Utilizzo di CHITOSANO come induttore di resistenza nelle piante alle malattie fungine e alle virosi

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Conflict of interest

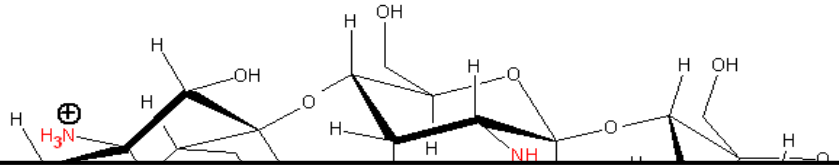
1. Probiotical S.p.A.
2. Hospira Inc.
3. SanitaNova s.r.l.
4. Functional Point s.r.l.
5. Winstar Medical AG

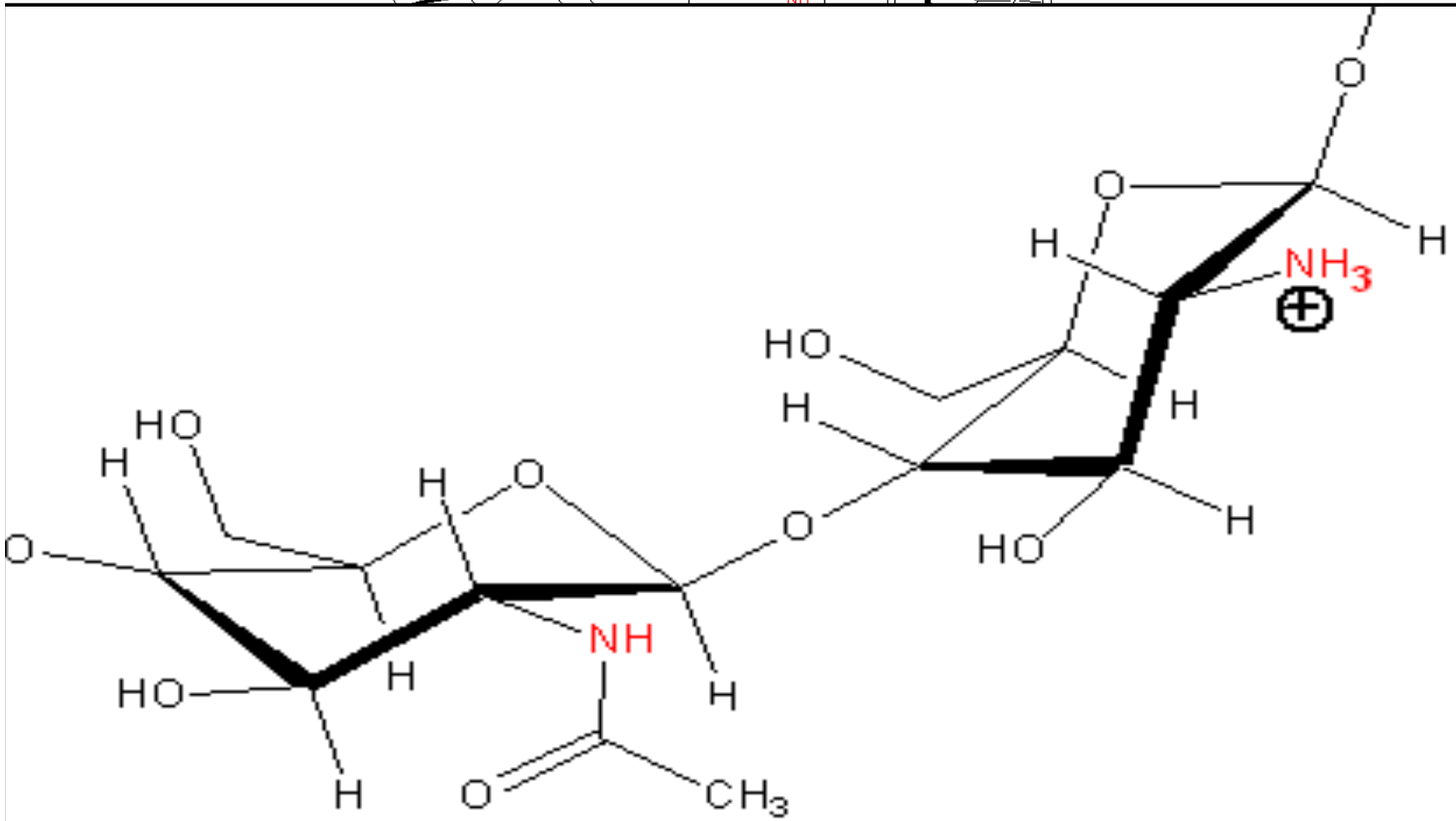
Outline

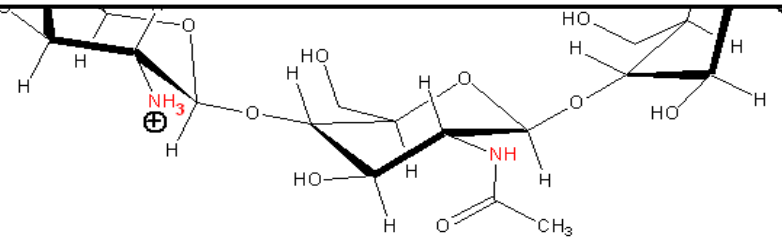
1. Overview on chitosan in crop protection
2. Antiviral activity of chitosan (case-studies and mechanisms)
3. Antifungal activity of chitosan (case-studies and mechanisms)
4. Fitness costs and crop yield
5. Antitranspirant activity of chitosan
6. Conclusions

1. Overview on chitosan in crop protection

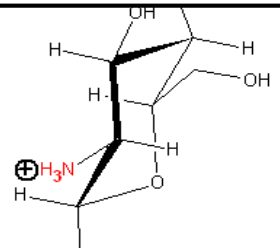
CHITOSANS

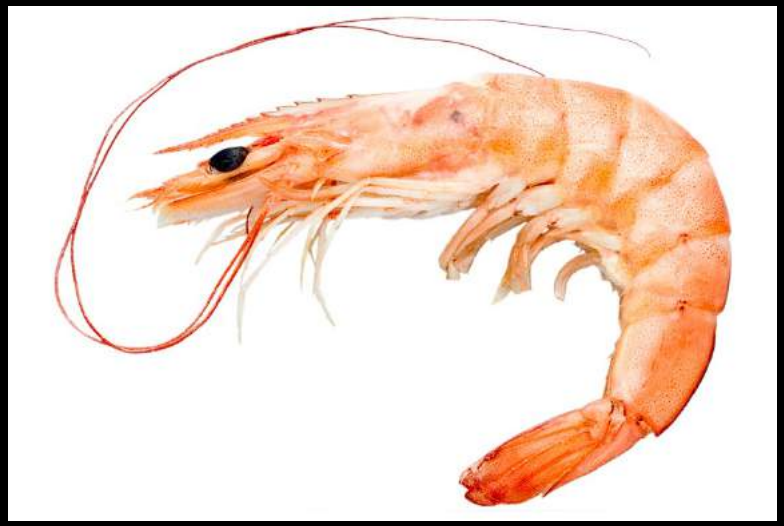






Chitosans (CHT) are natural, non-toxic and low expensive compounds obtained by deacetylation of chitin from the exoskeleton of crustaceans





Chitosan or chitosans?

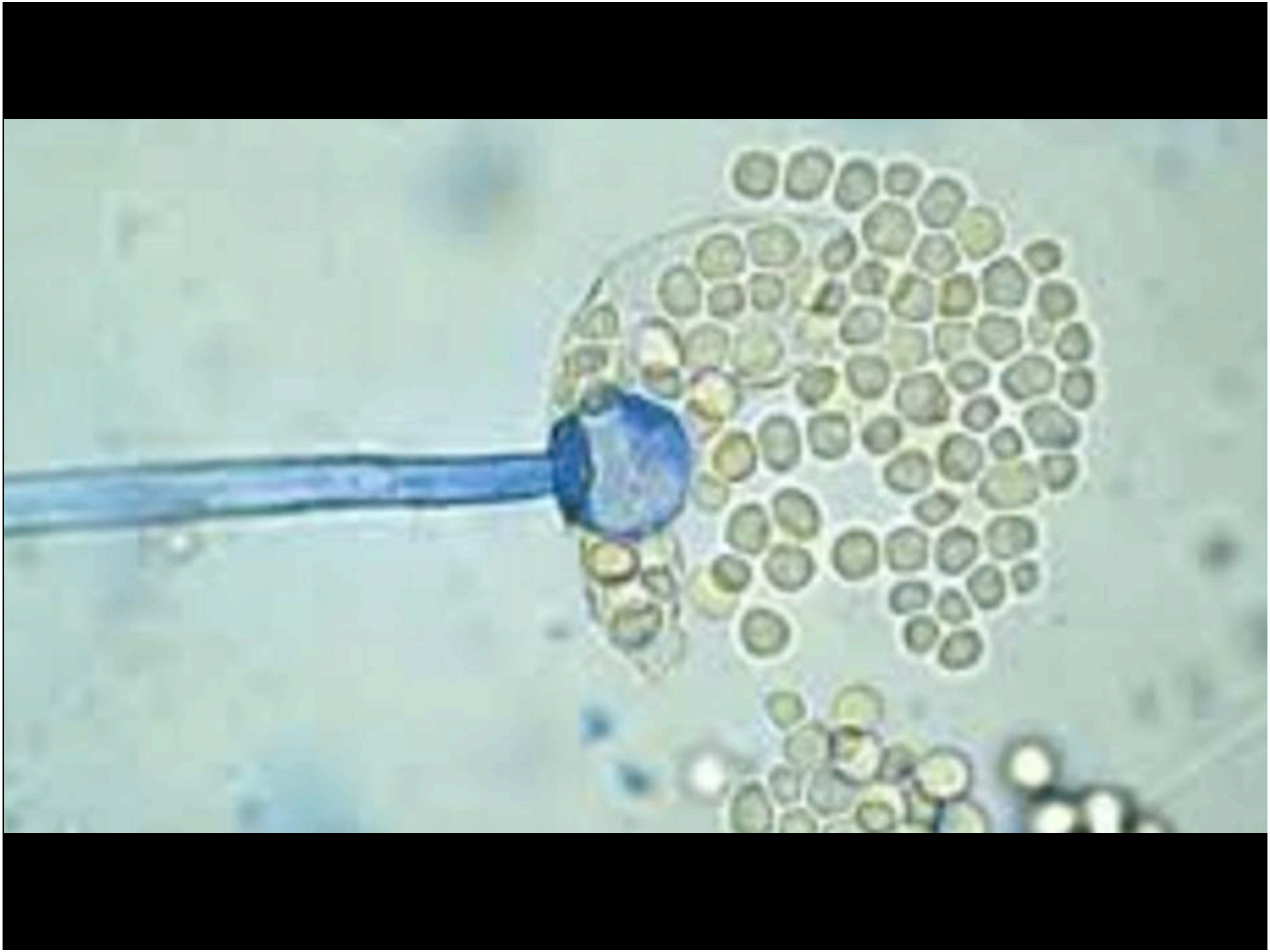
The deacetylation degree (DD), molecular weight (MW), polymerization degree (PD) and viscosity determine chitosan properties:

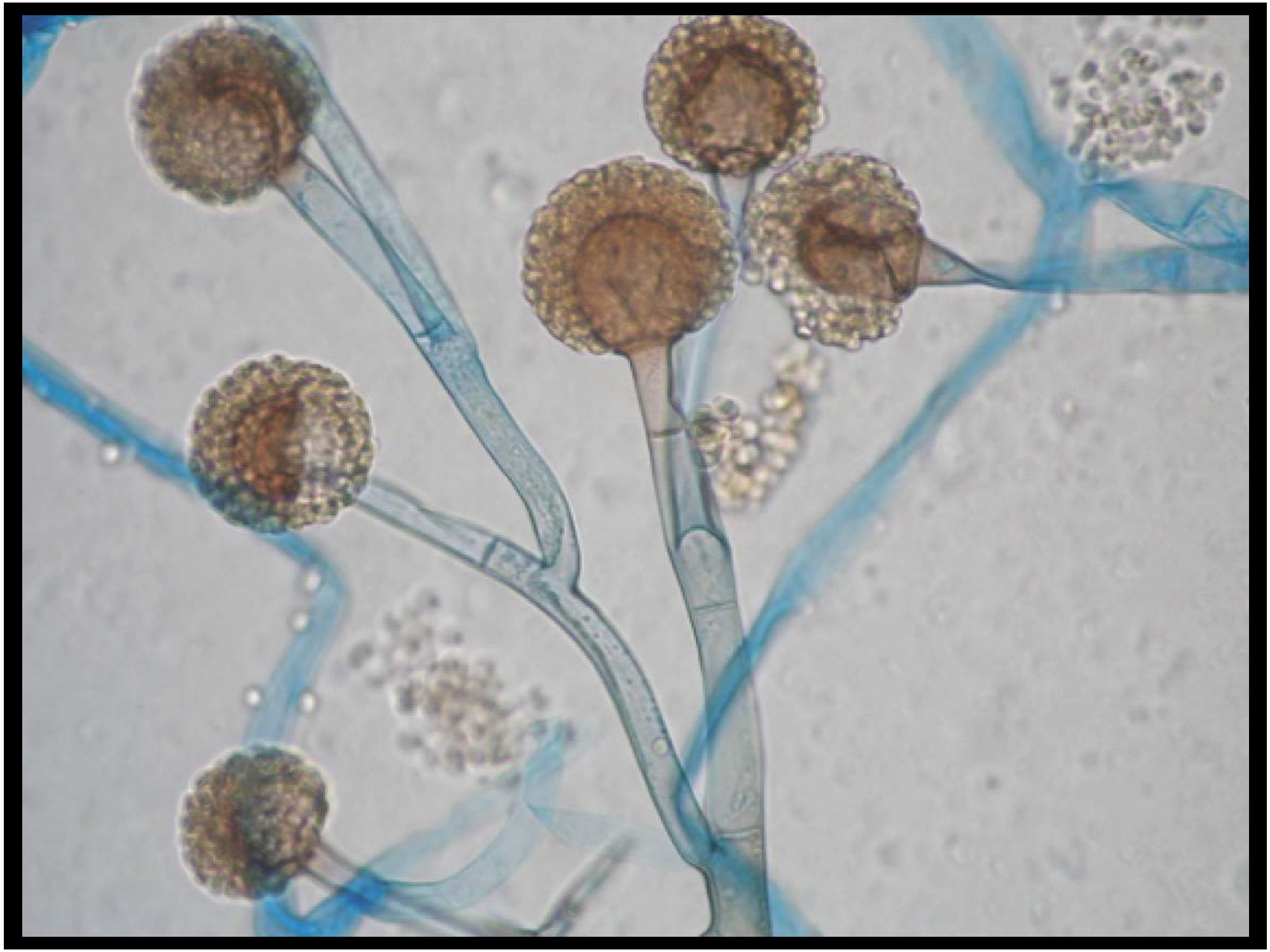
- different chitosans may cause very different physiological responses in a plant species

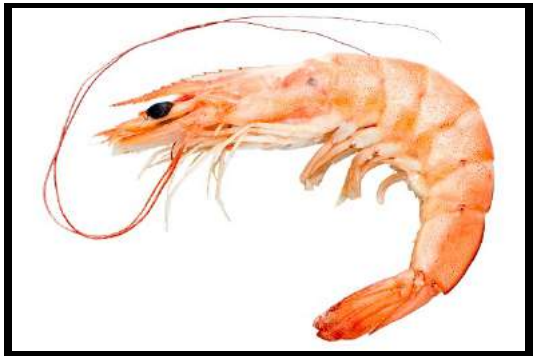
- as well as a specific chitosan may cause very different responses in different plant species

Commercial classification of chitosans

Polymers (souble in acids)	kDa (DD 80-85%)	Monomer units	Viscosity (mPa · s)
High MW	> 300	> 2000	> 800
Medium MW	190-310	1200-2000	200-800
Low MW	50-190	300-1200	20-200
Very low MW	10-50	10-300	6-20
Oligomers (soluble in water)	≤ 5	≤ 30	≤ 6







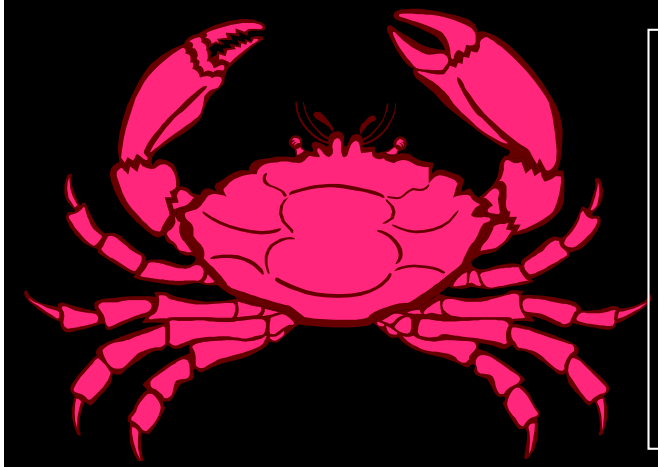
- The antifungal and antibacterial activities of chitosans are well-known and consist of more than one mechanism of action

- In addition, chitosans activate the plant immune system, acting as an elicitor

⇩

the only strategy effective against viruses!

Chitosan, besides a direct antimicrobial activity, induces in plants a great variety of defence mechanisms, such as:

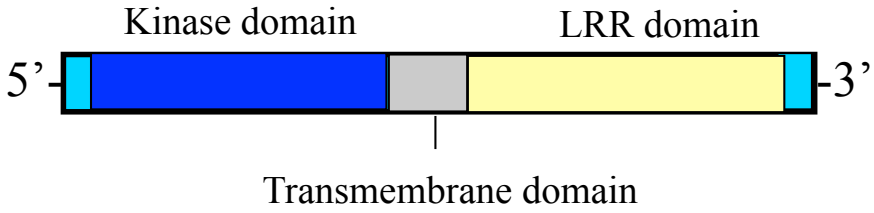


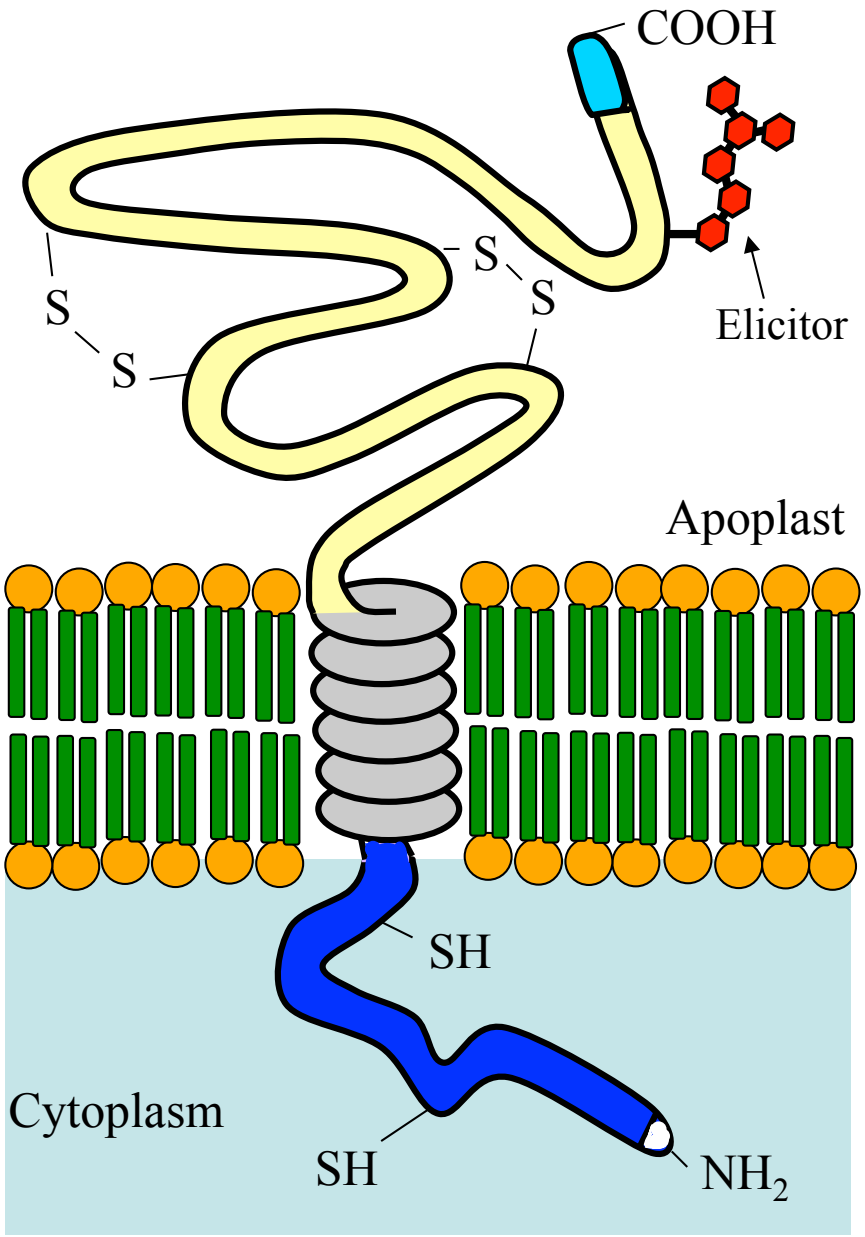
- salicylic acid and jasmonate biosyntheses
- stimulation of chitinases (PR proteins)
- accumulation of phytoalexins
- synthesis of proteinase inhibitors (PR proteins)
- increased lignification

⇩

leading to systemic acquired resistance (SAR), that prevent host colonization by different pathogens

Elicitor – receptor binding





To act as elicitor and induce plant resistance against viruses, chitosan should have:

- deacetylation degree around 80-90%

- low molecular weight (< 100kDa)

Oligomers are, usually, biologically more active than low MW polymers, but they must be used at lower concentration otherwise they can be highly phytotoxic

Oligomers with a homogeneous composition in terms of monomer number (i.e. 6-10) are also more difficult and expensive to prepare

Therefore, low MW polymers represent an acceptable compromise, at least for field treatments

[Plant Signaling & Behavior 4:1, 66-68; January 2009]; ©2009 Landes Bioscience

Chitosan as a MAMP, searching for a PRR

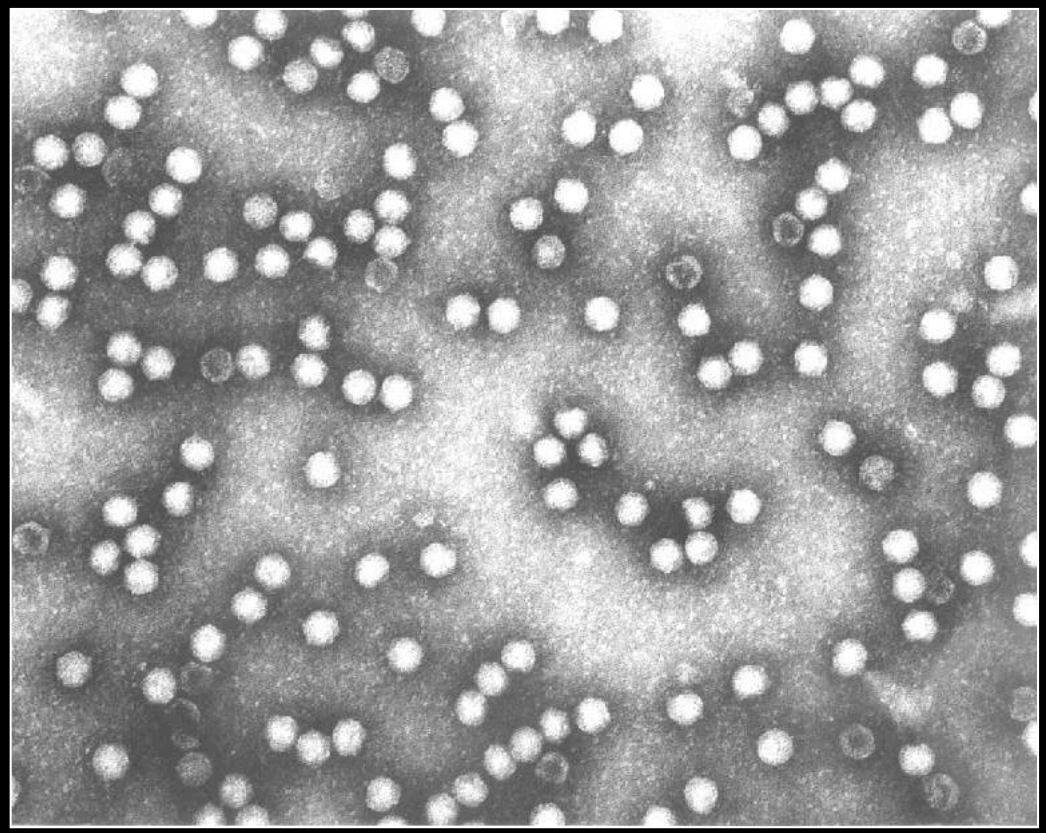
Marcello Iriti* and Franco Faoro

Istituto di Patologia Vegetale; Università di Milano and CNR; Istituto di Virologia Vegetale; Milano Italy

