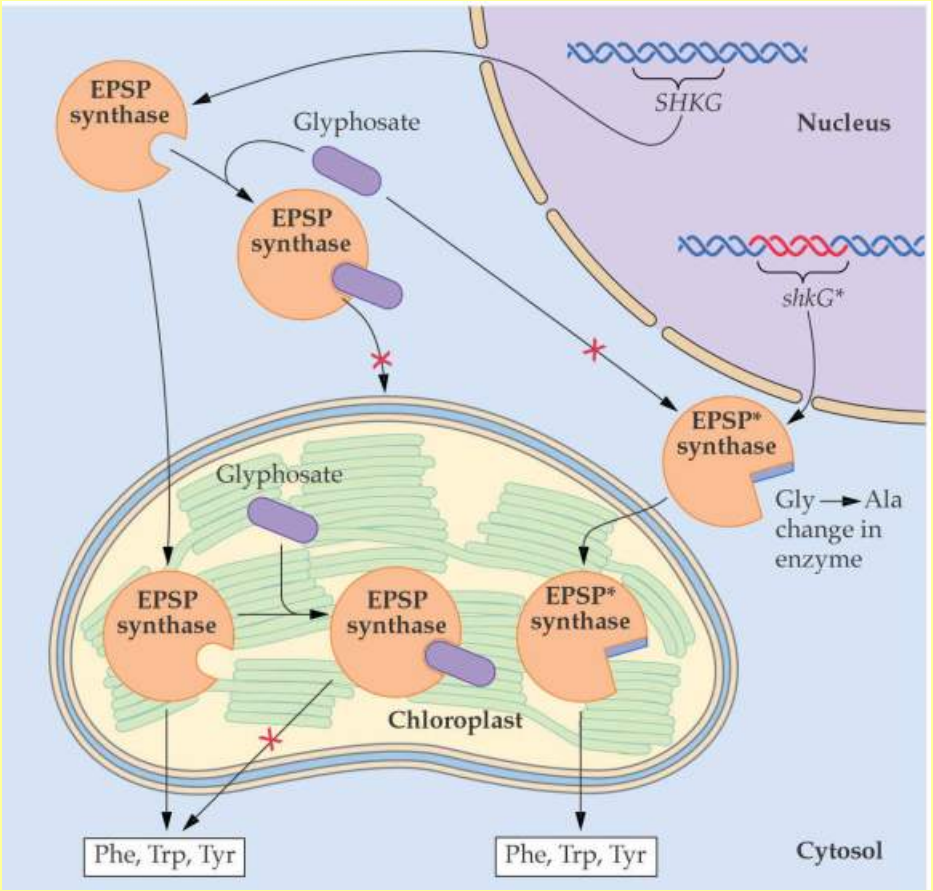


First, the RR transgene may not be fully effective:

Because:

- **It being a bacterial gene, it may suffer from some 'codon bias'**

- **The genomic location of the transgene, which will be different than the native EPSP synthase gene, may not be ideal for efficient transcription.**



First, the RR transgene may not be fully effective:

Because:

- **It being a bacterial gene, it may suffer from some ‘codon bias’**
- **The genomic location of the transgene, which will be different than the native EPSP synthase gene, may not be ideal for efficient transcription.**
- **The promoter used to derive the transgene may not be ideal either. While it may be fully effective in dealing with the constitutive requirements of shikimic acid pathway products, it may not respond appropriately or not at all to requirements imposed by pathogen challenge.**

(Defense responses in pants are inducible and highly localized both in space and time)

Second, even if everything is perfect with the transgene, there is very real possibility that a spike in the levels of *Pythium* and *Fusarium* in the vicinity of weed roots (rhizosphere) upon treatment with glyphosate may prove to be problematic, especially if environmental conditions are also not favorable for the plants.

It is well established that the inoculum level of a pathogen is directly proportional to the severity of the disease it inflicts on its host.

Published work on the suppression of soybean and wheat rust in response to glyphosate treatment in RR lines of these crop plants is a testimony that the transgene is not operating as the native gene.

Clearly, the physiology of the transgenic plants is changed in a way that translates into heightened resistance to these biotrophic pathogens.

However, the problem is that changes in plants that render them resistant to biotrophic pathogens, often enhance their susceptibility to necrotrophic pathogens like *Pythium* and *Fusarium*.

• There appears to be some sort of antagonism between mechanisms that confer resistance to these two classes of pathogens.

• Recent evidence indicates that an interplay between SA and JA (jasmonic acid) levels establish this antagonism.

• Thus, plants have to maintain a very fine balance of mechanisms so that they are able to contend with both types of pathogens.

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