- Calcium transient (Kauss, 1985, Zuppini et al., 2003)
- Plasma membrane H^+-ATPase inhibition (Amborabé, 2008)
- MAP-kinase activation (Lizama-Uc et al., 2007)
- Callose apposition (Conrath et al., 1989; Iriti et al., 2006; Iriti and Faoro, 2008)
- Reactive oxygen species (oxidative burst) (Lin et al., 2005; Iriti et al., 2006)
- HR / PCD (Iriti et al., 2006; Wang et al., 2008; Iriti and Varoni, 2015)
- Abscisic acid (Iriti and Faoro, 2008, Iriti et al., 2009)
- Jasmonate (Doares et al., 1995)
- Ethylene (Iriti et. al., 2010)
- Phytoalexins (Hadwiger et al., 1994; Chakraborty, 2008)
- Pathogenesis related (PR) proteins (Agrawal, 2002; Lin et al, 2005)

3. Antiviral activity of chitosan (case-studies and mechanisms)

Chitosan-induced systemic acquired resistance against plant viruses

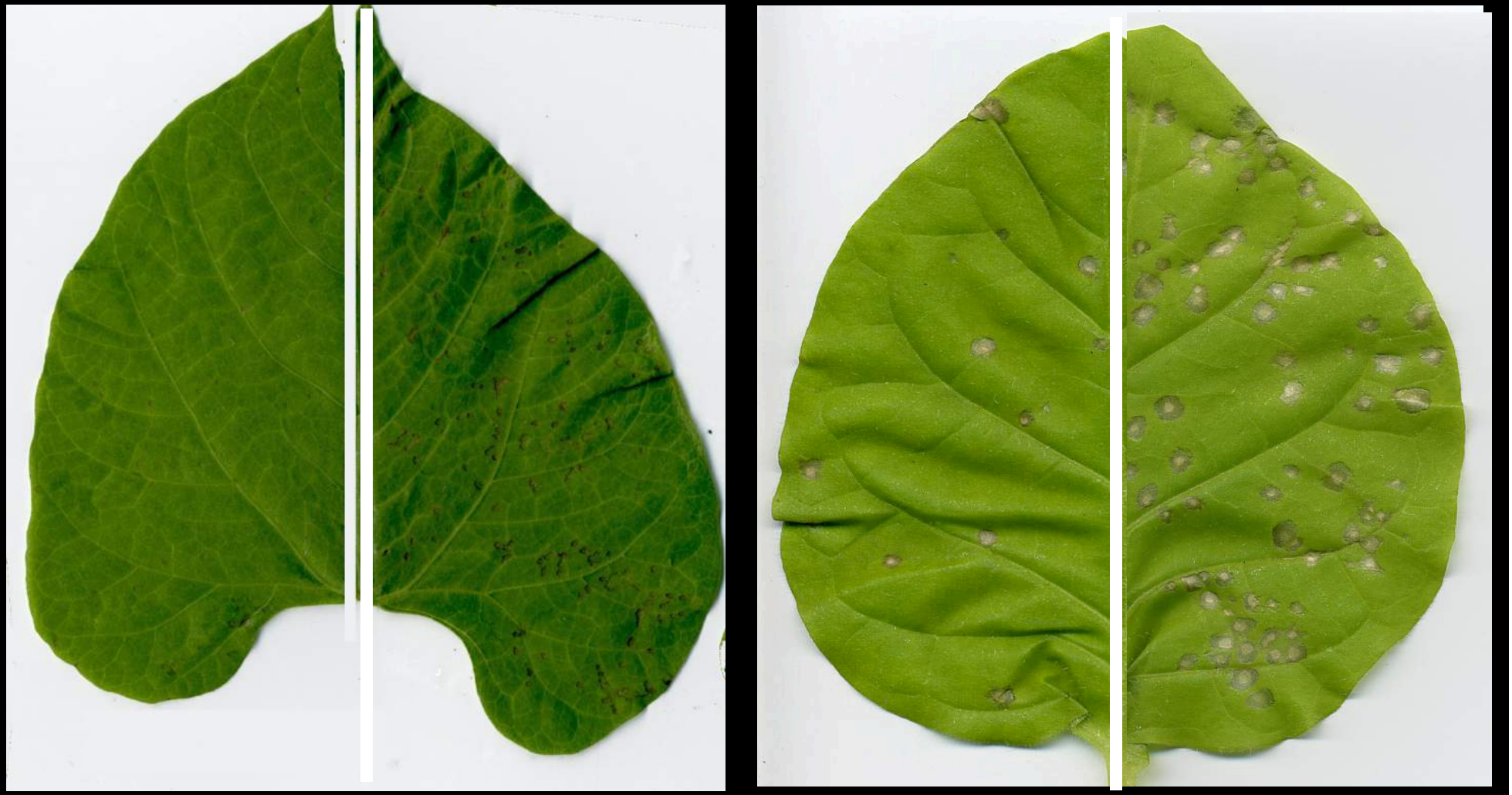


Chitosan antiviral activity is species-specific and strongly depends on its polymerisation and deacetylation degree

Influence of chitosan concentration on induced resistance (same virus, different host)

Bean

Tobacco



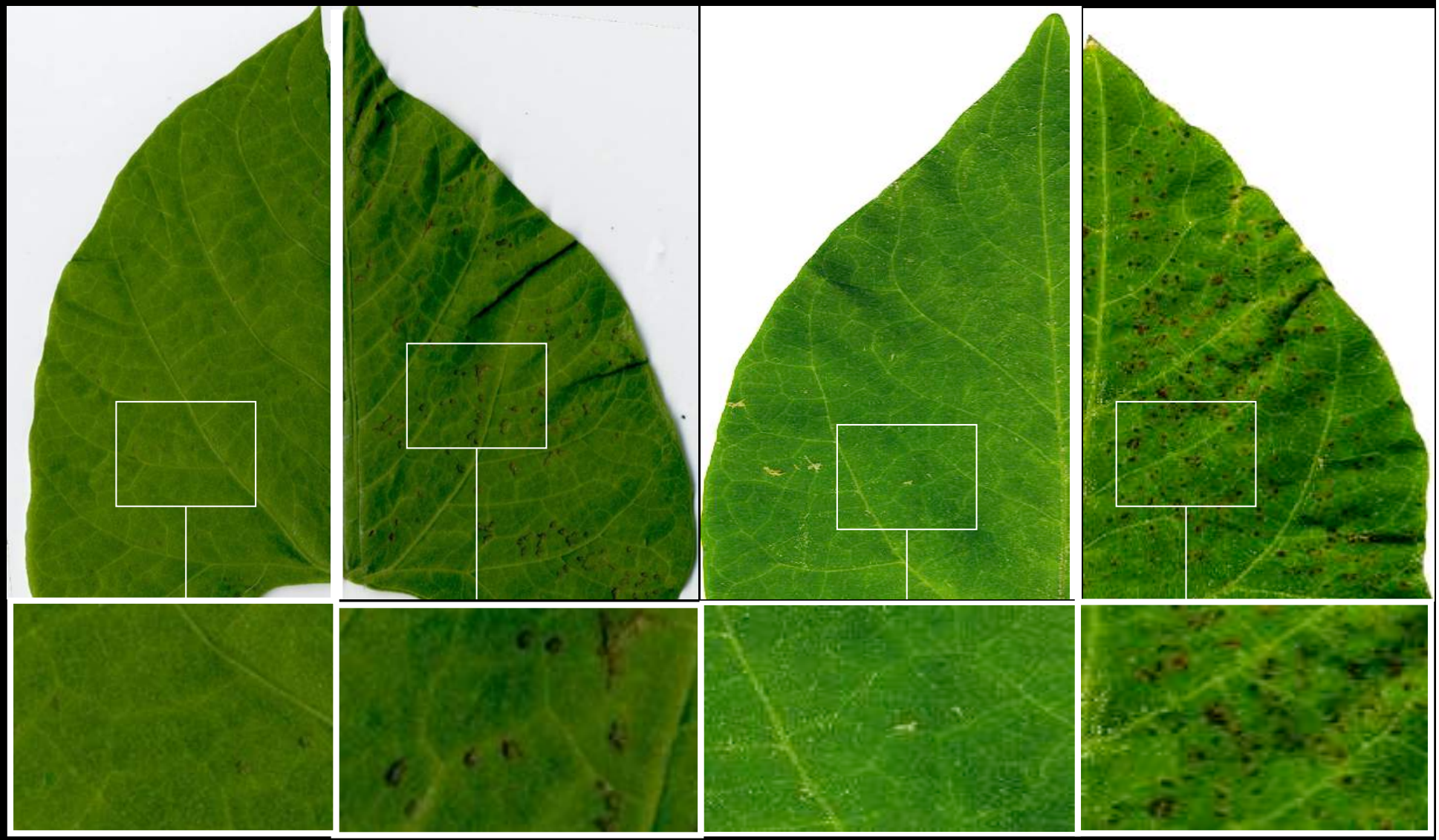
Chitosan 0.05% + TNV 48 h later

Water + TNV 48 h later

Chitosan 0.2% + TNV 48 h later

Water + TNV 48 hr later

Influence of chitosan concentration on induced resistance (different virus, same host)

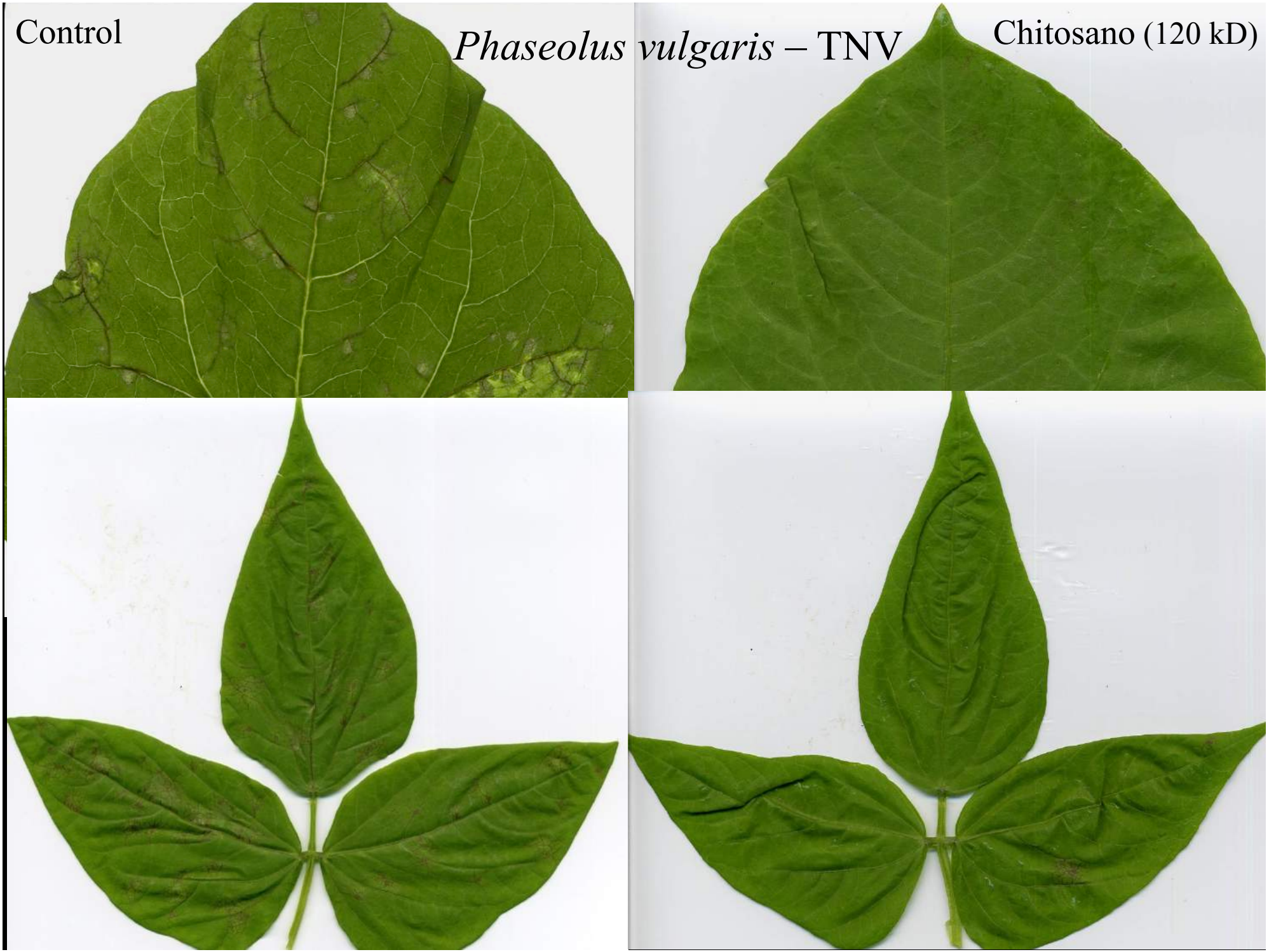


Chitosan 0.05 % + TNV 48 h later

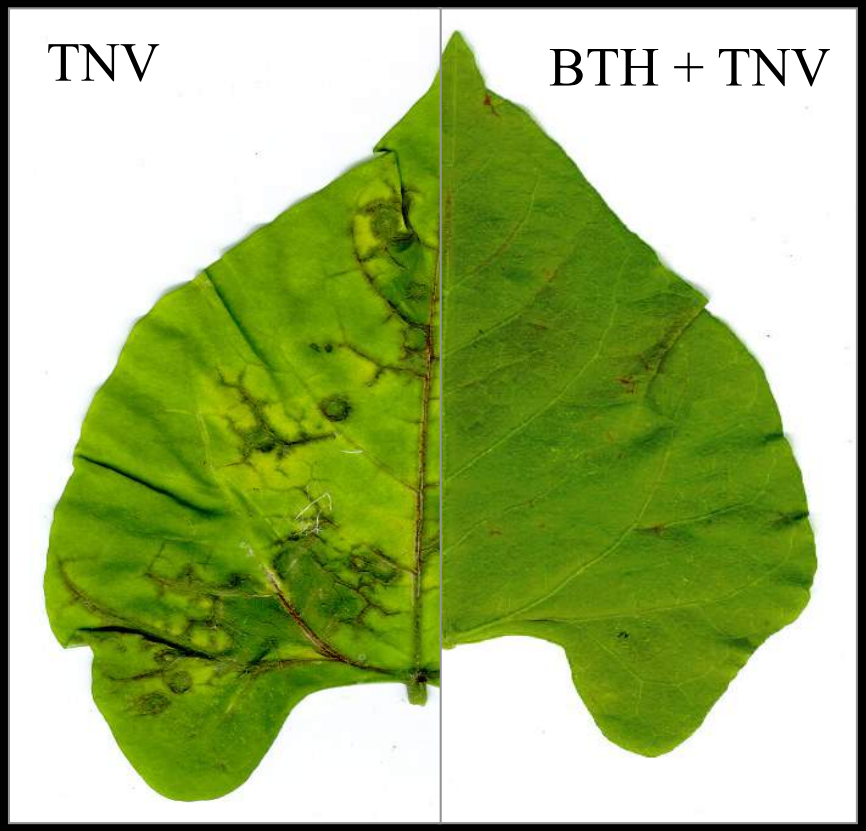
Water + TNV 48 h later

Chitosan 0.15 % + TBSV 48 h later

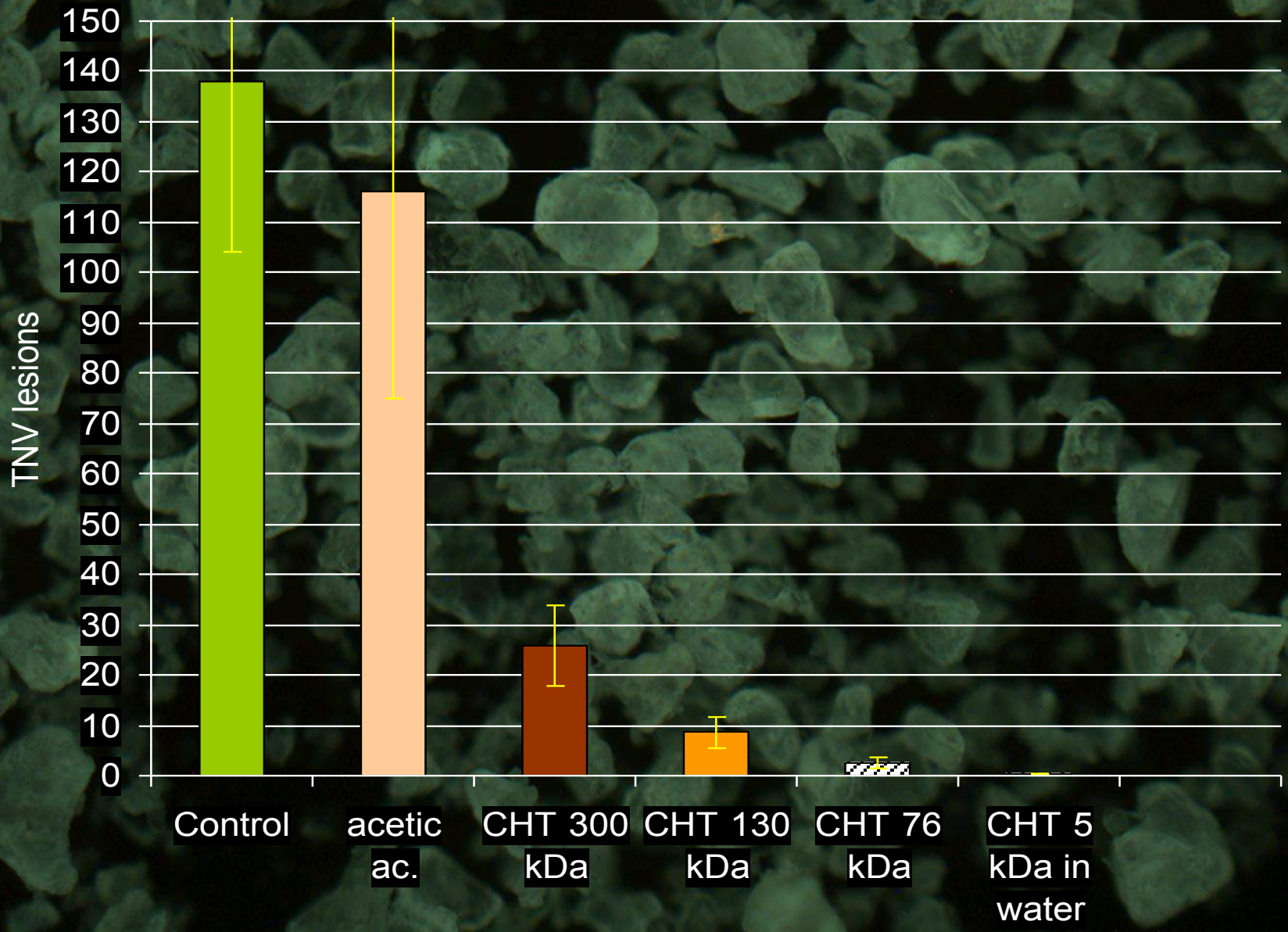
Water + TBSV 48 h later


Control
Phaseolus vulgaris – TNV
Chitosano (120 kD)

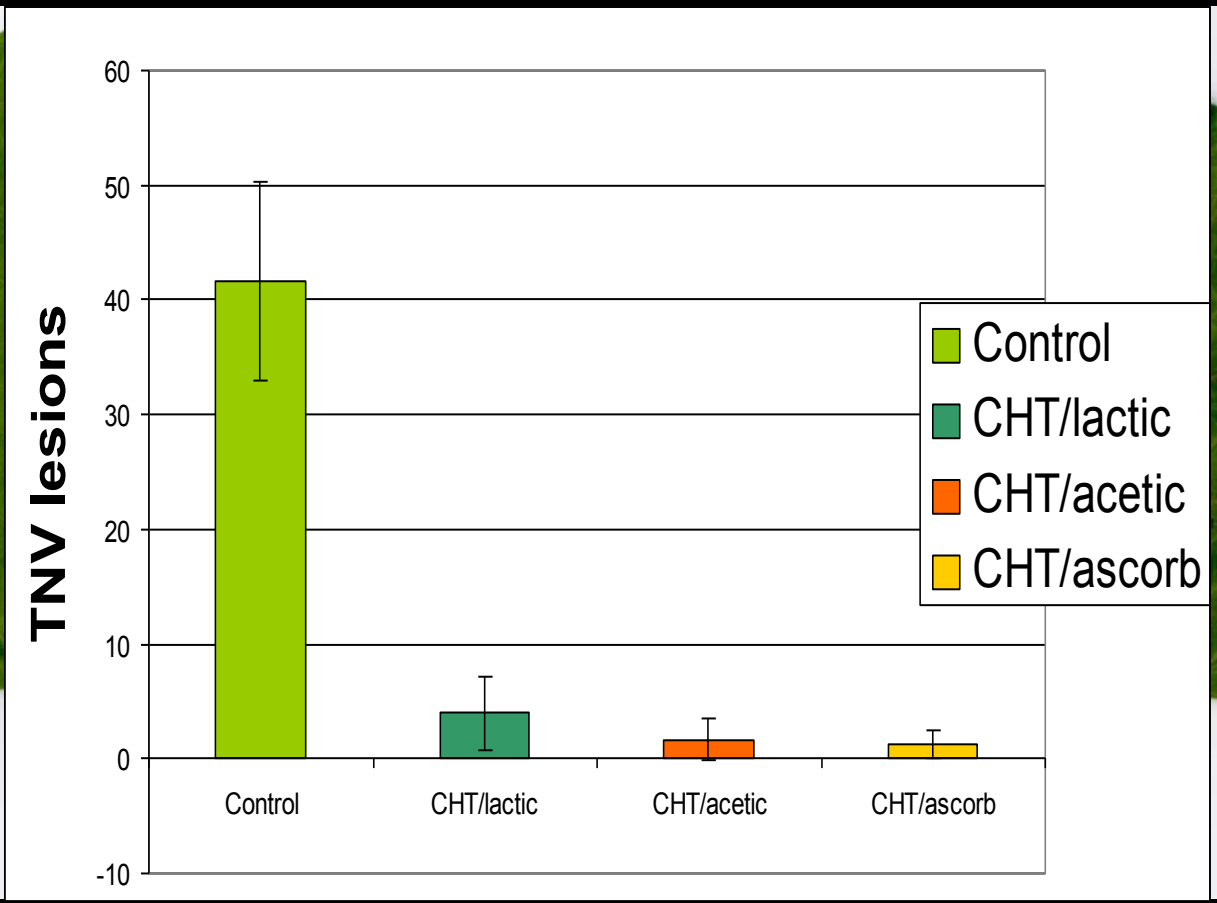
BTH and TNV



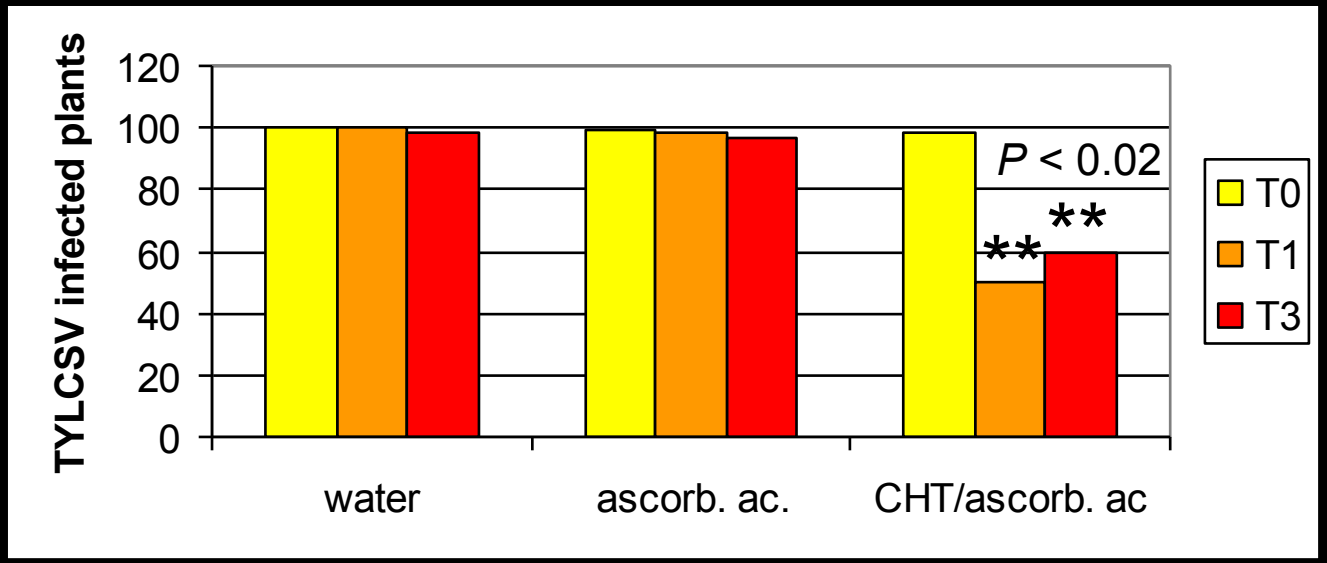
Influence of the MW on chitosan-induced resistance in bean to TNV

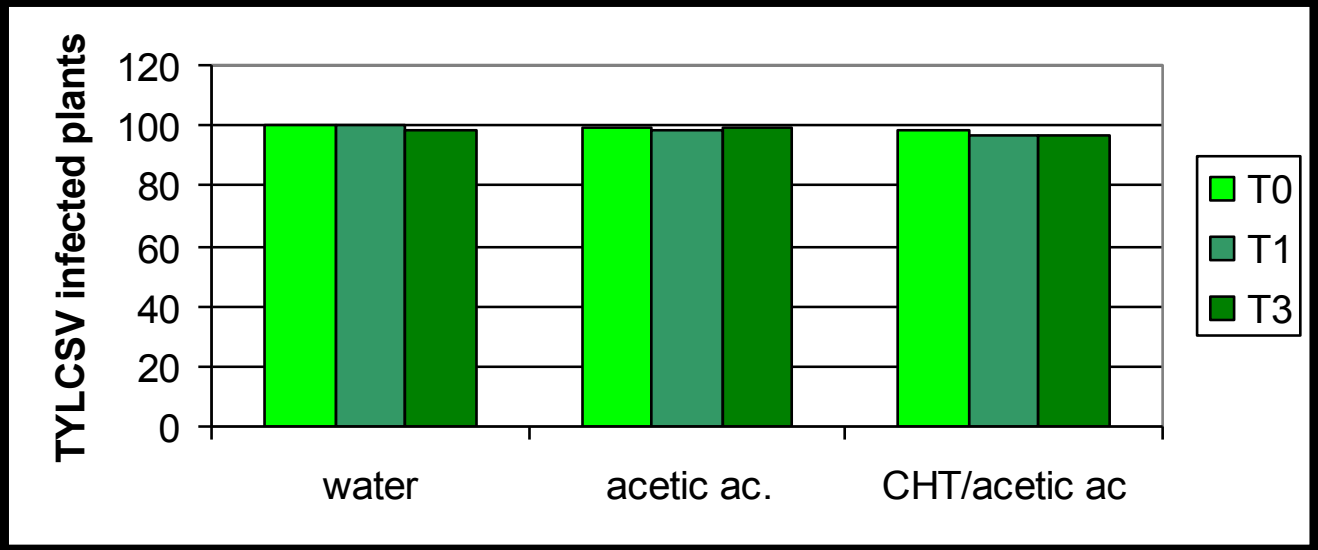


Influence of the solvent on chitosan-induced resistance in bean to TNV



Influence of the solvent on chitosan-induced resistance to TYLCSV in tomato (inoculation with the vector *Bemisia tabaci*)



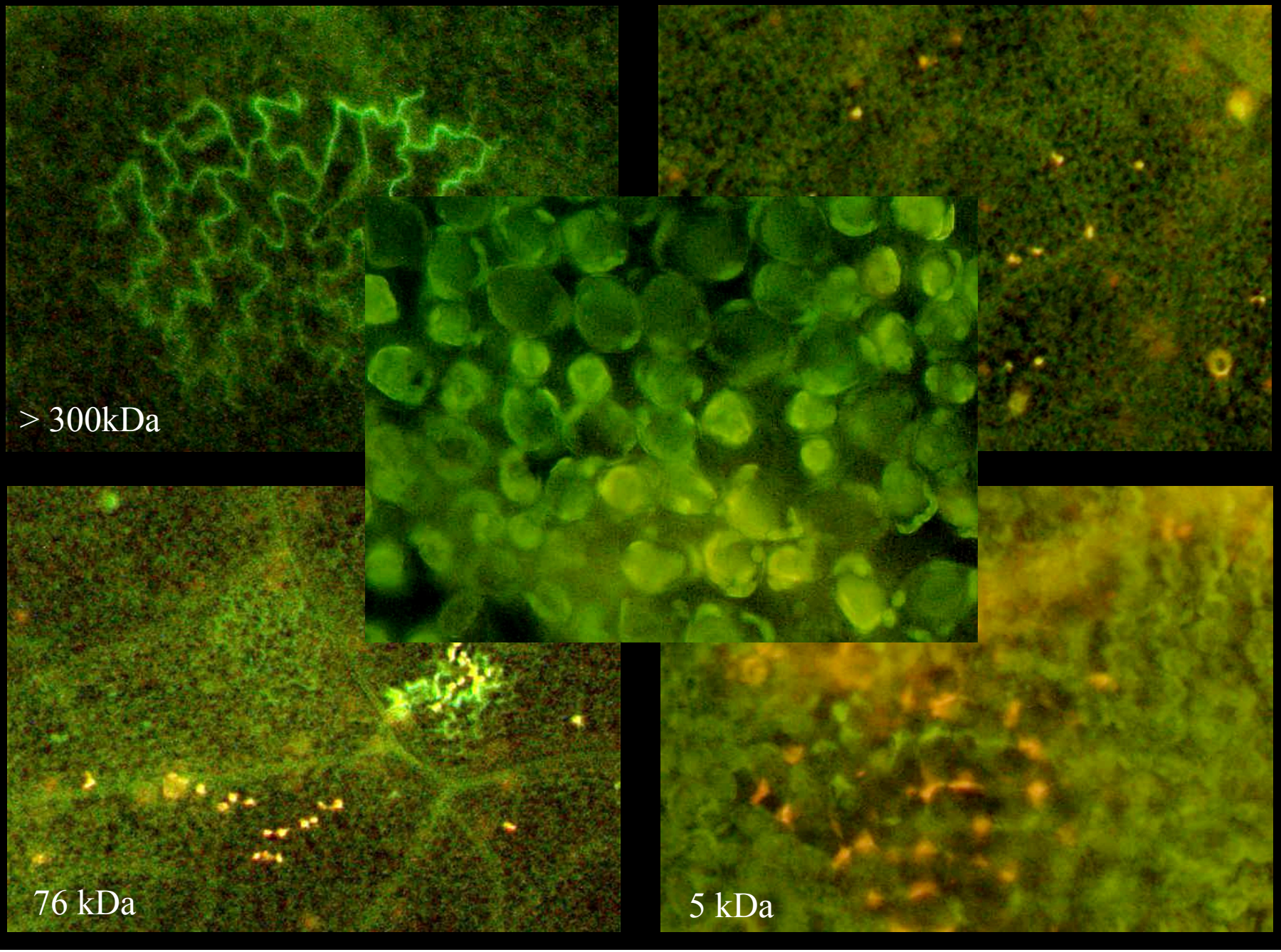


Tomato plants were sprayed with a 0.05% chitosan 40 kDa;

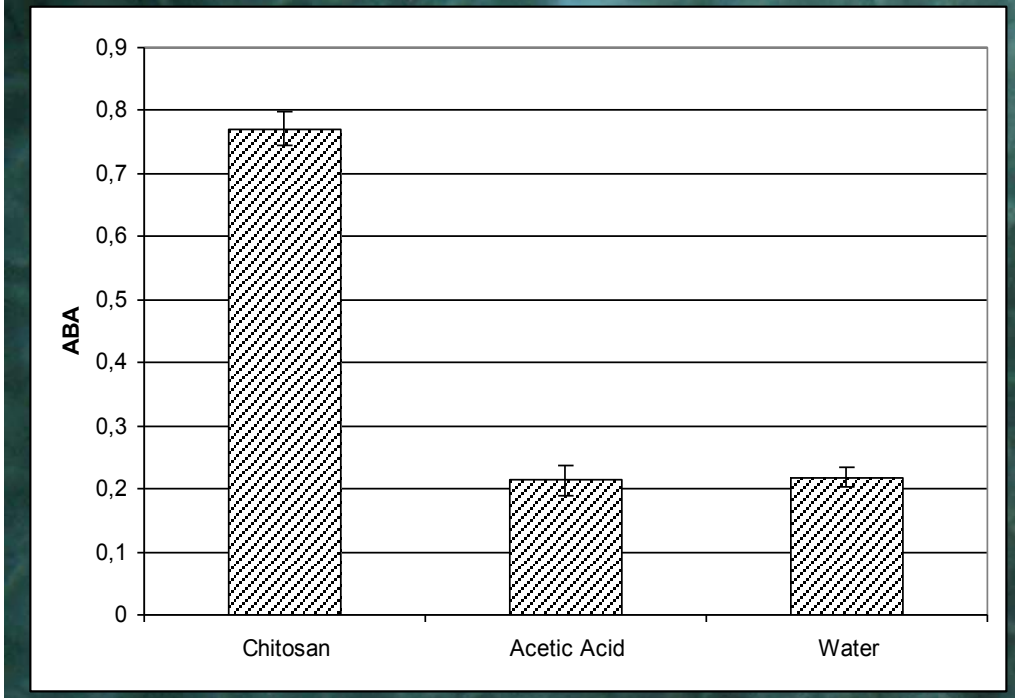
after:
2 h (T0),
48 h (T1),
96 h (T2)
each plant was infested with 7 virulent *Bemisia*;
insects were let feeding for 24 h,
then killed;
virus transmission was assessed by Elisa after 3 weeks.

Courtesy of Dr. Piero Caciagli (CNR)

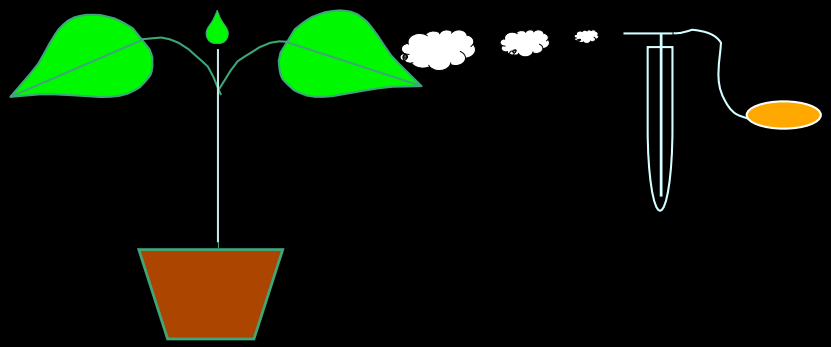
Effects of a 0.15% chitosan solution in acetic acid on bean leaf



Chitosan-induced callose synthesis is mediated by ABA

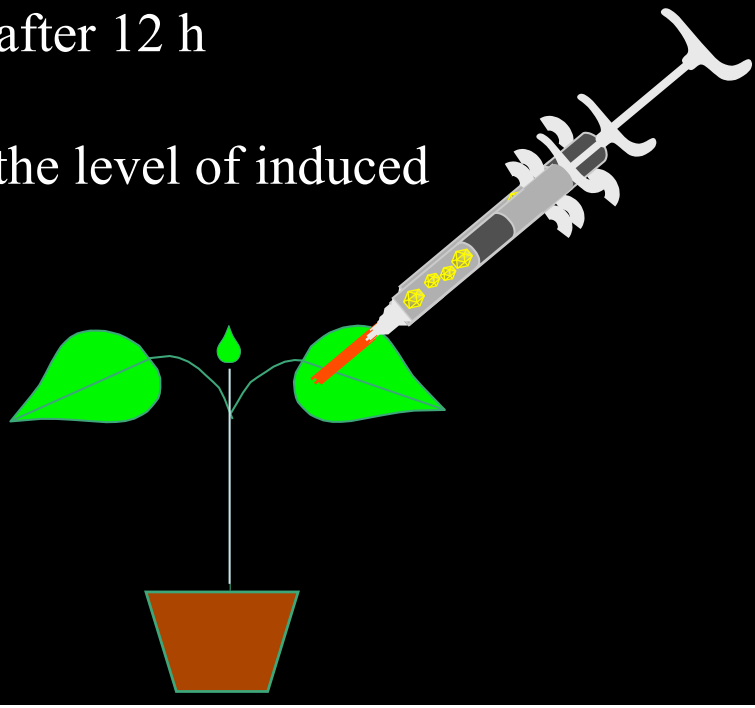


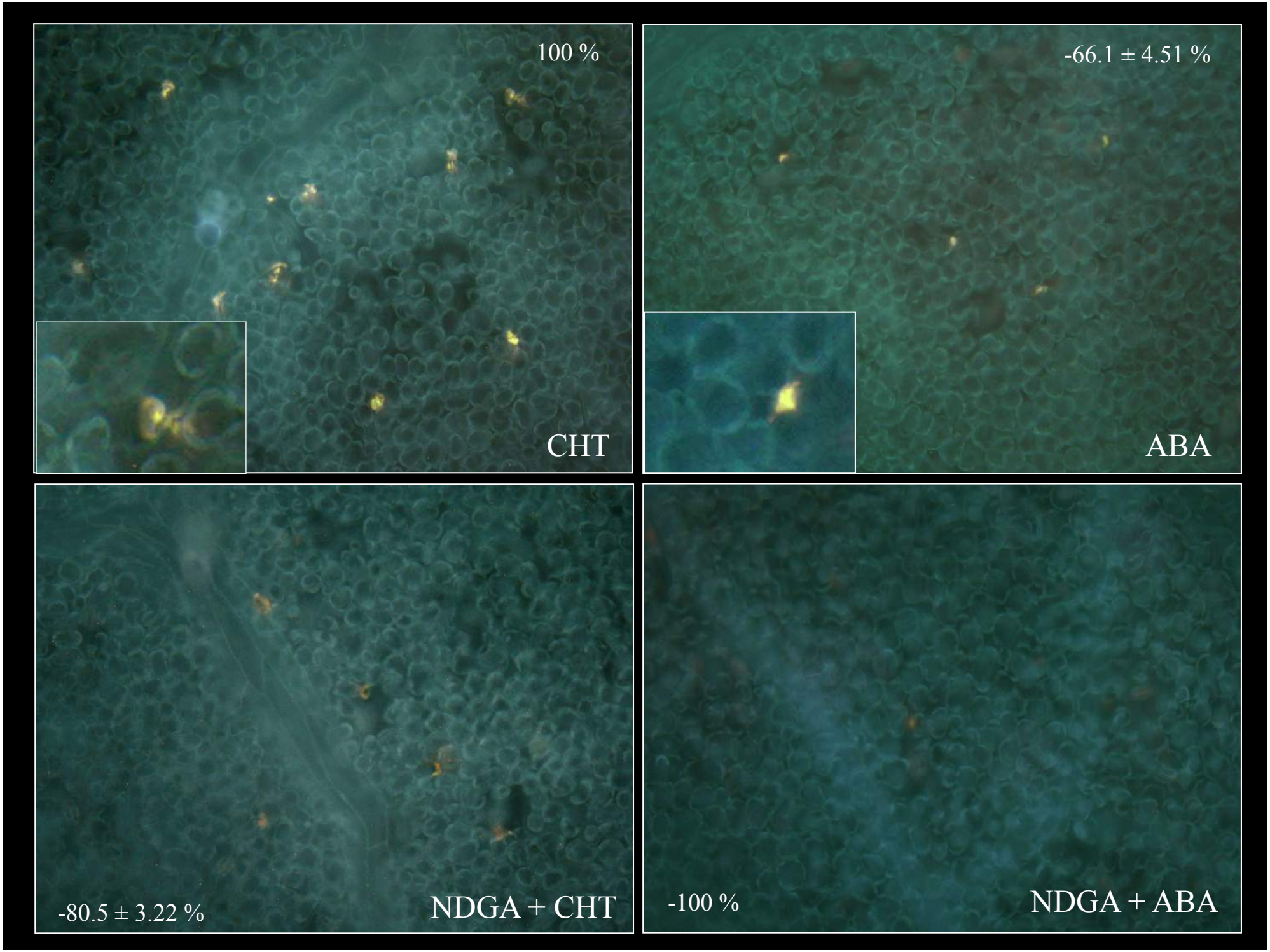
(Iriti and Faoro 2008, Plant Physiol. Biochem. 46: 1106–1111)

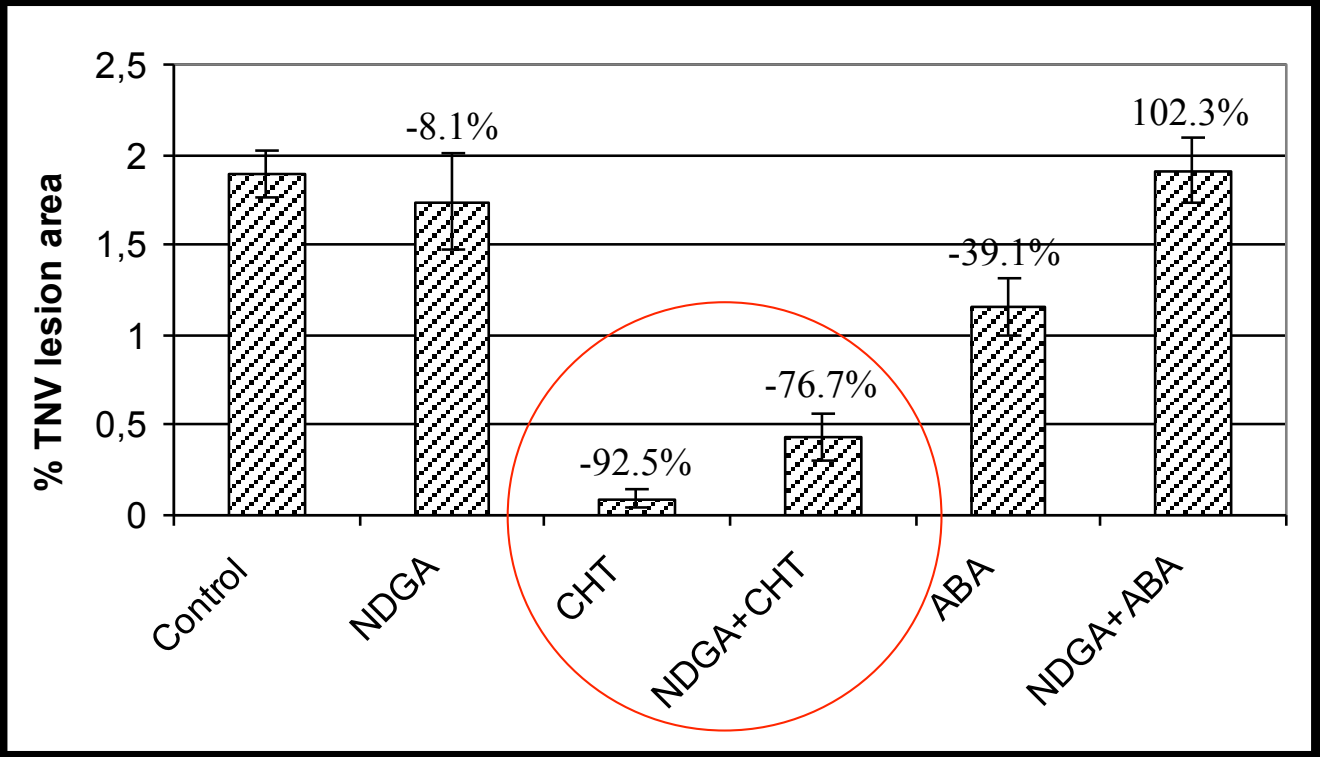


- water (control)
- CHT 0.15% (Fluka low MW in AA 0.01M)
- AA (0.01M pH 5.6)
- 0.1 mM ABA
- 10 mM NDGA + (after 2h) 0.15% CHT
- 10 mM NDGA + (after 2h) 0.1 mM ABA
- 10 mM NDGA

- plants were sprayed with CHT in presence or not of an ABA inhibitor (nordihydroguaiaretic acid, NDGA)
- callose apposition was assessed with aniline blue after 12 h
- plants were challenged after 24 hr with TNV and the level of induced resistance assessed as % of necrotic lesion areas.



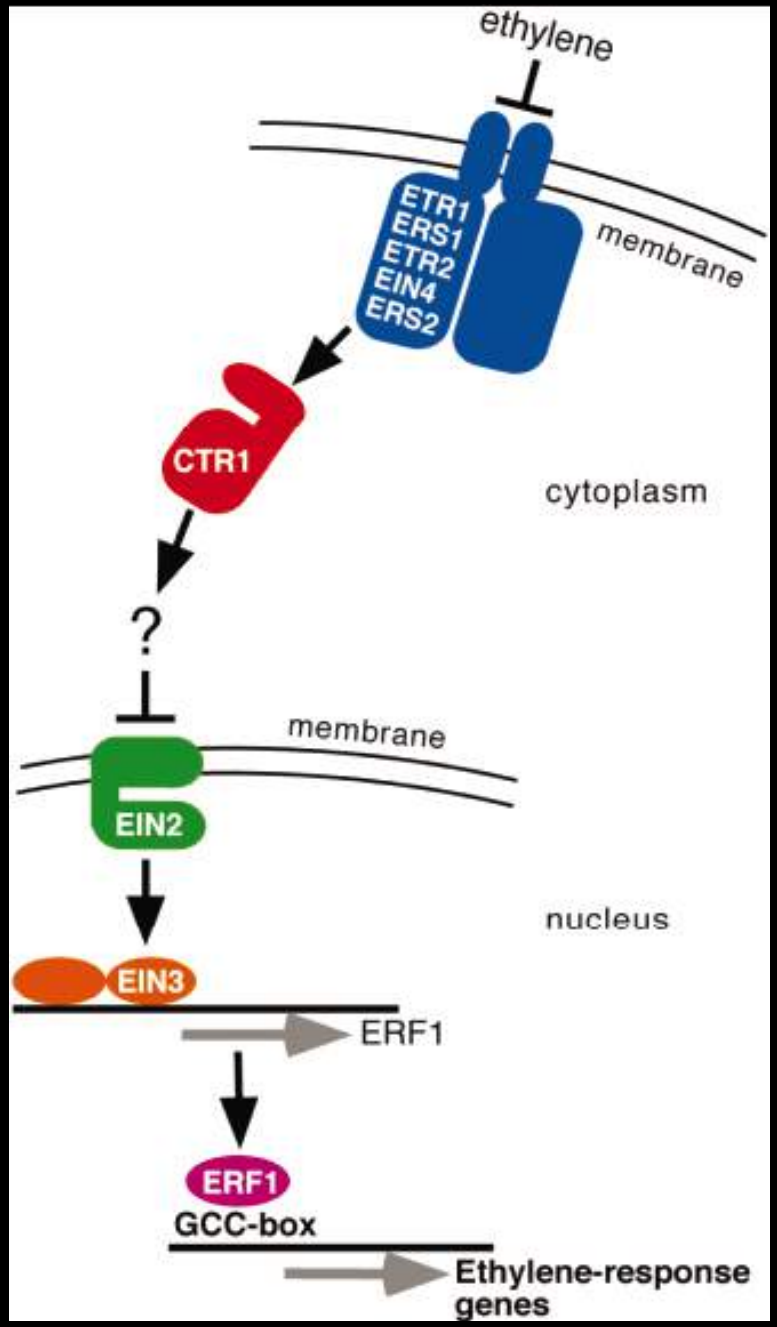

100 %
CHT
-66.1 ± 4.51 %
ABA
-80.5 ± 3.22 %
NDGA + CHT
-100 %
NDGA + ABA

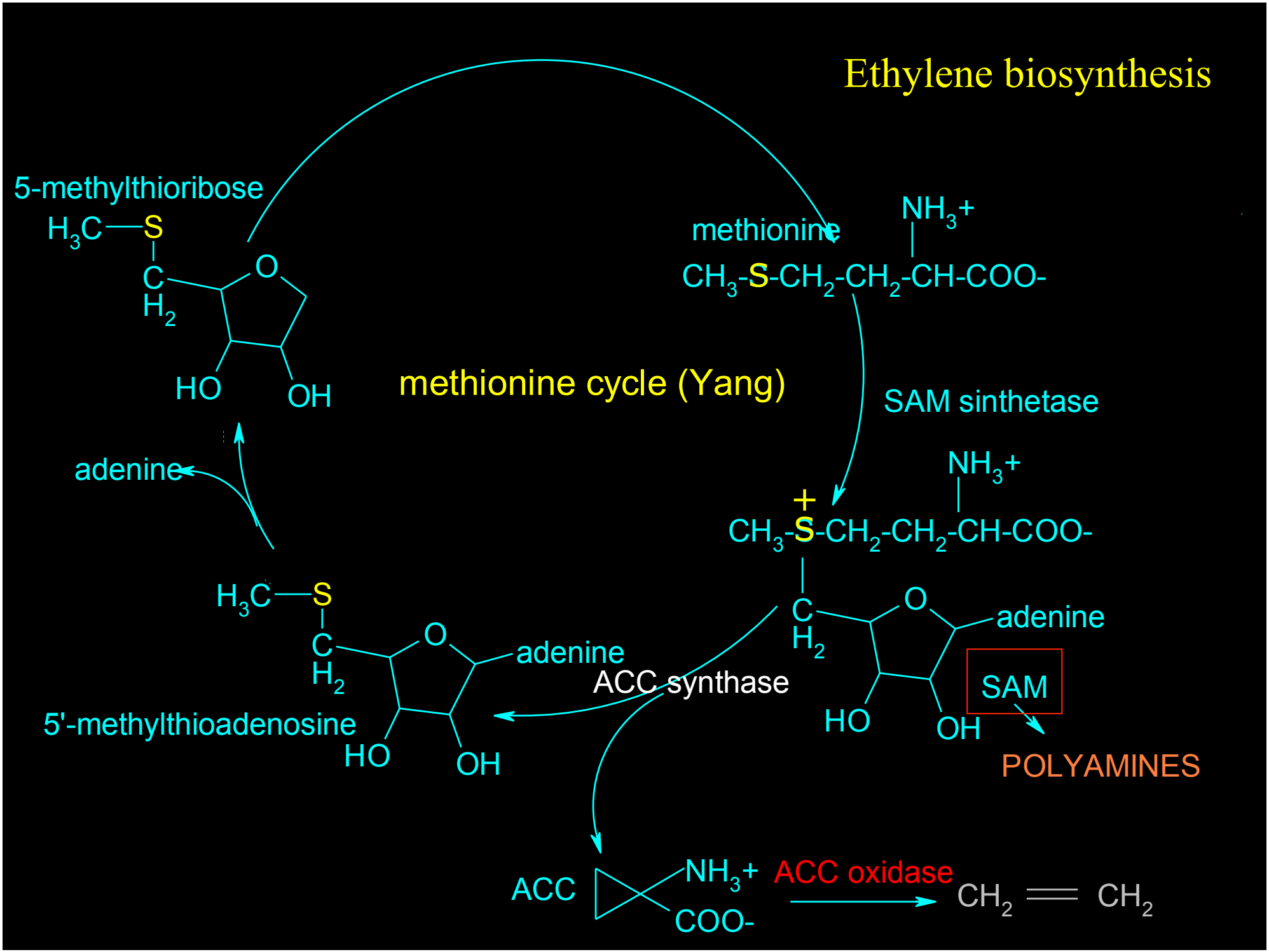


- Counteracting callose sinthesis by ABA inhibition, the plant resistance degree to the virus only lowered to a small extent, thus it is likely that CHT antiviral activity mostly resides in the induction of PCD.

- Callose deposition would slowdown cell-to-cell movement of the virus from the first infected cells, allowing PCD to be mounted before excessive spreading of the infection. This view is also suggested by the lower resistance to TNV induced by ABA treatment.

ETHYLENE


ethylene
ETR1
ERS1
ETR2
EIN4
ERS2
membrane
CTR1
cytoplasm
?
membrane
EIN2
nucleus
EIN3
ERF1
ERF1
GCC-box
Ethylene-response genes


Ethylene biosynthesis
5-methylthioribose
methionine
methionine cycle (Yang)
SAM sinthetase
adenine
5'-methylthioadenosine
ACC synthase
SAM
POLYAMINES
ACC
ACC oxidase

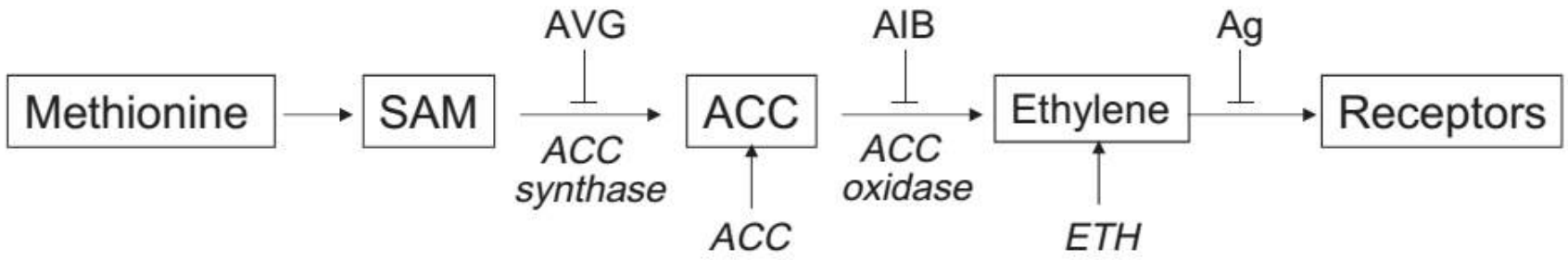
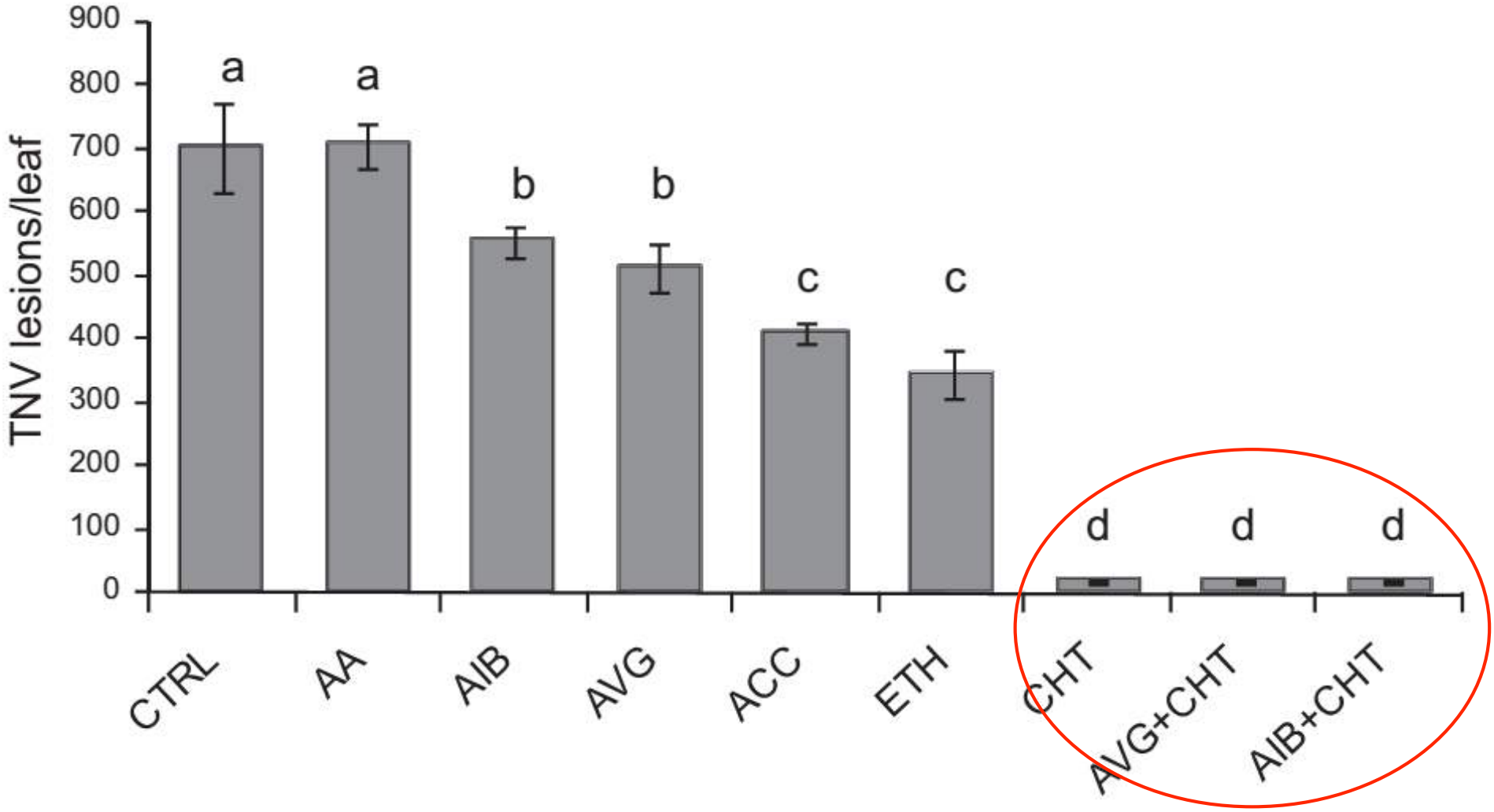


Fig. 1. Ethylene biosynthesis from methionine. Aminoethoxyvinylglycine (AVG) inhibits ACC (1-aminocyclopropane-1-carboxylic acid) synthase, which converts S-adenosylmethionine (SAM) to ACC; ACC oxidase generates ethylene from ACC and is inhibited by 2-aminoisobutyric acid (AIB). ACC and ethephon (ETH) function as ethylene precursor and donor, respectively, whereas ethylene perception is inhibited by $AgNO_3$ (Ag).

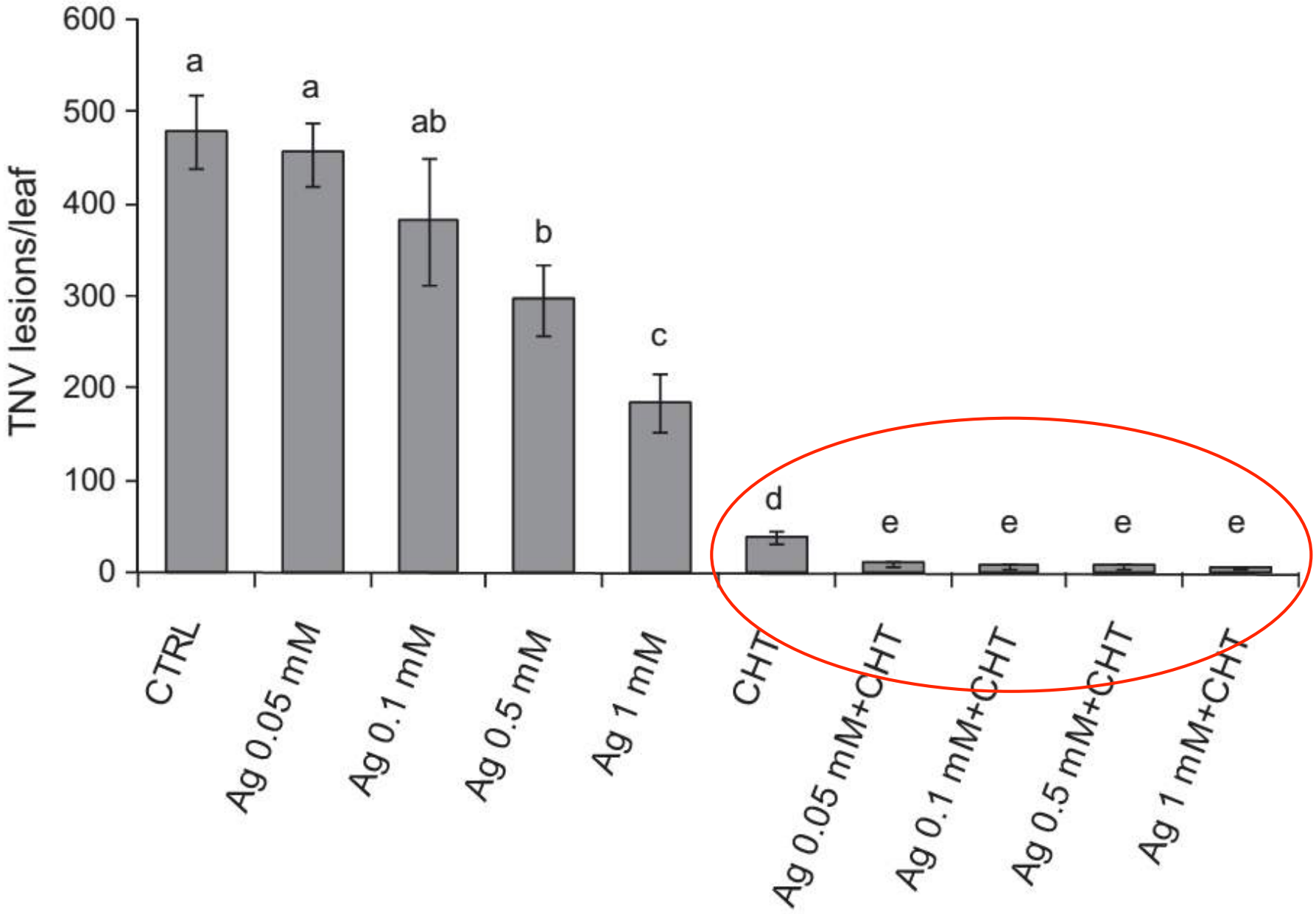
Table 1
Chemical approach: list of treatments and their activity.

Treatment	Activity
2-Aminoisobutyric acid (AIB)	ACC oxidase inhibitor
Aminoethoxyvinylglycine (AVG)	ACC synthase inhibitor
1-Aminocyclopropane-1-carboxylic acid (ACC)	Ethylene precursor
Ethephon (ETH)	Ethylene donor
Argentic nitrate ($AgNO_3$)	Inhibitor of ethylene perception
Chitosan (CHT)	Resistance inducer
Acetic acid (AA)	Solvent of CHT
Water	Untreated control

Effects of 5mM AIB, 0.2mM AVG (ethylene inhibitors), 2mM ACC (Ethylene precursors) and 5mM ETH (Ethylene donor) on the 0.15% CHT antiviral activity

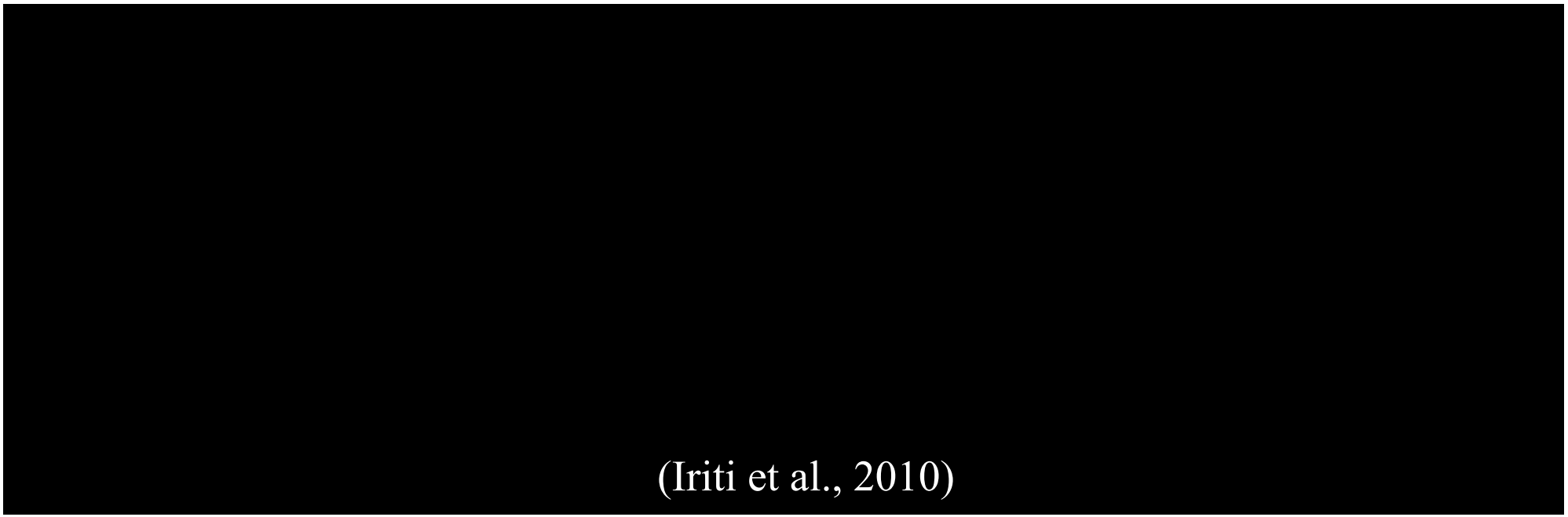


Effects of $AgNO_3$ (receptor inhibitor) on the CHT antiviral activity



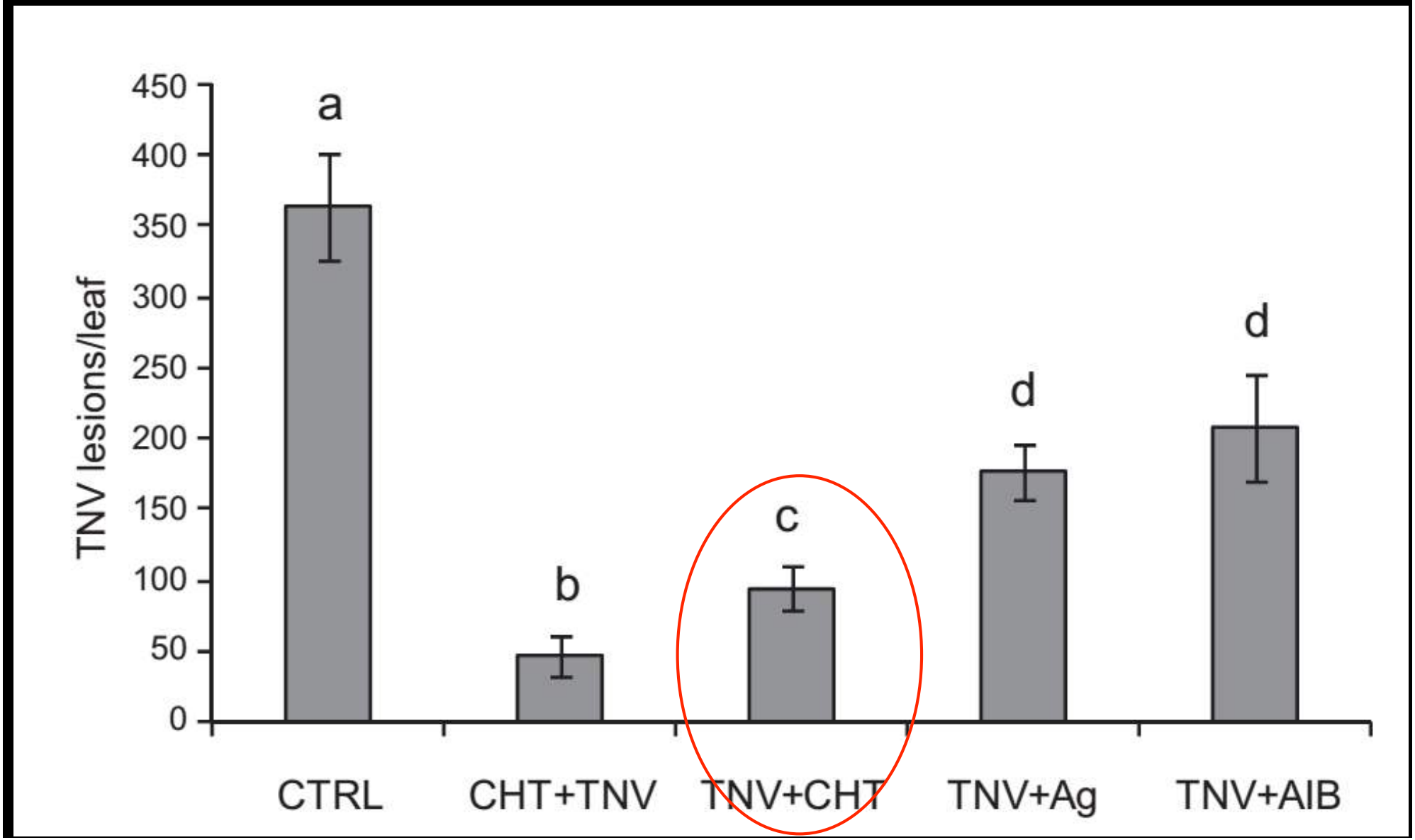
3. Results

3.1. CHT does not exert direct viral activity and must enter stomata to induce resistance to TNV

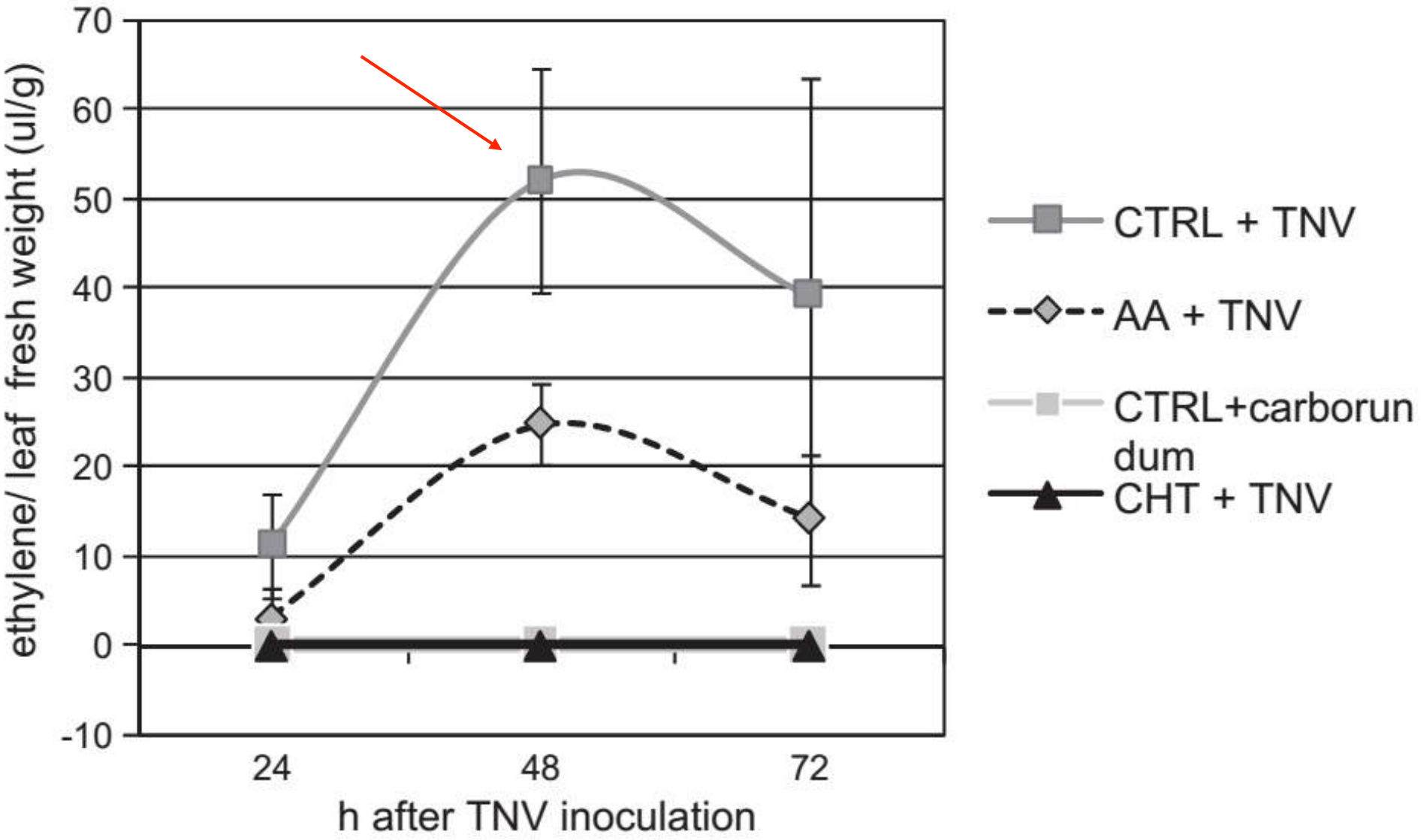


(Iriti et al., 2010)

Effects of ethylene inhibitors (0.1mM $AgNO_3$ and 5mM AIB) on the disease development and CHT curative efficacy

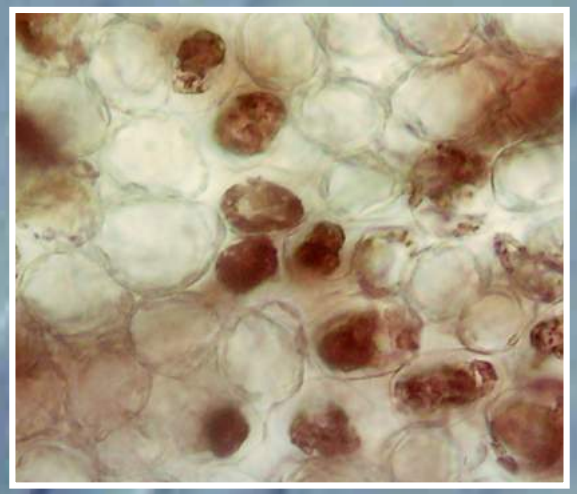


Ethylene production

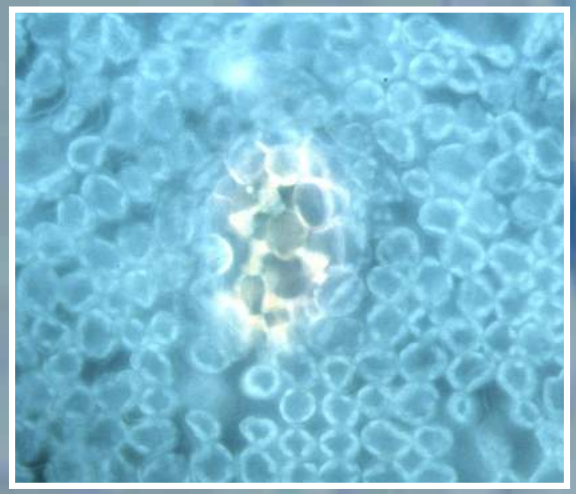


Chitosan action mechanism as elicitor

microburst due to H_2O_2 deposition

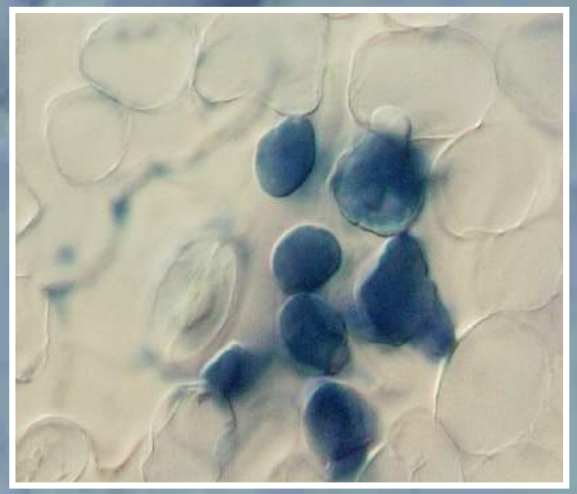


callose apposition

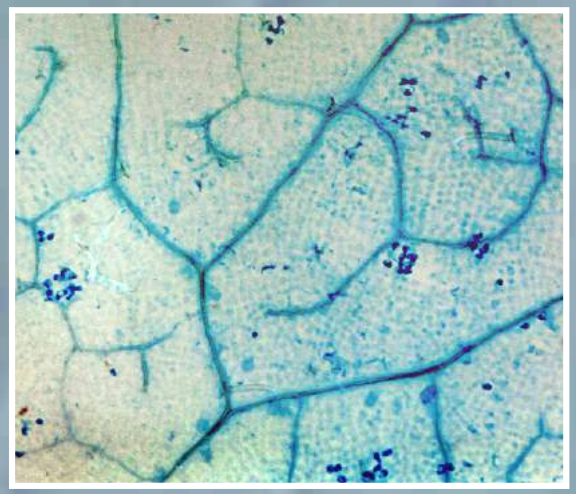


Ca^{++} influx

PCD

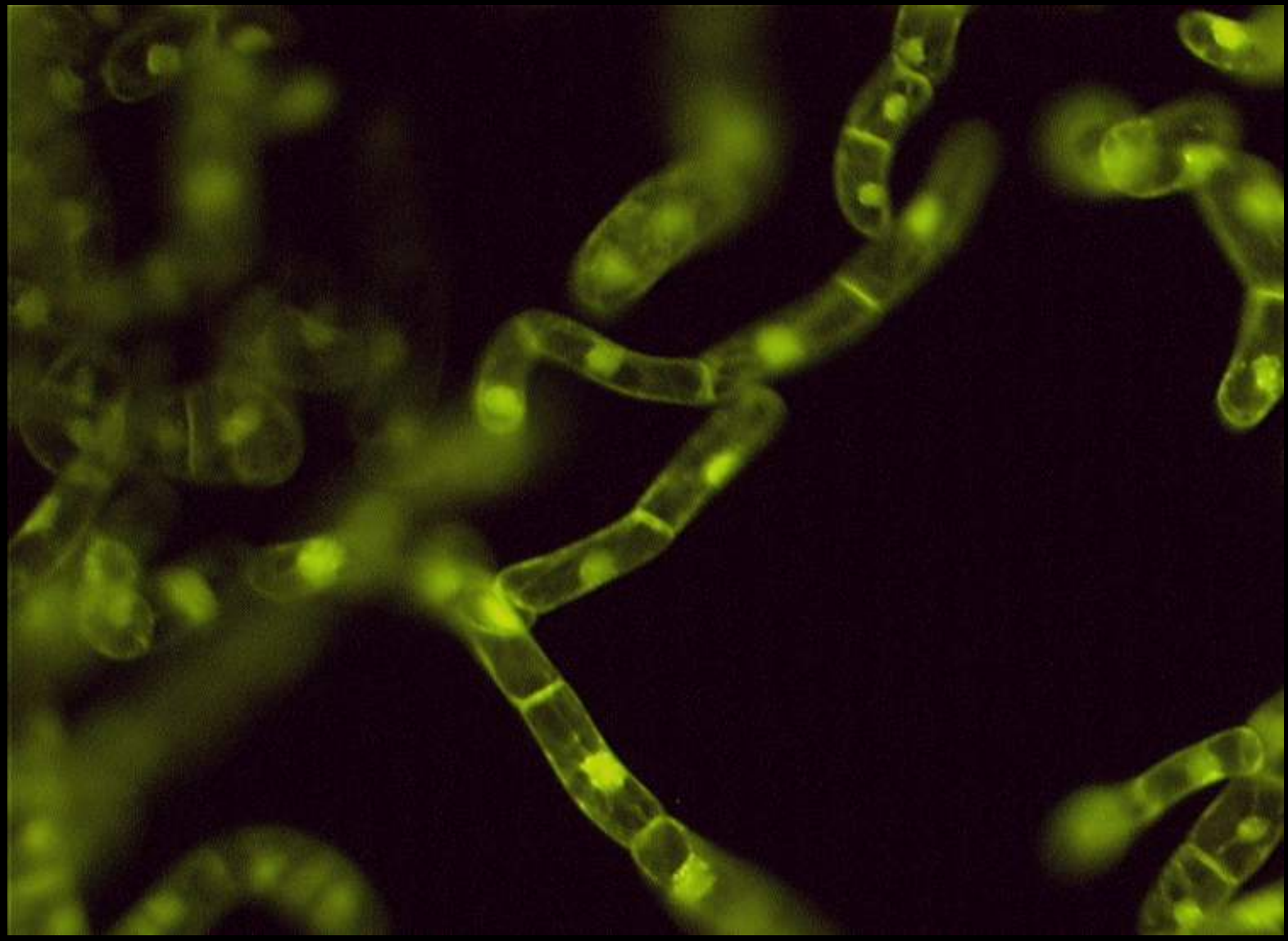


microHR-like network

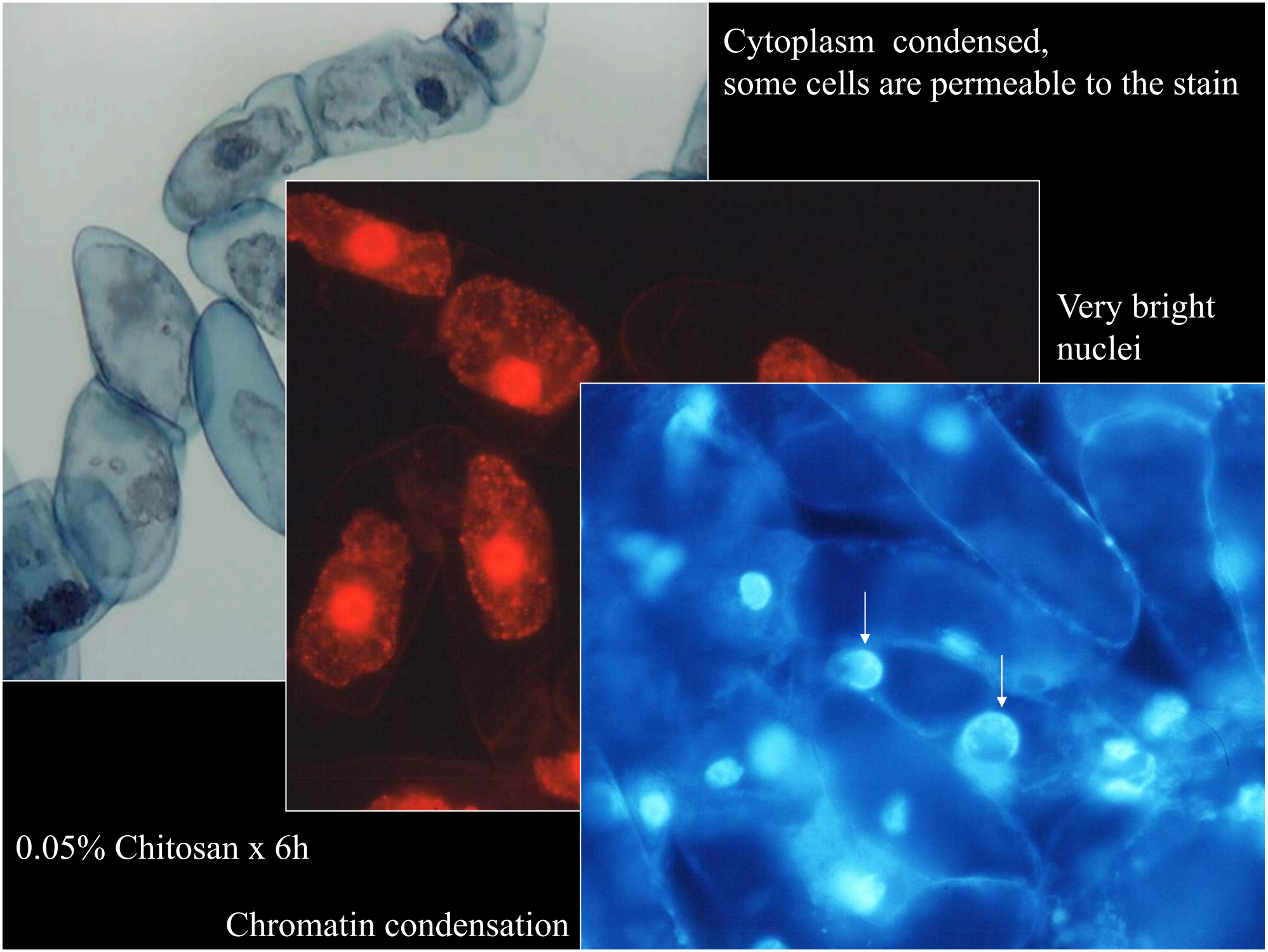


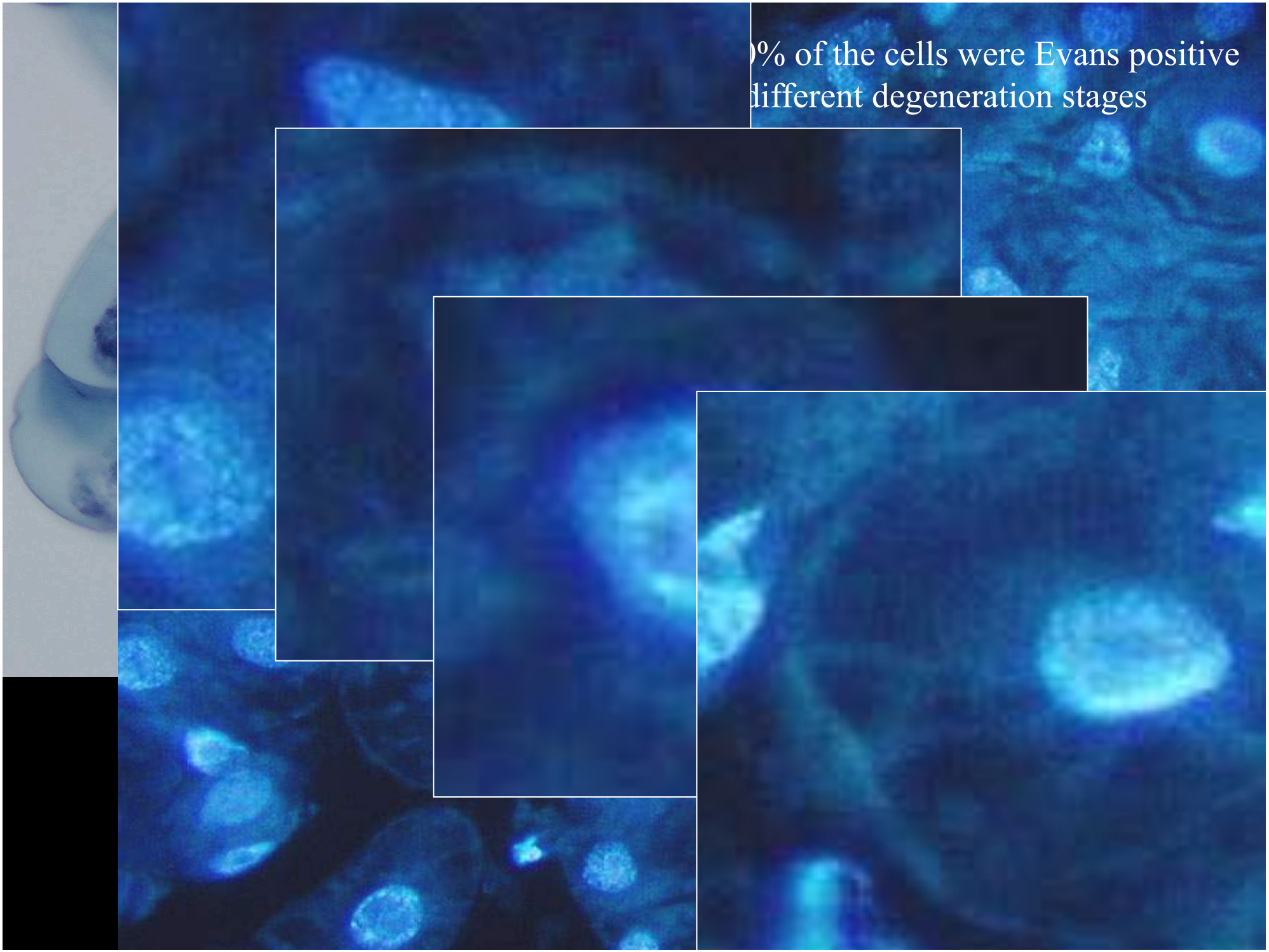
SAR

Hypersensitive response/Programmed cell death (HR\PCD)

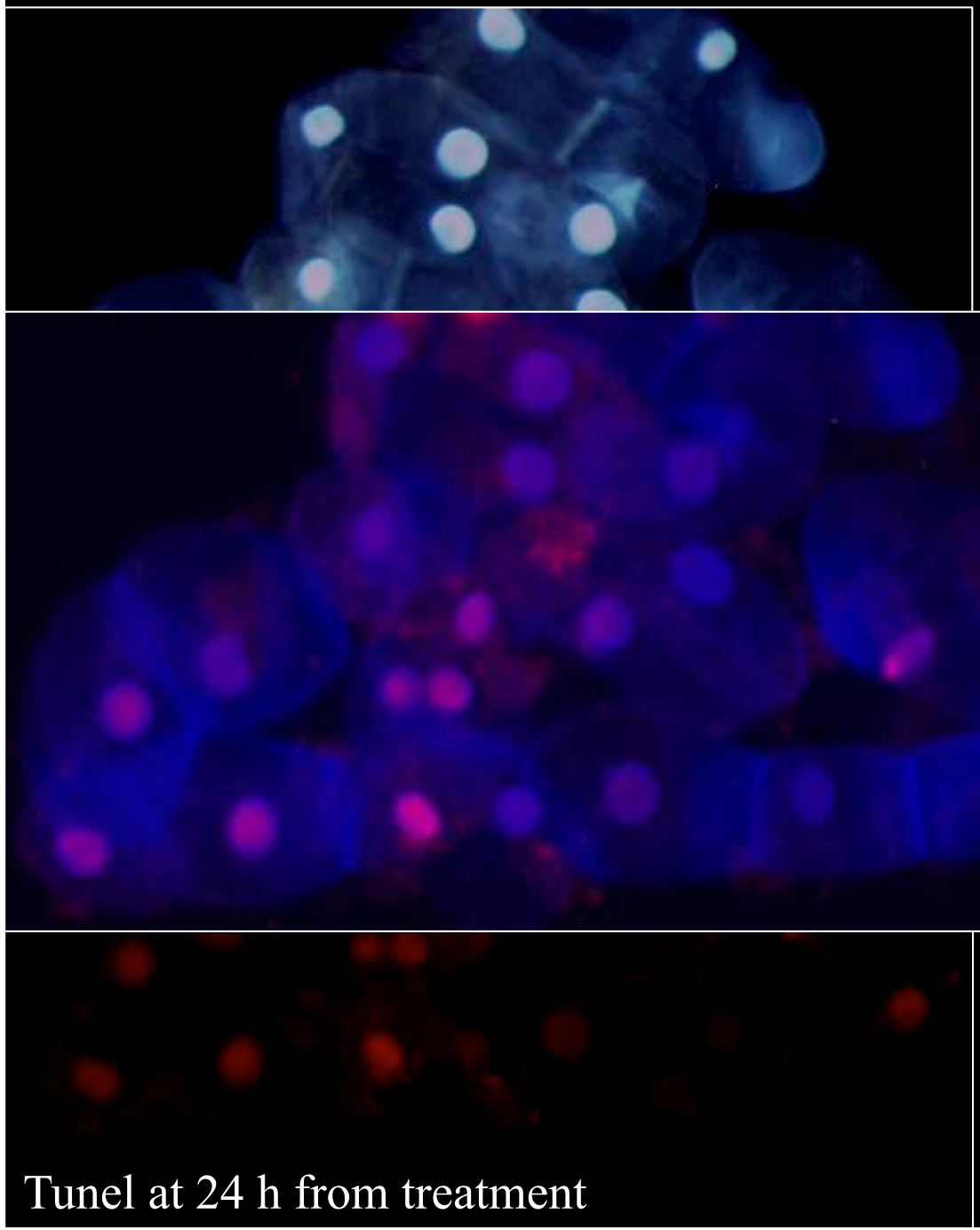


(Iriti et al., 2006; Iriti and Varoni, 2015)

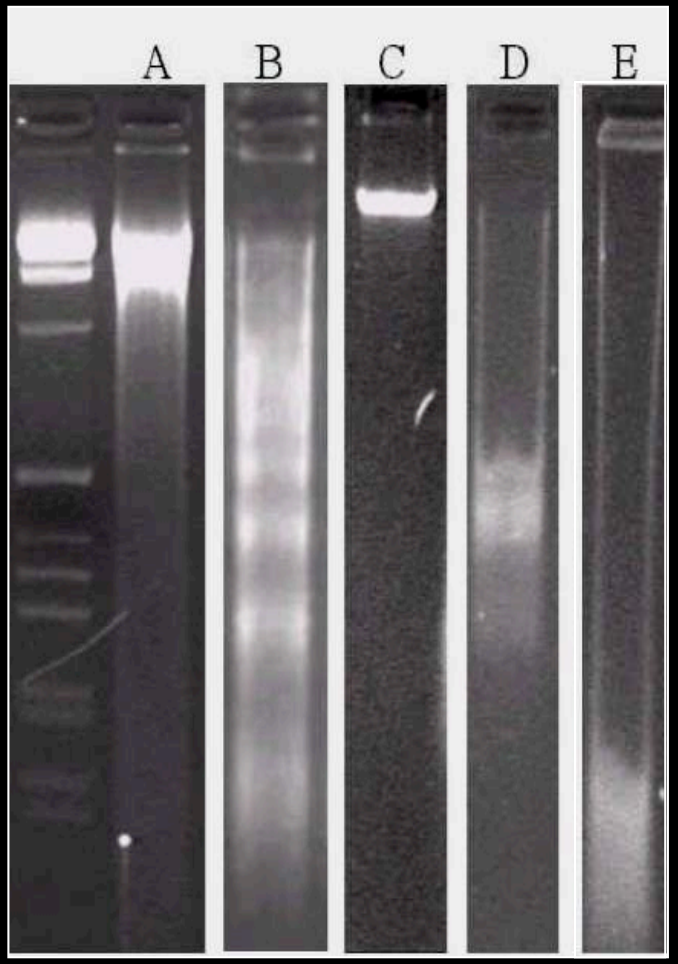

Cytoplasm condensed,
some cells are permeable to the stain
Very bright
nuclei
0.05% Chitosan x 6h
Chromatin condensation


% of the cells were Evans positive
different degeneration stages

Tunel and DNA laddering confirm chitosan-induced PCD

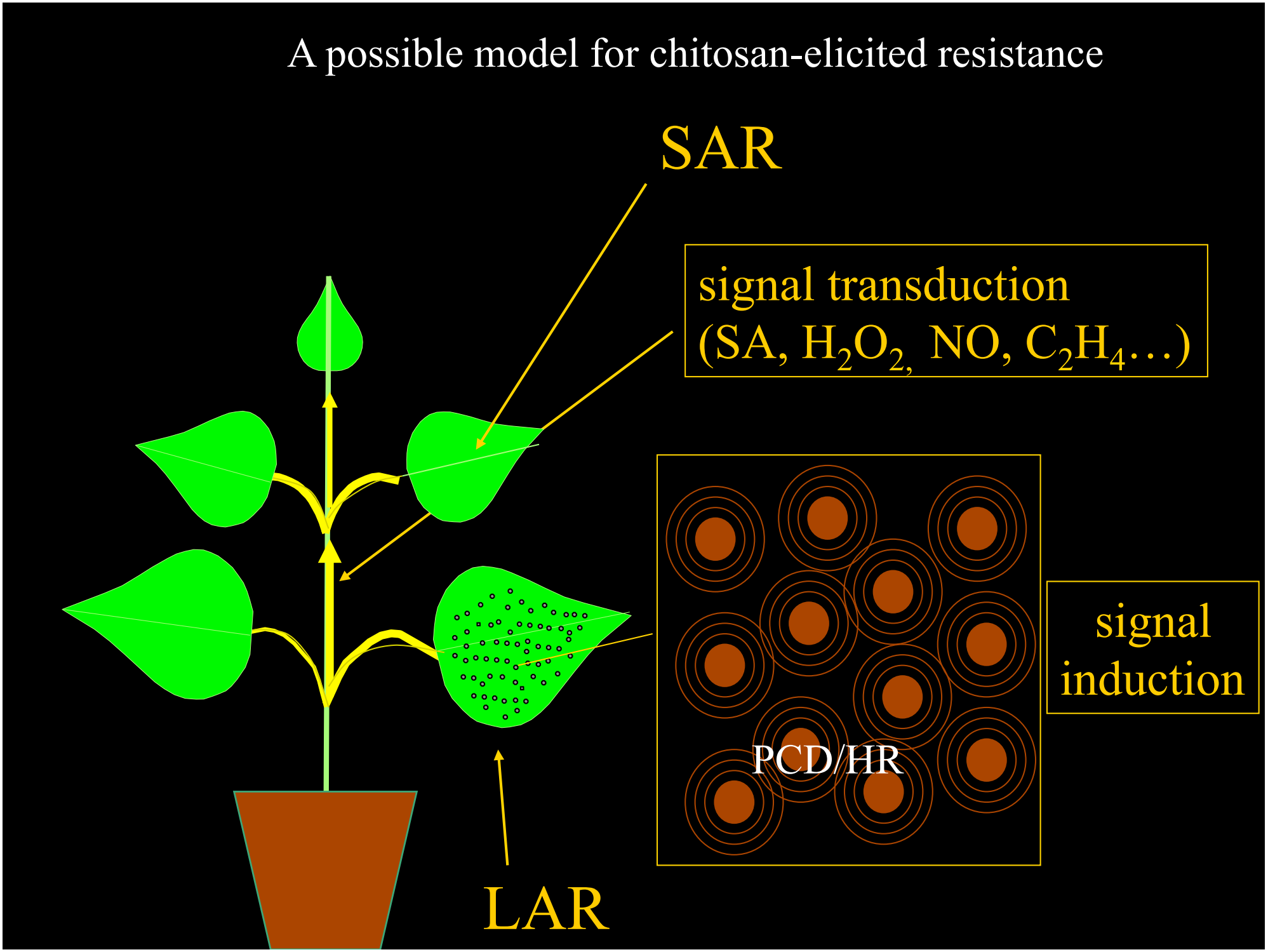


Tunel at 24 h from treatment

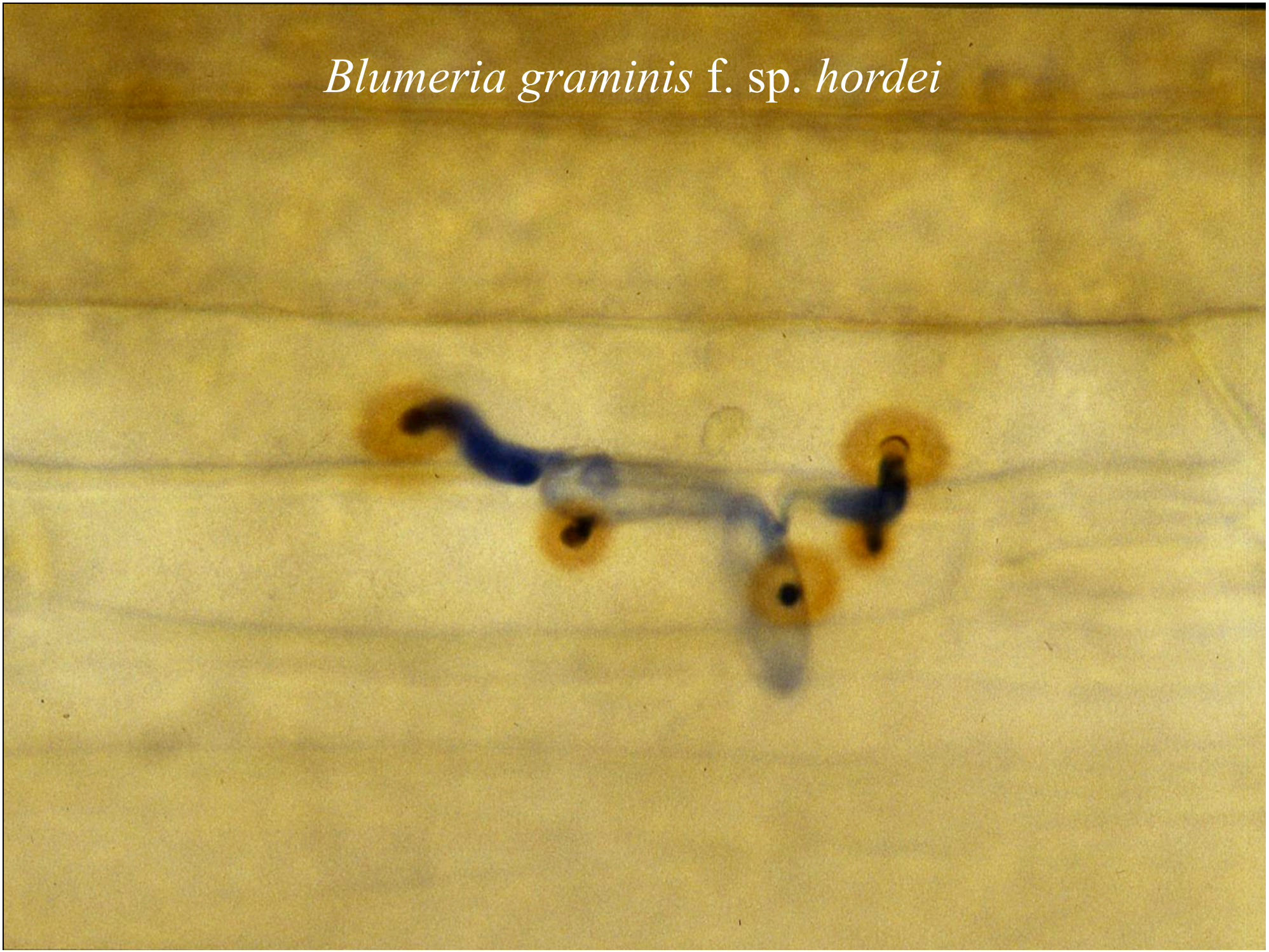


A = control
B = 0.05% chitosan
in 0.05% acetic acid
C = 0.05% acetic acid
D = 0.05% chitosan
in 0.05% acetic acid
E = boiled cells

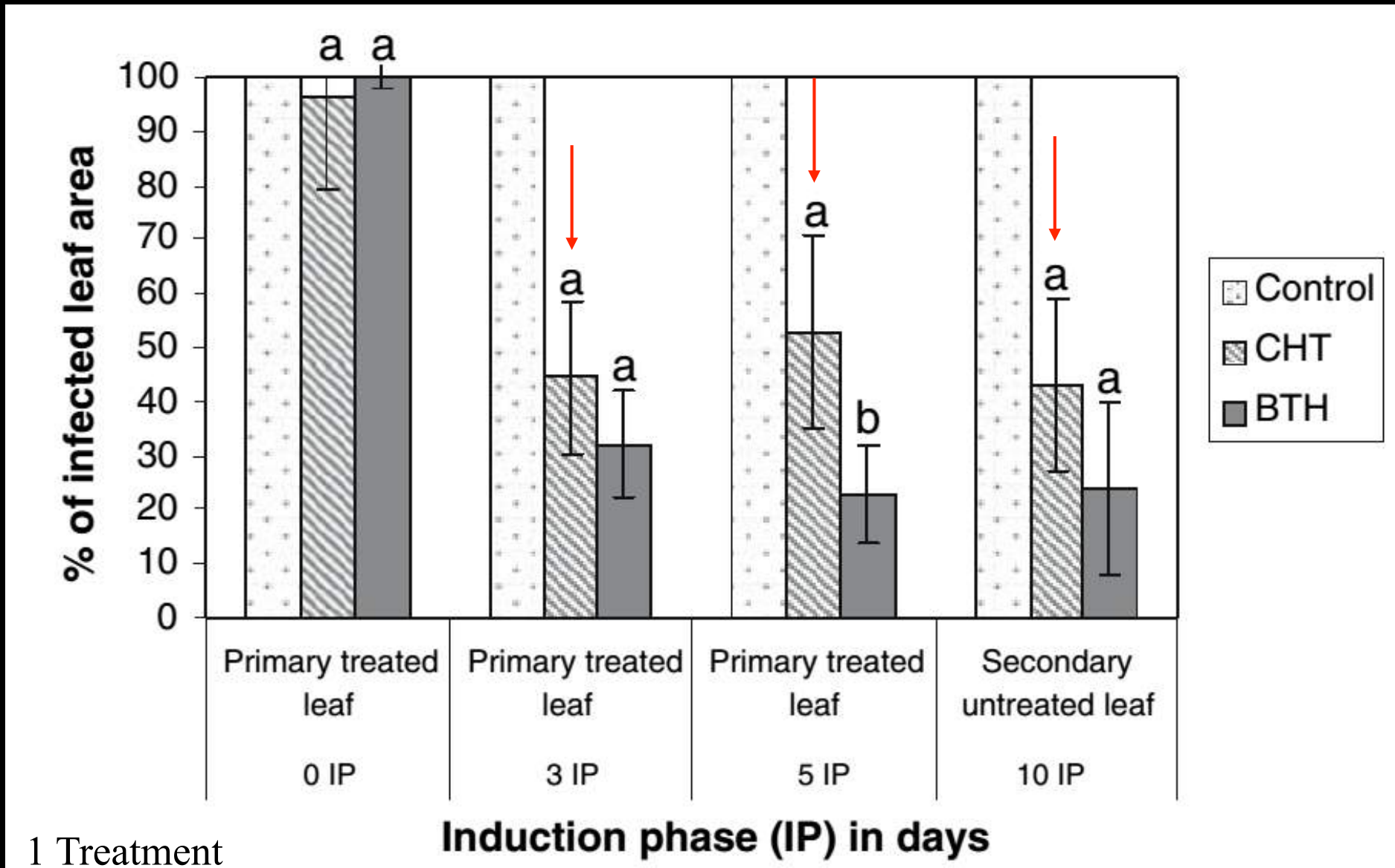
A possible model for chitosan-elicited resistance



3. Antifungal activity of chitosan (case studies and mechanisms)

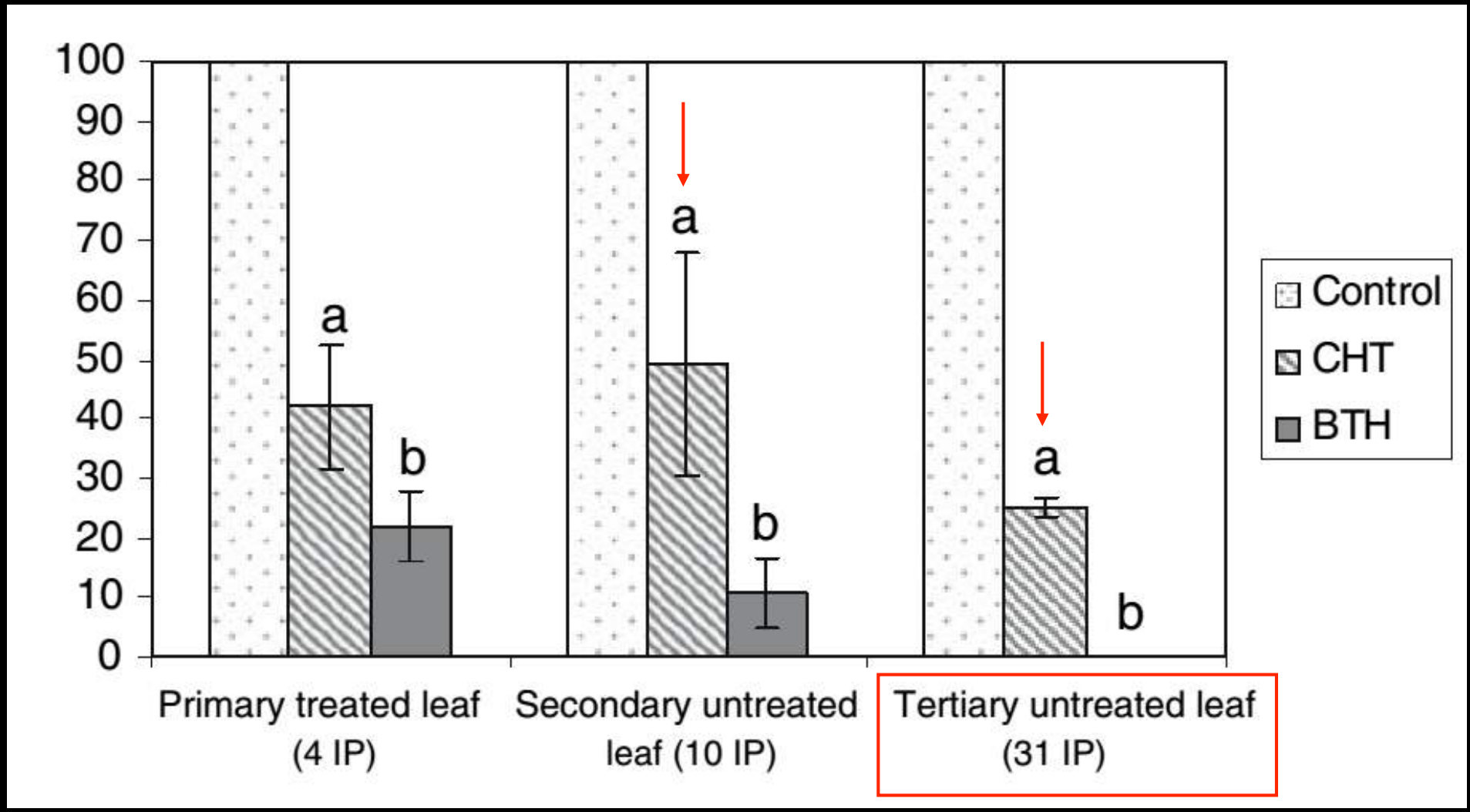

Blumeria graminis f. sp. *hordei*

Evaluation of local (LAR) and systemic (SAR) acquired resistance induced by treatments with BTH or CHT after different induction phases

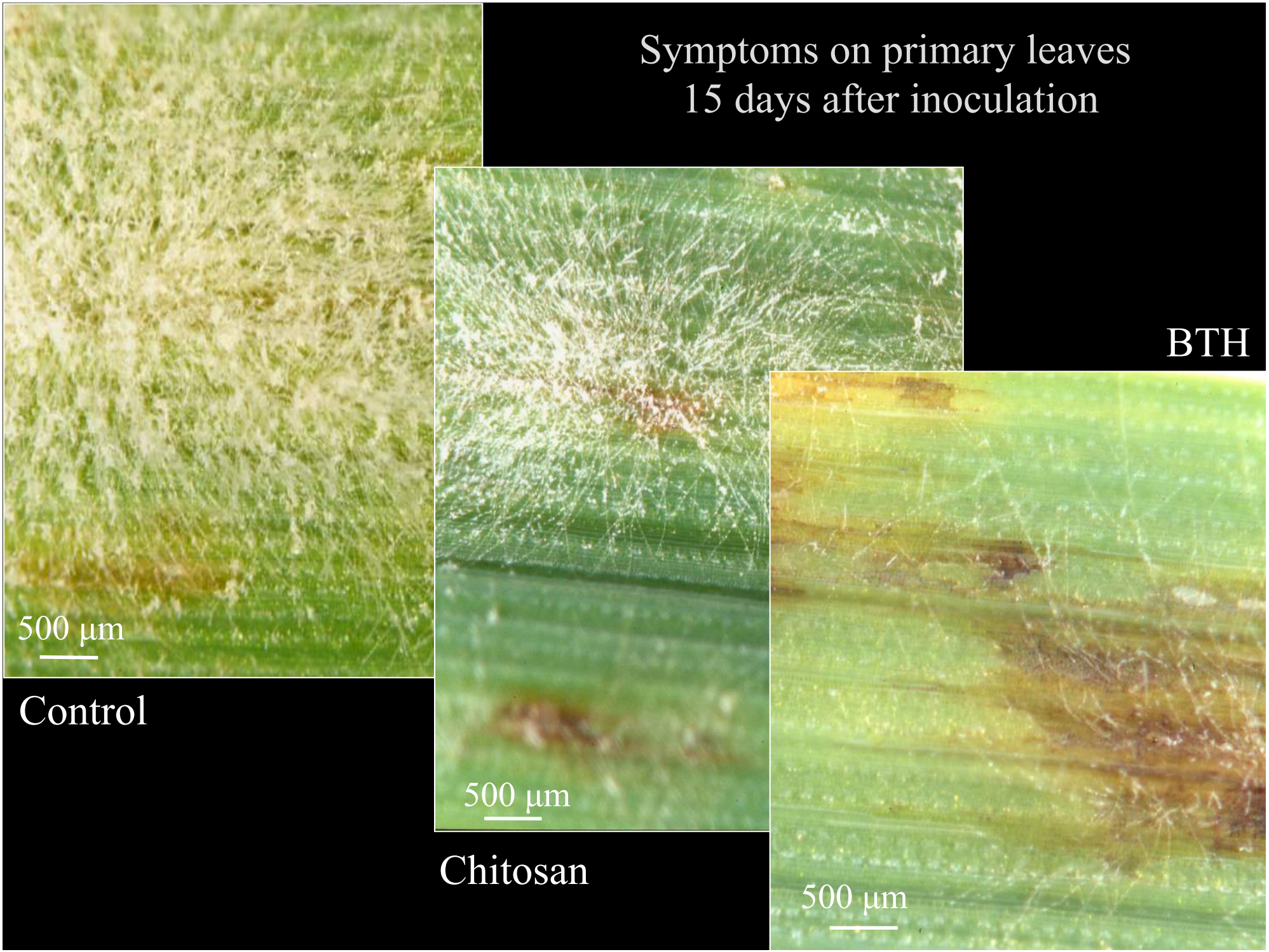


1 Treatment

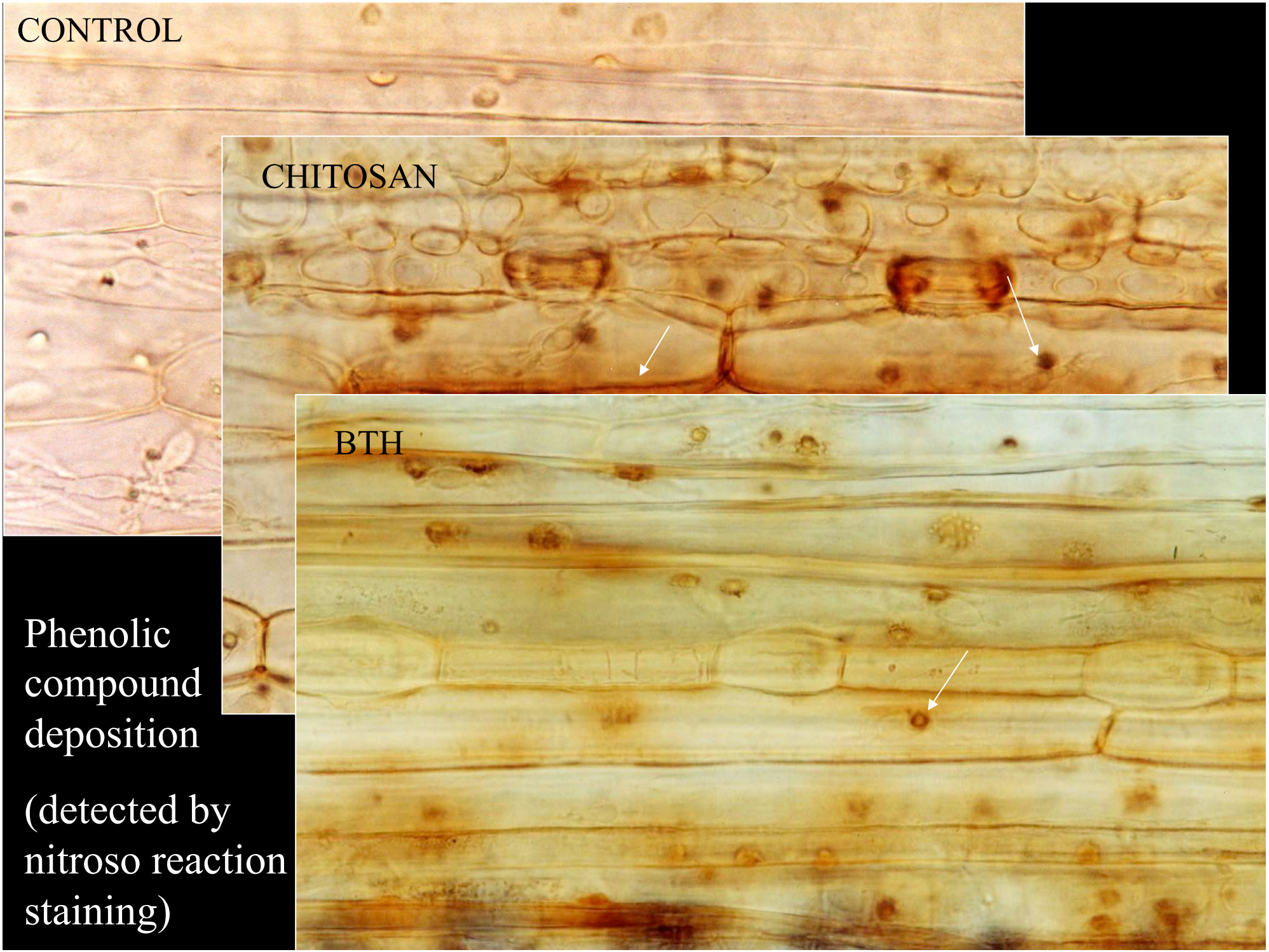
Evaluation of local (LAR) and systemic (SAR) acquired resistance induced by treatments with BTH or CHT after different induction phases



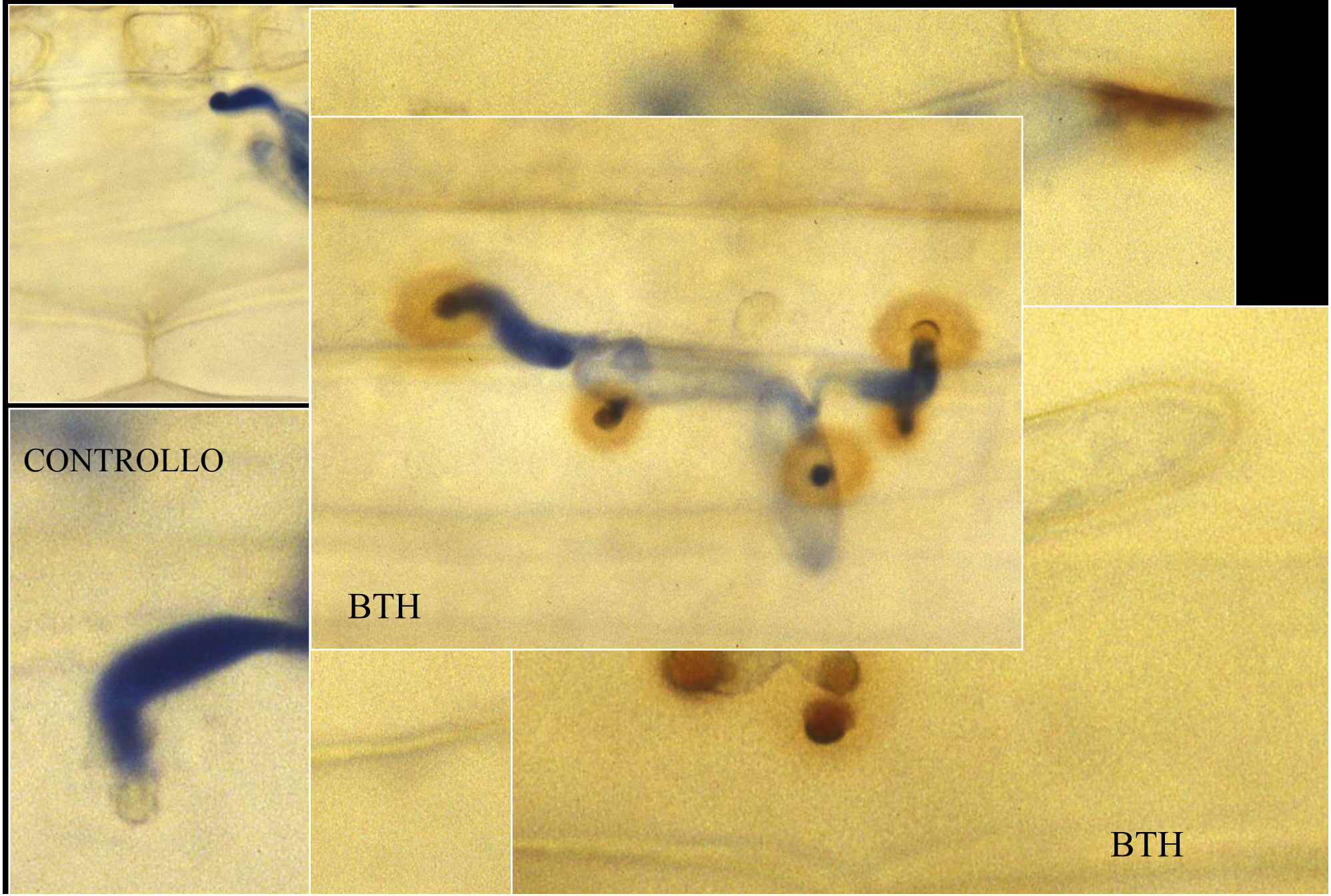
2 Treatments


Symptoms on primary leaves
15 days after inoculation
BTH
500 µm
Control
500 µm
Chitosan
500 µm

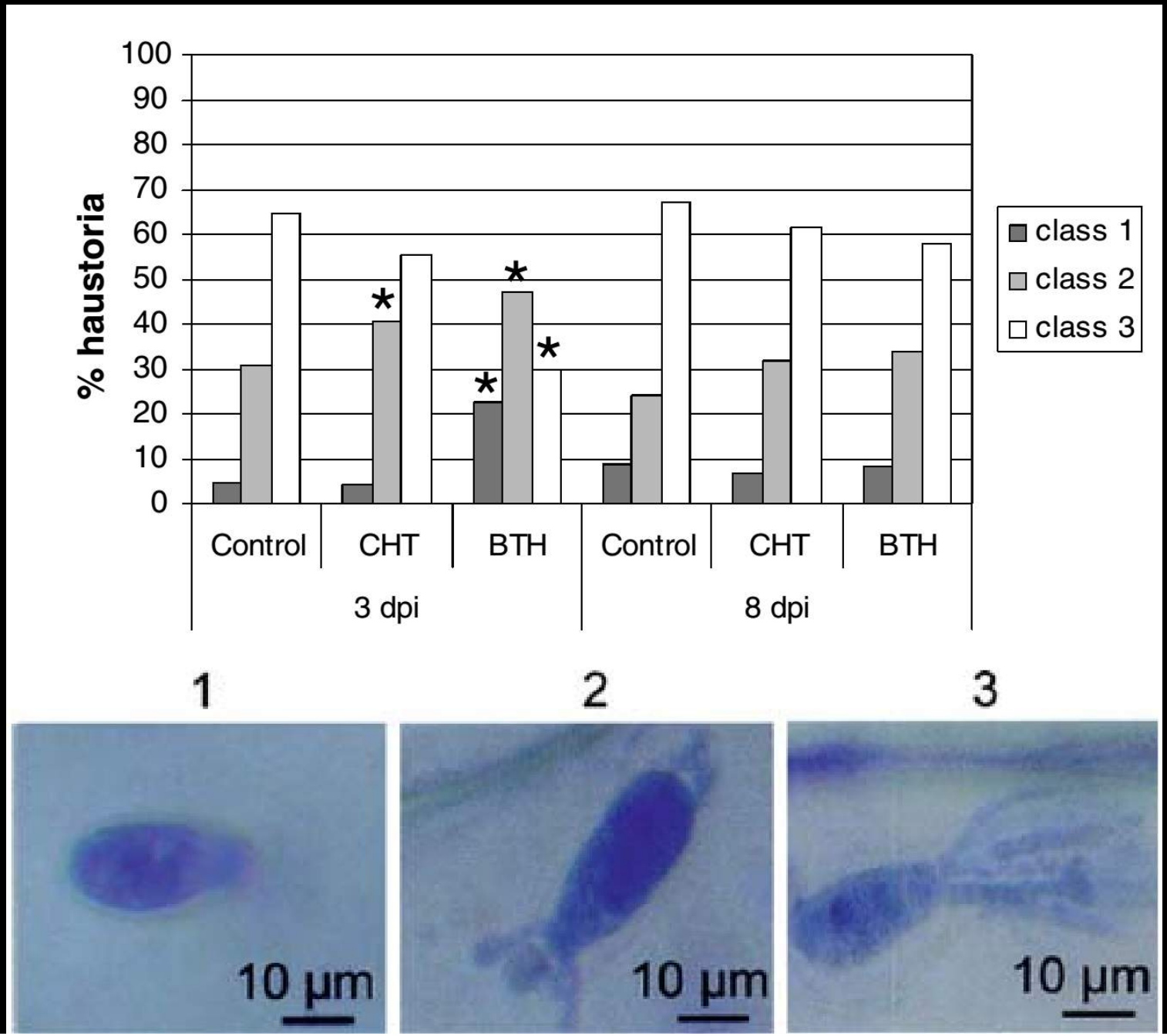

Primary germ tube
Papilla
Conidium
Secondary germ tube
10 µm
Aniline blue staining
In presence of 2-deoxy-D-glucose (DDG)


CONTROL
CHITOSAN
BTH
Phenolic compound deposition
(detected by nitroso reaction staining)

Localization of H_2O_2 with 3-3’-diaminobenzidine (DAB)



Haustorium development at 4 dpi



Montepulciano d'Abruzzo – *Erysiphe necator*

(Iriti et al., 2011)

CHT efficacy on powdery mildew

Table 2. Disease incidence (expressed as % of infected leaves or bunches) and severity (expressed as % of infection according to Townsend–Heuberger formula) in the untreated parcels of Montepulciano d'Abruzzo grapevines or in plots treated with Kendal Cops® at different concentrations and/or in combination with fungicides (Fs) (Kc-H, Kc-L, Kc-L/F, and Kc + F), or treated with Fs alone.

Treatments	26 June				1 August			
	Leaves		Bunches		Leaves		Bunches	
	% incidence	% severity	% incidence	% severity	% incidence	% severity	% incidence	% severity
Kc-H	4.67cd†	0.11c	37.78c	0.72c	12.67cd	0.84c	46.67cd	2.39d
Kc-L	17.53b	0.54b	73.33b	3.61b	52.67b	12.62b	77.78b	10.94b
Kc-L/F	8.00c	0.30bc	28.89cd	0.64c	44.00b	5.42bc	73.33b	9.89bc
Kc + F	2.00cd	0.07c	15.56de	0.36c	38.67bc	3.59bc	62.22bc	4.22cd
F	1.33d	0.02c	4.44e	0.11c	0.67d	0.02c	28.89d	0.92d
Untreated	98.00a	47.30a	100.00a	87.50a	100.00a	87.50a	100.00a	87.50a

Data were collected the 26 June and the 1 August 2007. †Different letters indicate means significantly different at $P < 0.05$ (Tukey test).

CHT effects on grape polyphenols

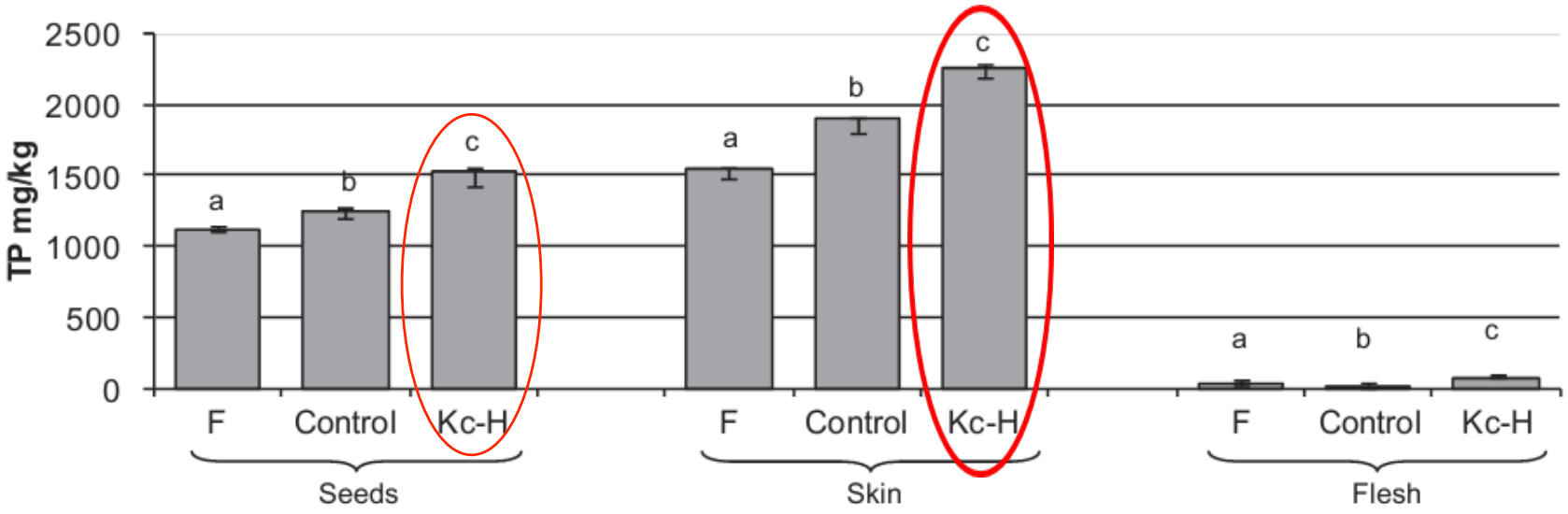


Figure 1. Total polyphenols (TPs) of different grape berry tissues treated with conventional fungicides (Fs) or Kendal Cops® at 10 mL/L (Kc-H) and the untreated control. Samples were collected at complete veraison and TP content was measured by the Folin–Ciocalteau colorimetric assay. Results are mean ± standard deviation of three independent extractions. Bars carrying different letters indicate means significantly different at $P < 0.05$ (Fisher's least significant difference test).

CHT effects on wine polyphenols

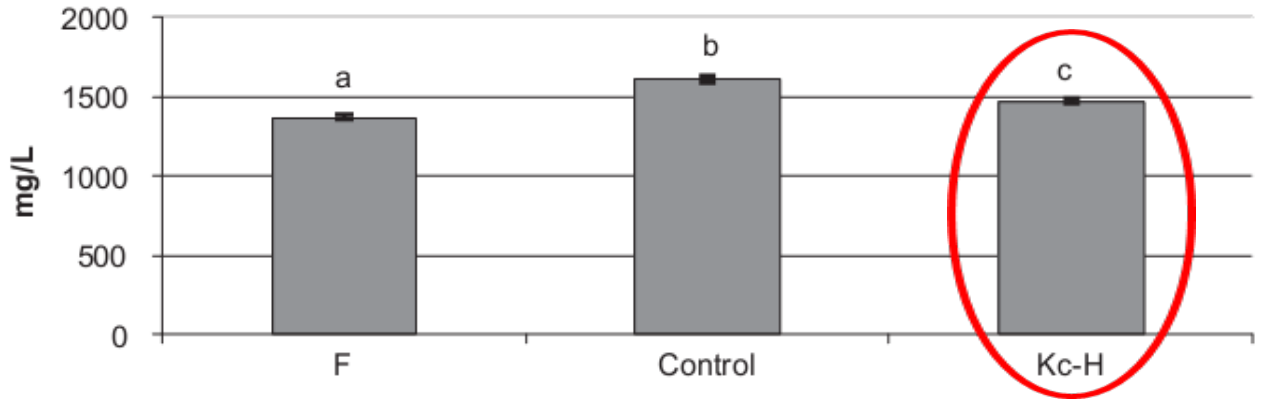


Figure 2. Total polyphenols (TPs) of wines obtained from grapes treated with conventional fungicides (Fs) or Kendal Cops®, at 10 mL/L (Kc-H) and from relative untreated bunches, as control. TP content was measured by the Folin–Ciocalteau colorimetric assay. Results, expressed as gallic acid mEq, are mean ± standard deviation of three replicates. Bars carrying different letters indicate means significantly different at $P < 0.05$ (Fisher's least significant difference test).

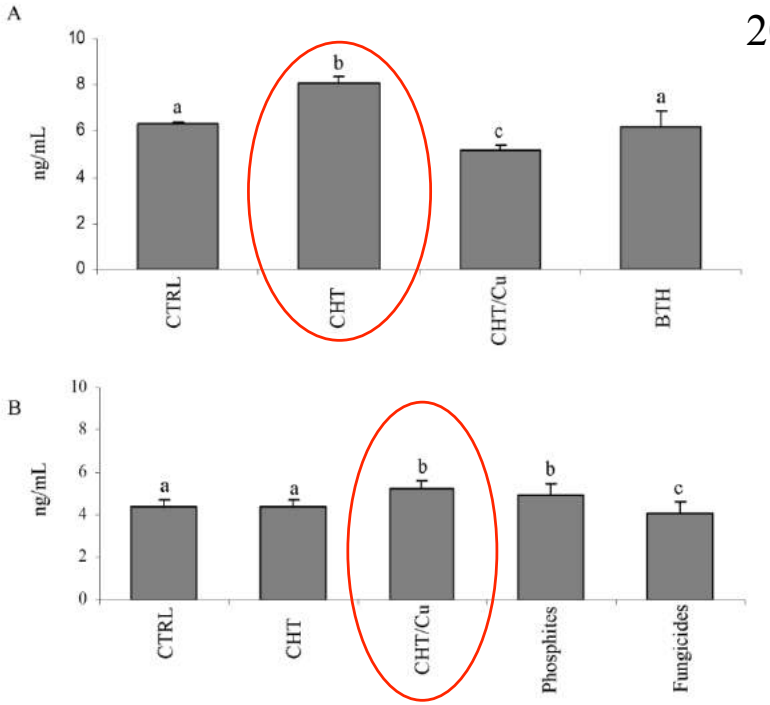
Antiradical capacity

Table 3. Antiradical activity of grape berry tissues and wines made from grapes treated with fungicides, Kendal Cops® at 10 mL/L (Kc-H) and untreated, measured by DPPH (2,2-diphenyl-picrylhydrazyl free radical) scavenging test.

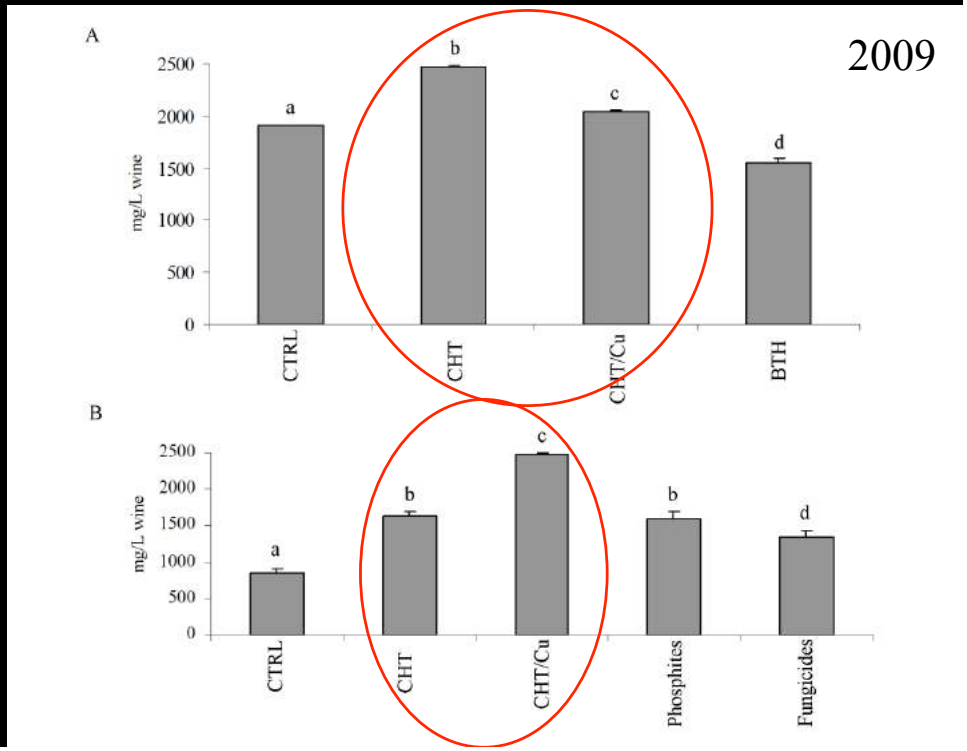
	Sample	DPPH (IC_{50}) µM
Reference compounds	Trolox	12.9 ± 0.82†
	Ascorbic acid	9.65 ± 0.63
	Quercetin	4.37 ± 0.59
Seeds	Fungicides	5.94 ± 1.25a‡
	Untreated control	5.24 ± 0.59b
	Kc-H	3.88 ± 0.87c
Skin	Fungicides	12.1 ± 0.57a
	Untreated control	12.2 ± 1.20a
	Kc-H	10.2 ± 1.13b
Wines	Fungicides	21.7 ± 1.38a
	Untreated control	17.2 ± 0.73b
	Kc-H	16.9 ± 1.55b

†Values are mean ± standard deviation of three independent extractions. ‡Different letters indicate means significantly different at $P < 0.05$ (Fisher's least significant difference test).

Groppello

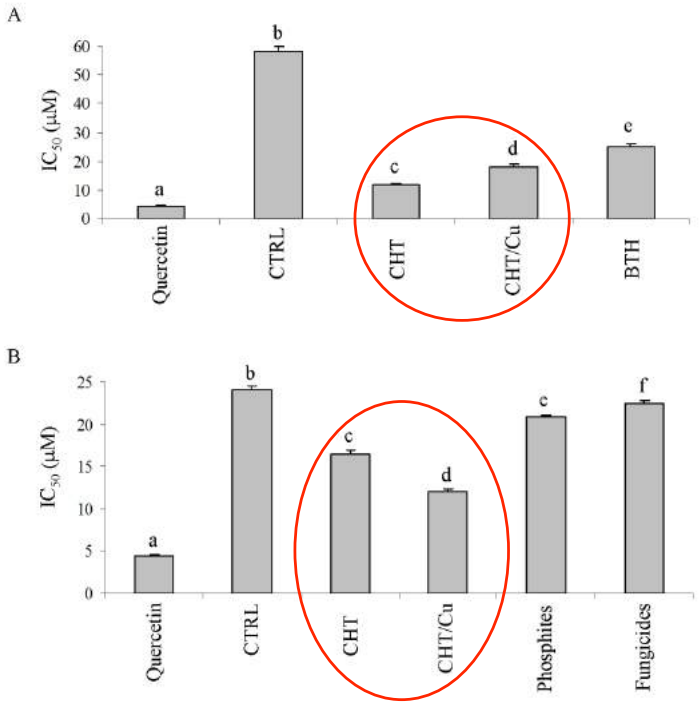


Melatonin concentration (ng/mL wine) measured by UPLCMS/MS in experimental (A) Groppello and (B) Merlot wines produced by the microvinification of grapes from different phytoiatric treatments (Vitalini et al., 2011)



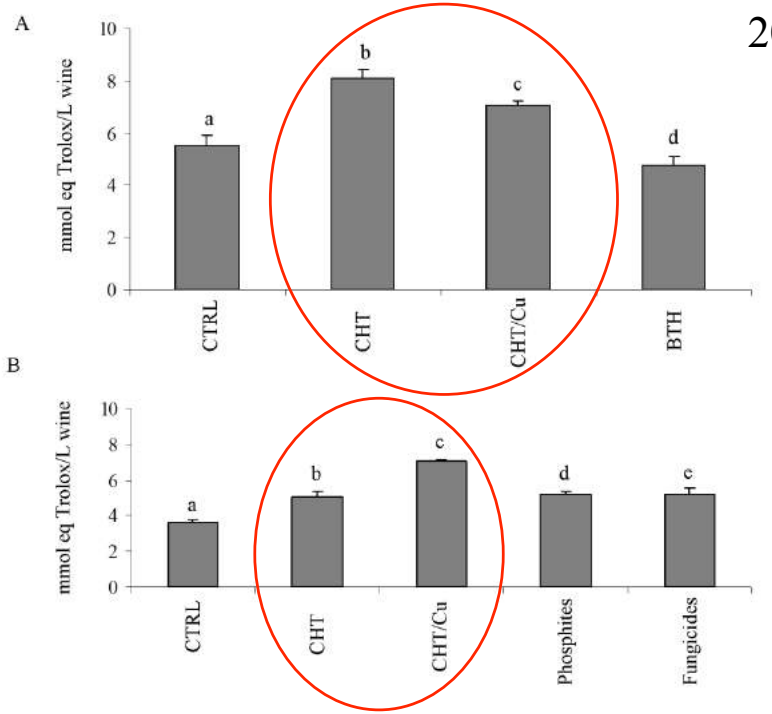
Total **polyphenol** content (mg/L wine) measured by the Folin-Ciocalteu colorimetric assay in experimental (A) Groppello and (B) Merlot wines produced by microvinification of grapes from different phytoiatric treatments (Vitalini et al., 2011)

2009



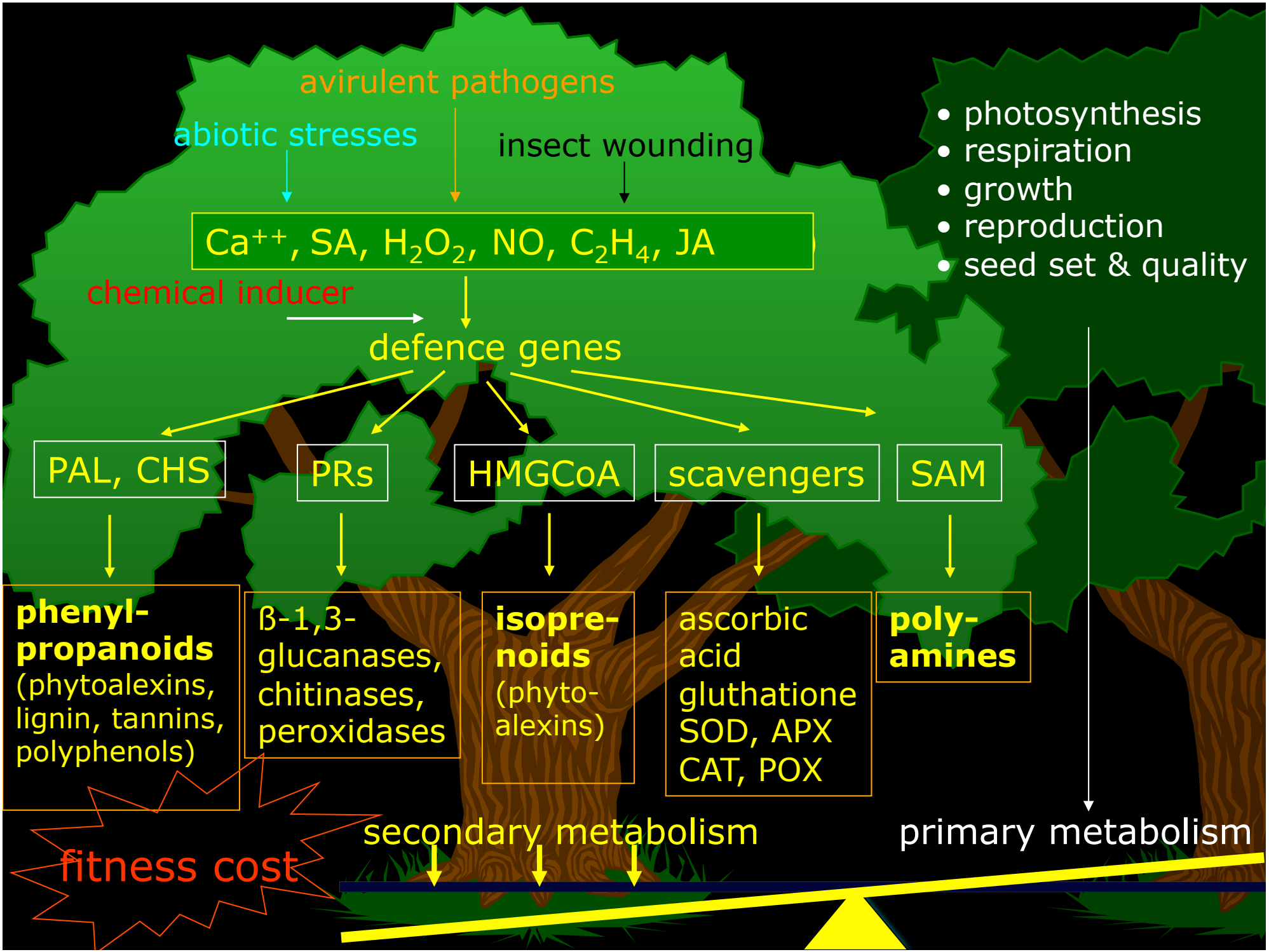
DPPH (2,2-diphenyl-picrylhydrazyl) radical-scavenging activity (IC_{50}) of (A) Groppello and (B) Merlot wines obtained by microvinification of grapes from different phytoiatric treatments (Vitalini et al., 2011)

2009



ABTS [2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)] radical-scavenging activity (TEAC, mmol eq Trolox/L wine) of (A) Groppello and (B) Merlot wines obtained by microvinification of grapes from different phytoiatric treatments (Vitalini et al., 2011)

4. Fitness costs and crop yield


avirulent pathogens
abiotic stresses
insect wounding
Ca^{++}, SA, H_2O_2, NO, C_2H_4, JA
chemical inducer
defence genes
PAL, CHS
PRs
HMGCoA
scavengers
SAM
phenyl-propanoids (phytoalexins, lignin, tannins, polyphenols)
ß-1,3-glucanases, chitinases, peroxidases
isopre-noids (phyto-alexins)
ascorbic acid gluthatione SOD, APX CAT, POX
poly-amines
• photosynthesis
• respiration
• growth
• reproduction
• seed set & quality
fitness cost
secondary metabolism
primary metabolism

Fitness costs

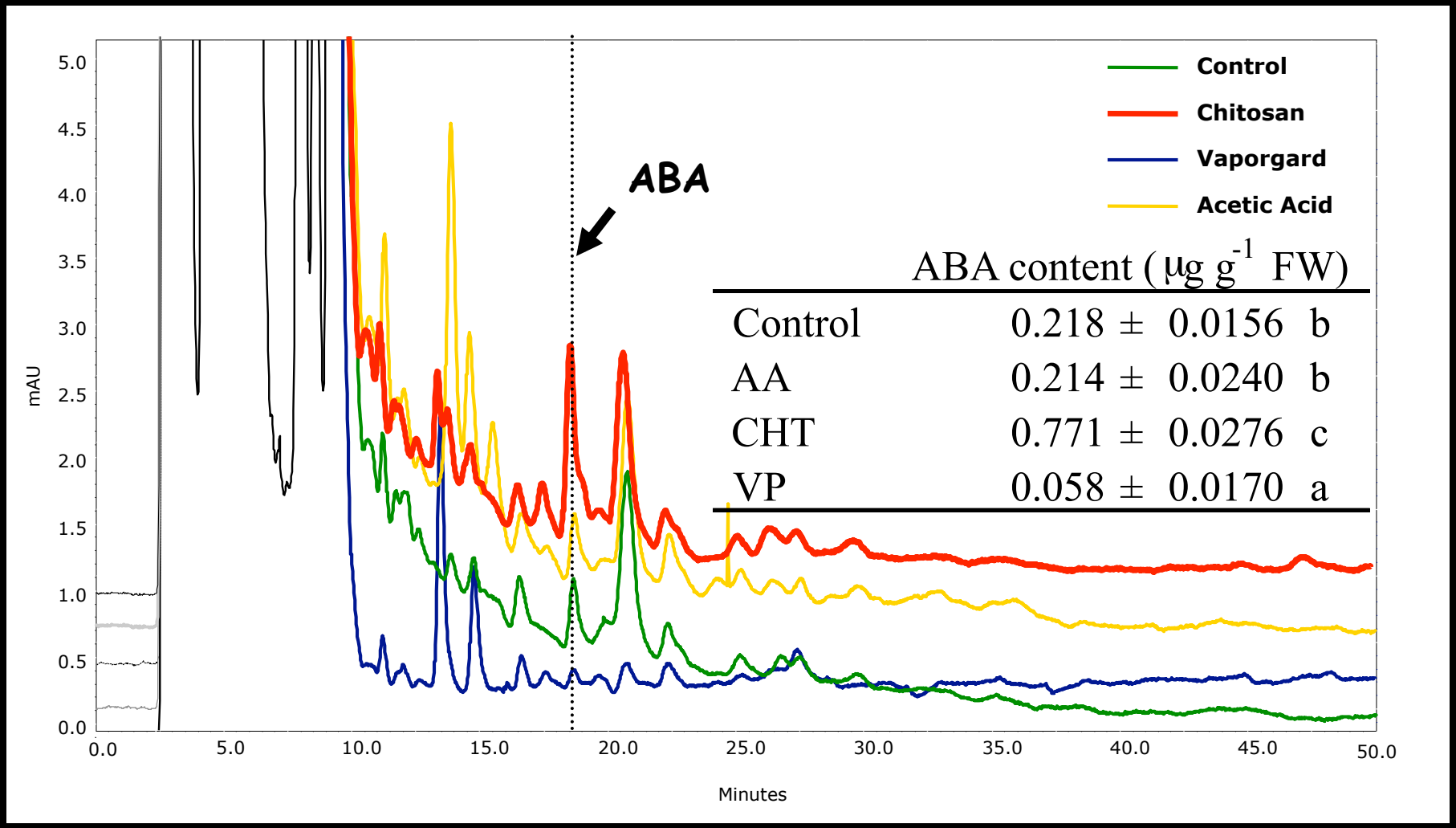
Table 4
Crop yield parameters of bean (*Phaseolus vulgaris* L.) plants treated with 0.05% chitosan (CHT) or water (CTRL), every 10 day in an open filed trial in enemy-free conditions. Values are means ± SE of data collected from two independent plots.

Treatments	Number of pods/ plant	Number of seeds/ pod	Number of seeds/ plant	Dry weight pods/ plant (g)	Dry weight seeds/ pod (g)	Dry weight/plant (g)	100-Seed dry weight (g)
CTRL	5.60 ± 0.29	4.17 ± 0.09	23.43 ± 1.31	0.65 ± 0.03	2.06 ± 0.07	0.49 ± 0.01	52.05 ± 0.75
CHT	6.13 ± 0.41*	4.30 ± 0.10	25.87 ± 2.07*	0.66 ± 0.03	2.12 ± 0.06	0.50 ± 0.01	51.93 ± 0.36

* Indicates significant difference at $p < 0.05$ (Tukey's test).

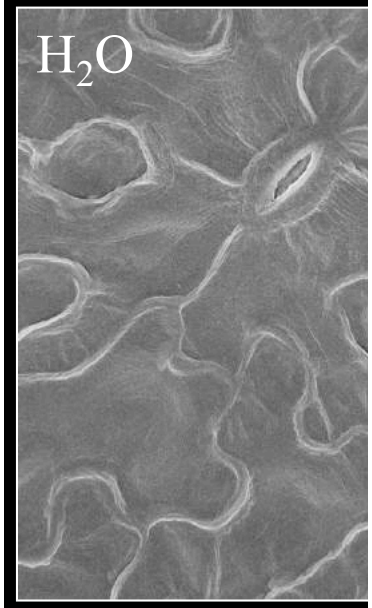
5. Antitranspirant activity of chitosan

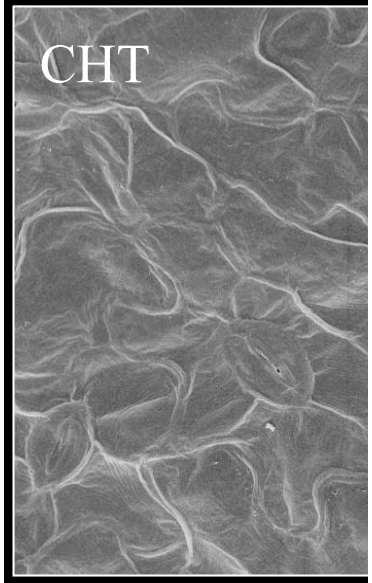
CHT-induced antitranspirant activity, in bean plants, is mediated by ABA, that raises over three-folds in treated leaves, 24 h after foliar treatment

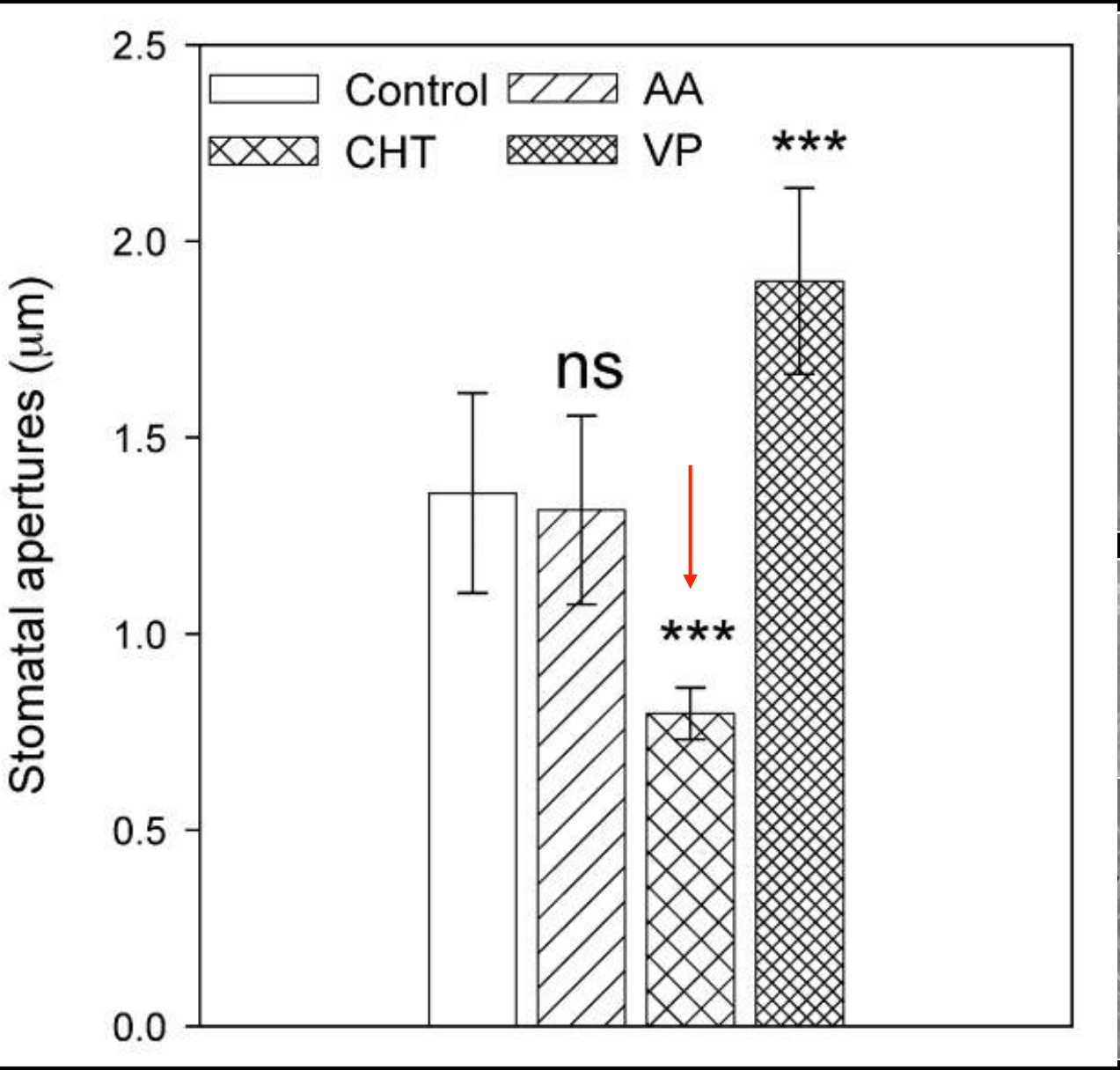


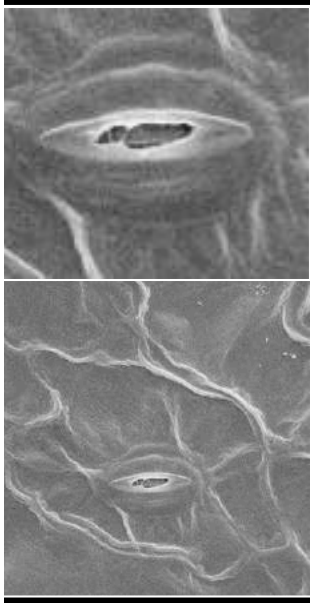
ABA content ($\mu g\ g^{-1}$ FW)		
Control	0.218 ± 0.0156	b
AA	0.214 ± 0.0240	b
CHT	0.771 ± 0.0276	c
VP	0.058 ± 0.0170	a

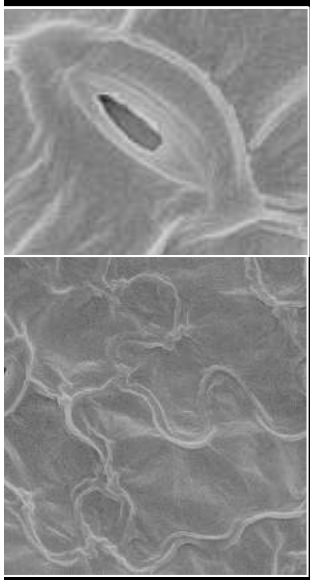
SEM analysis and stomatal aperture of treated bean leaves 24 h after treatment



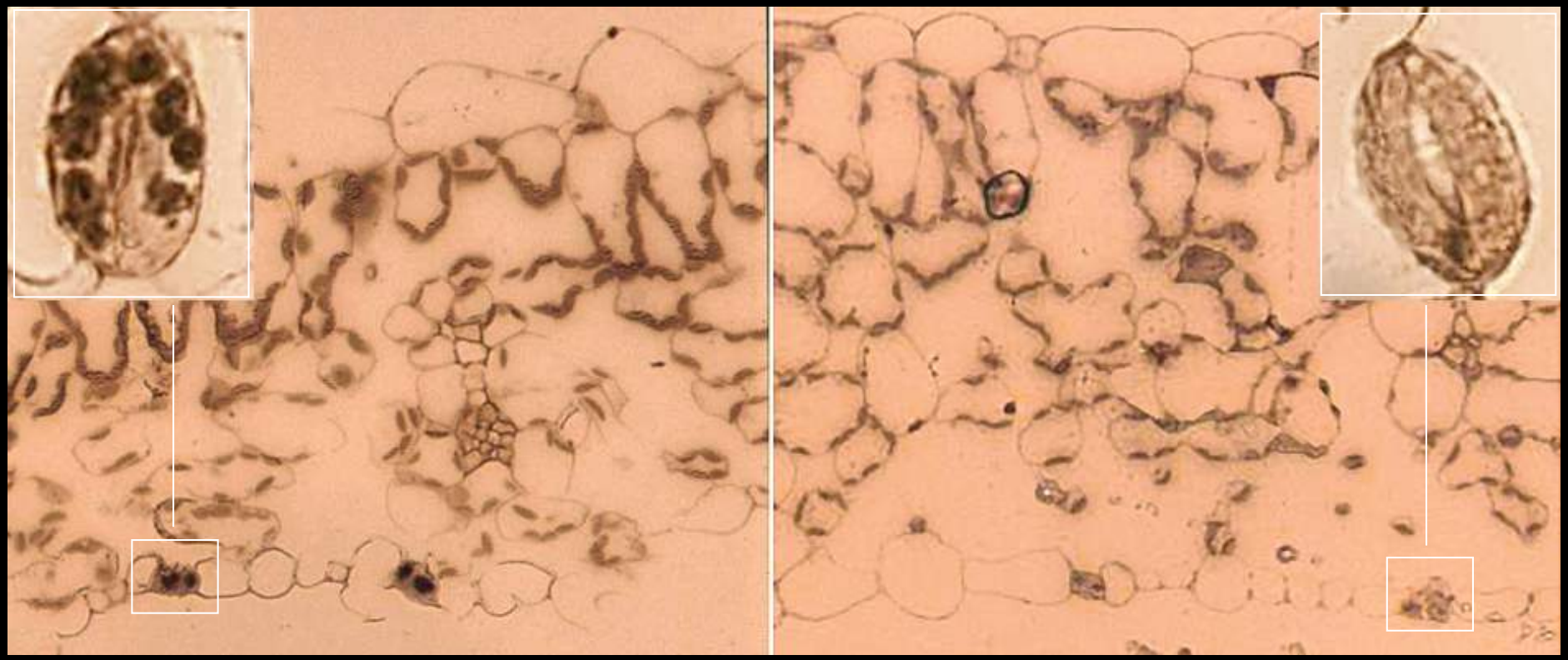








CHT elicits a H_2O_2 burst in treated tissues

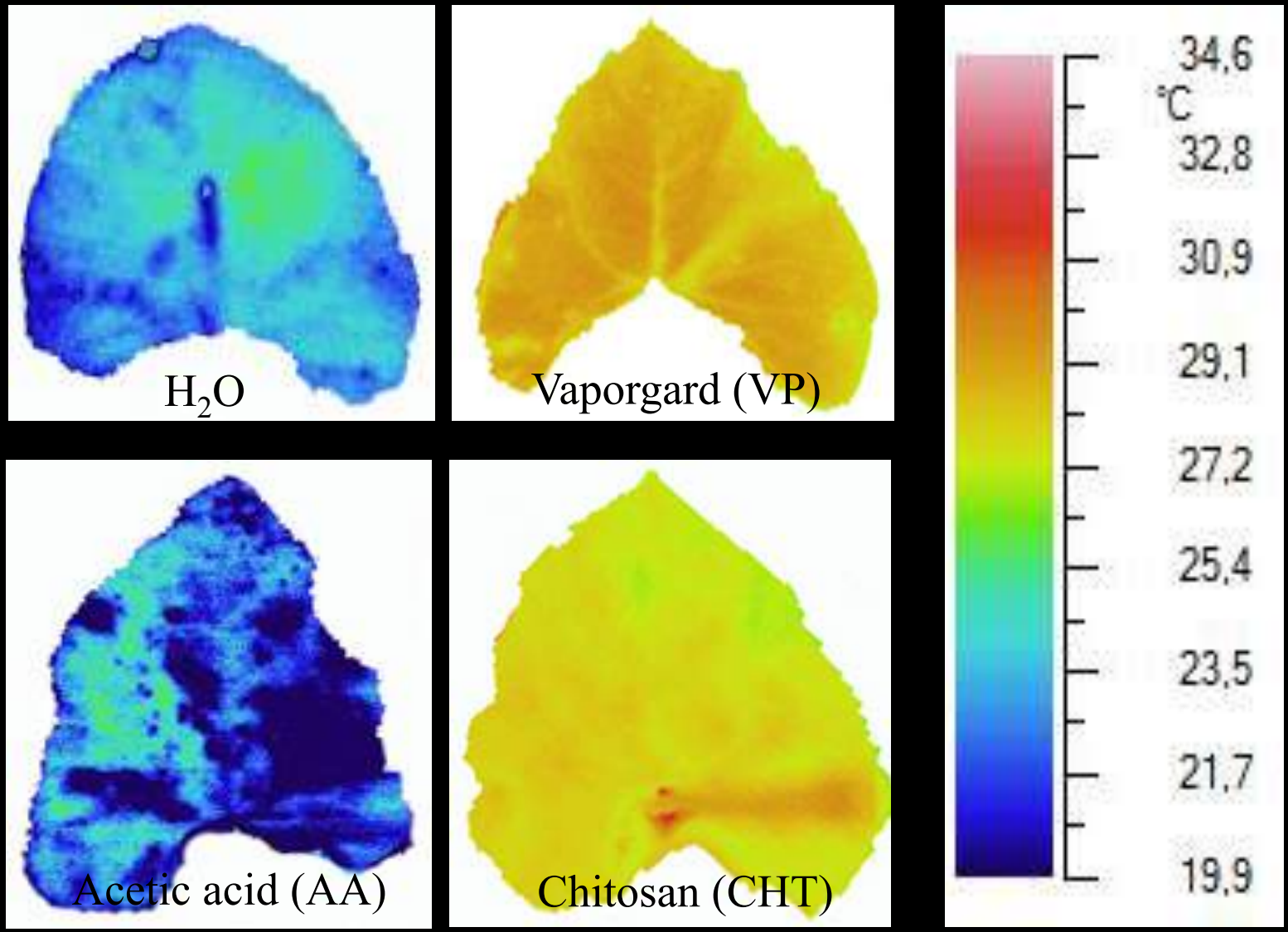


CHT

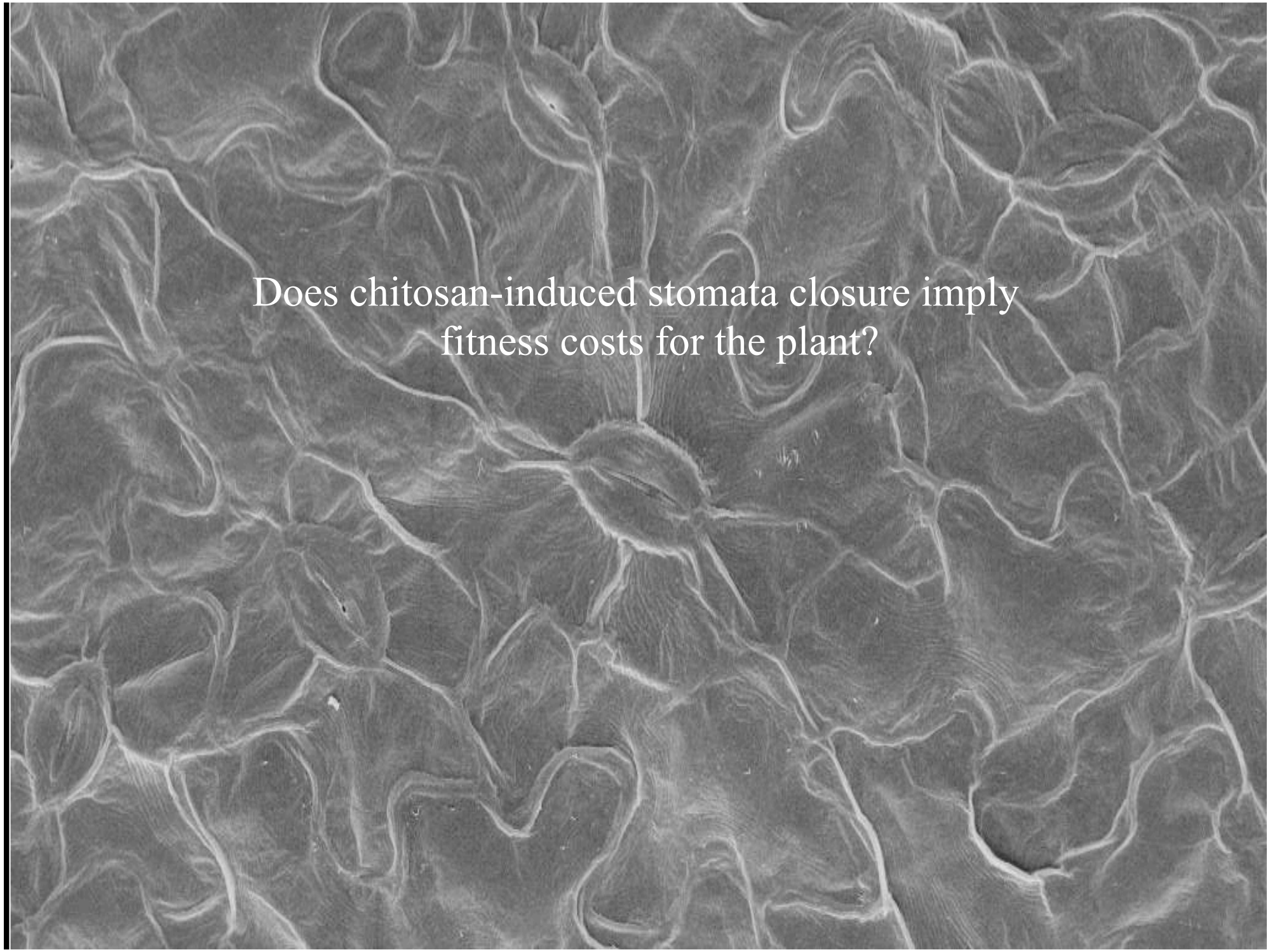
CONTROL

DAB staining

A comparison with Vaporgard®, a commercial antritranspirant, by infrared termography

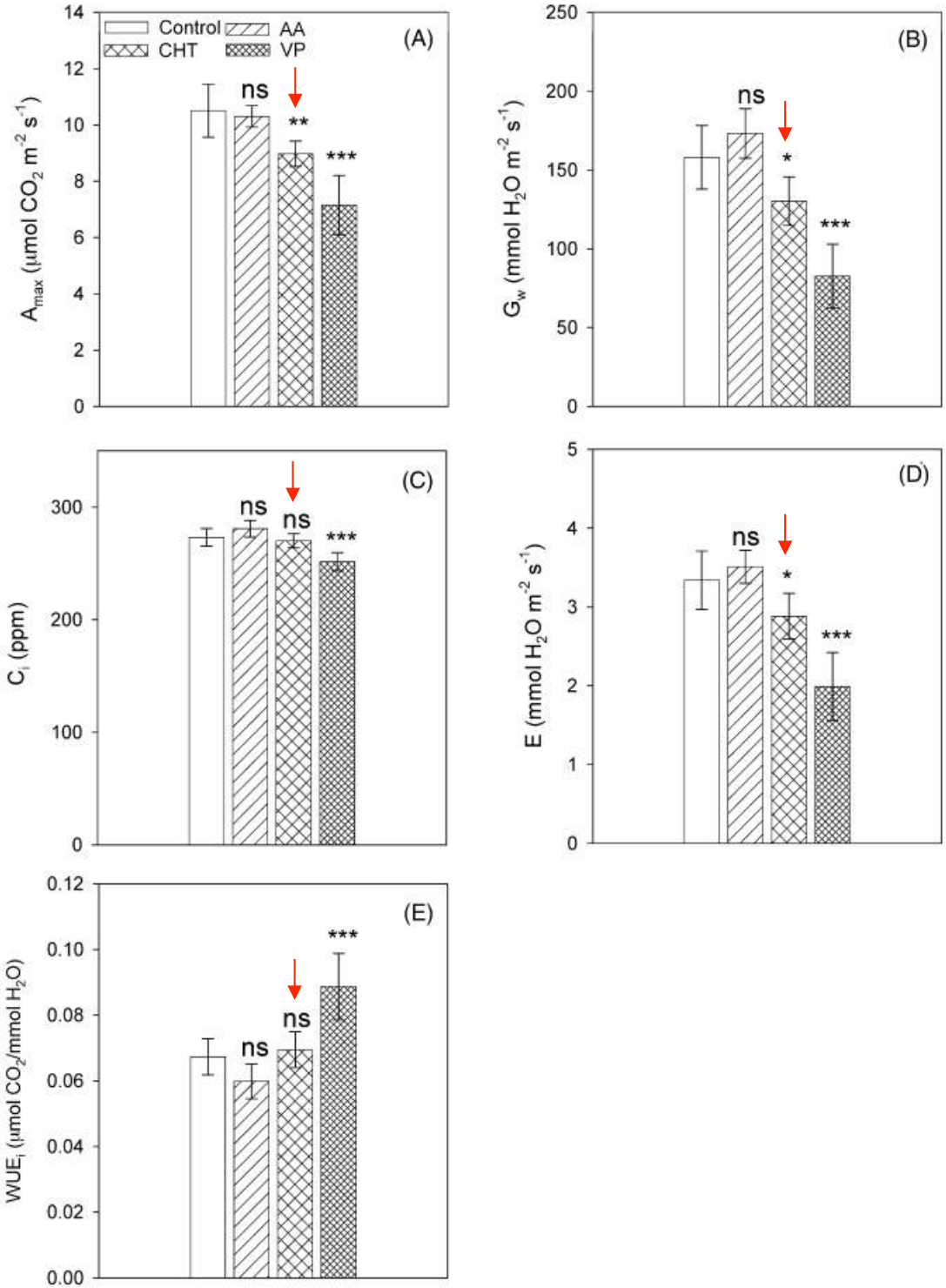


(Iriti et al., Environ. Exp. Bot., 2009)

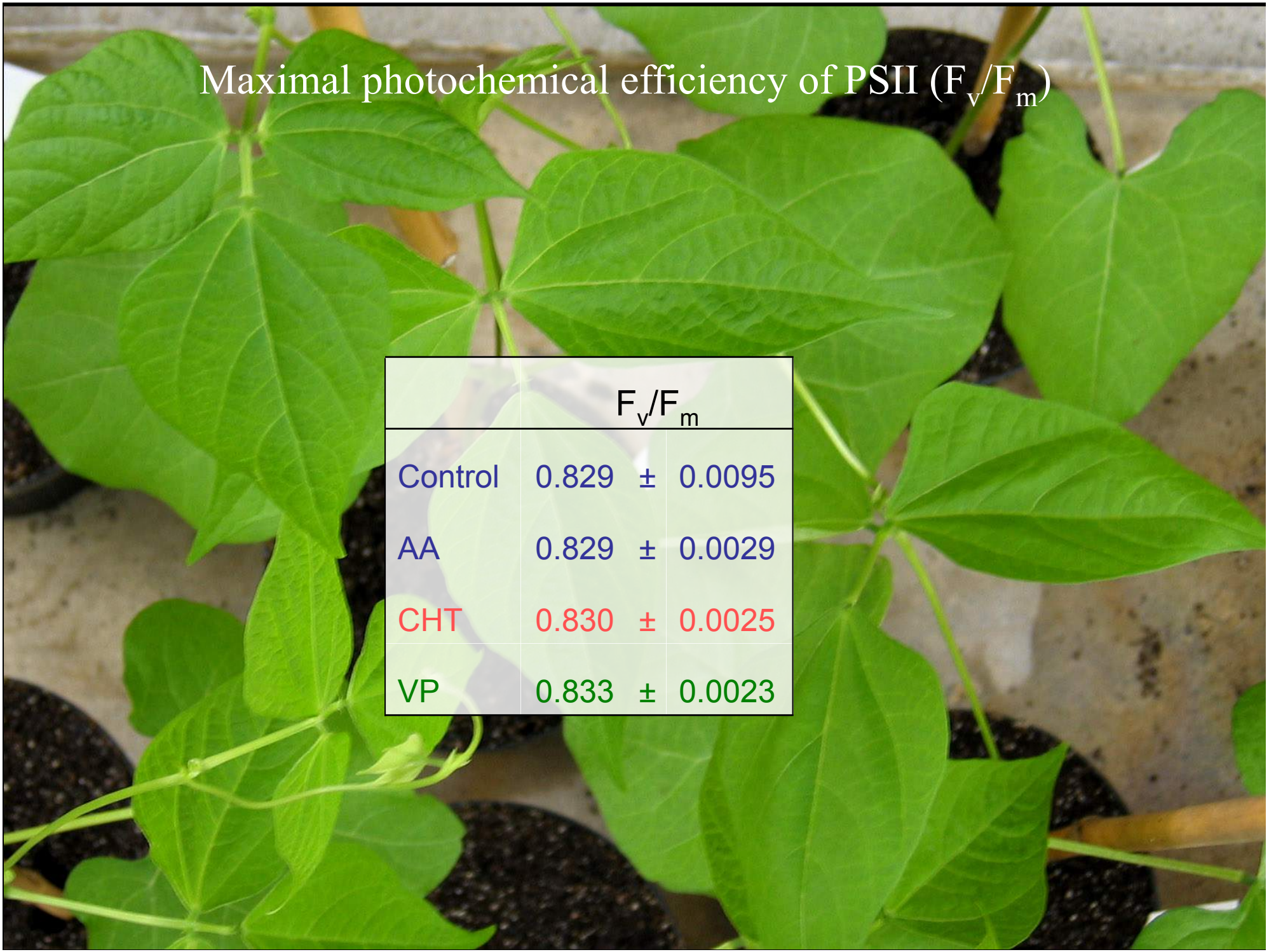

Does chitosan-induced stomata closure imply
fitness costs for the plant?

Eco-physiological parameters of primary bean leaves at 24 h after treatments measured with a gas-analyzer (Li-6400)

(A) Maximal photosynthetic activity (Amax);
(B) stomatal conductance to water vapour (Gw);
(C) intercellular CO_2 concentration (Ci);
(D) transpiration rate (E);
(E) intrinsic water use efficiency (WUEi),



Maximal photochemical efficiency of PSII (F_v/F_m)



	F_v/F_m		
Control	0.829	±	0.0095
AA	0.829	±	0.0029
CHT	0.830	±	0.0025
VP	0.833	±	0.0023

6. Conclusions

• CHT seems to be effective in viral disease control

• CHT efficacy depends on its chemico-physical properties (MW, DD, PD) and varies in different patho-systems (plant-pathogen interactions)

• CHT may increase the levels of bioactive phytochemicals in plant ('beneficial' side effects); the healthy potential of plant foods may benefit from this strategy

• CHT treatment does not incur fitness costs and yield in plant, probably due to the activation of priming mechanisms as opposed to the direct resistance

• The possibility of improving tolerance to drought deserves particular attention for a sustainable agriculture in the context of a climate changing scenario

• Stomatal closure and plant immunity!

Many thanks for the kind attention