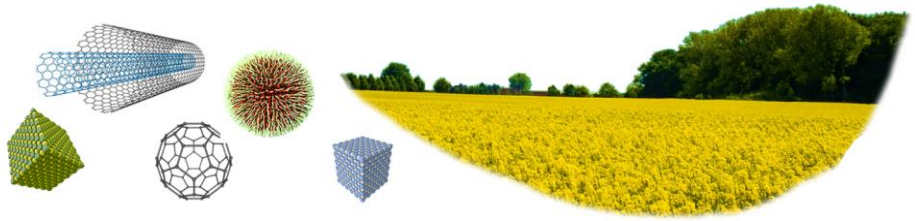


2nd Summer School
"Nanotechnology in Agriculture"
29-30 June, 2023
University of Udine

Session 6. Nano-Enabled Agriculture and Circular Economy.

Recycling of waste compounds: chitosan of biological origin as a raw material for ENMs production

Dr. Dora Scarpin

Department of Agrifood, Environmental and Animal Sciences - DI4A
University of Udine

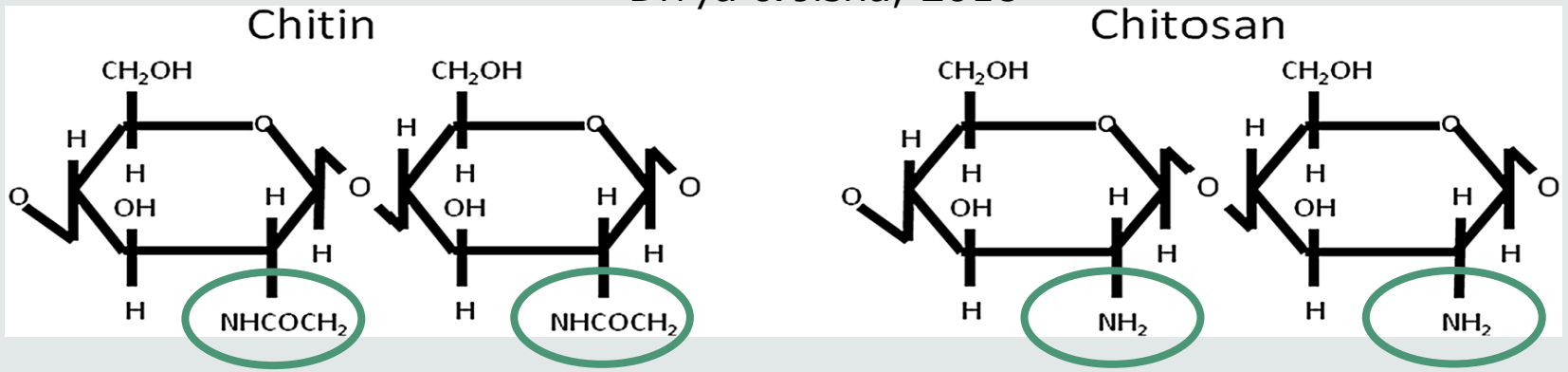
scarpin.dora@spes.uniud.it

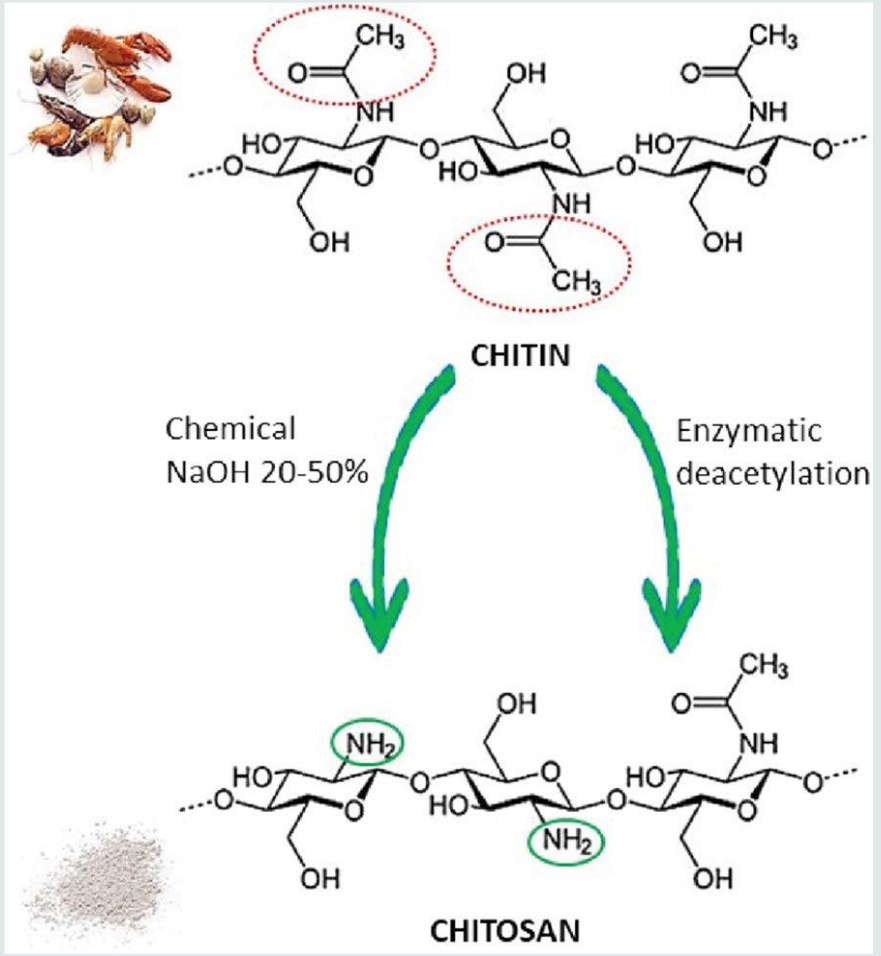
Chitosan: what is it?

A biopolymer of glucosamine and N-acetyl glucosamine residues

(a de-acetylated product of chitin)

Divya & Jisha, 2018





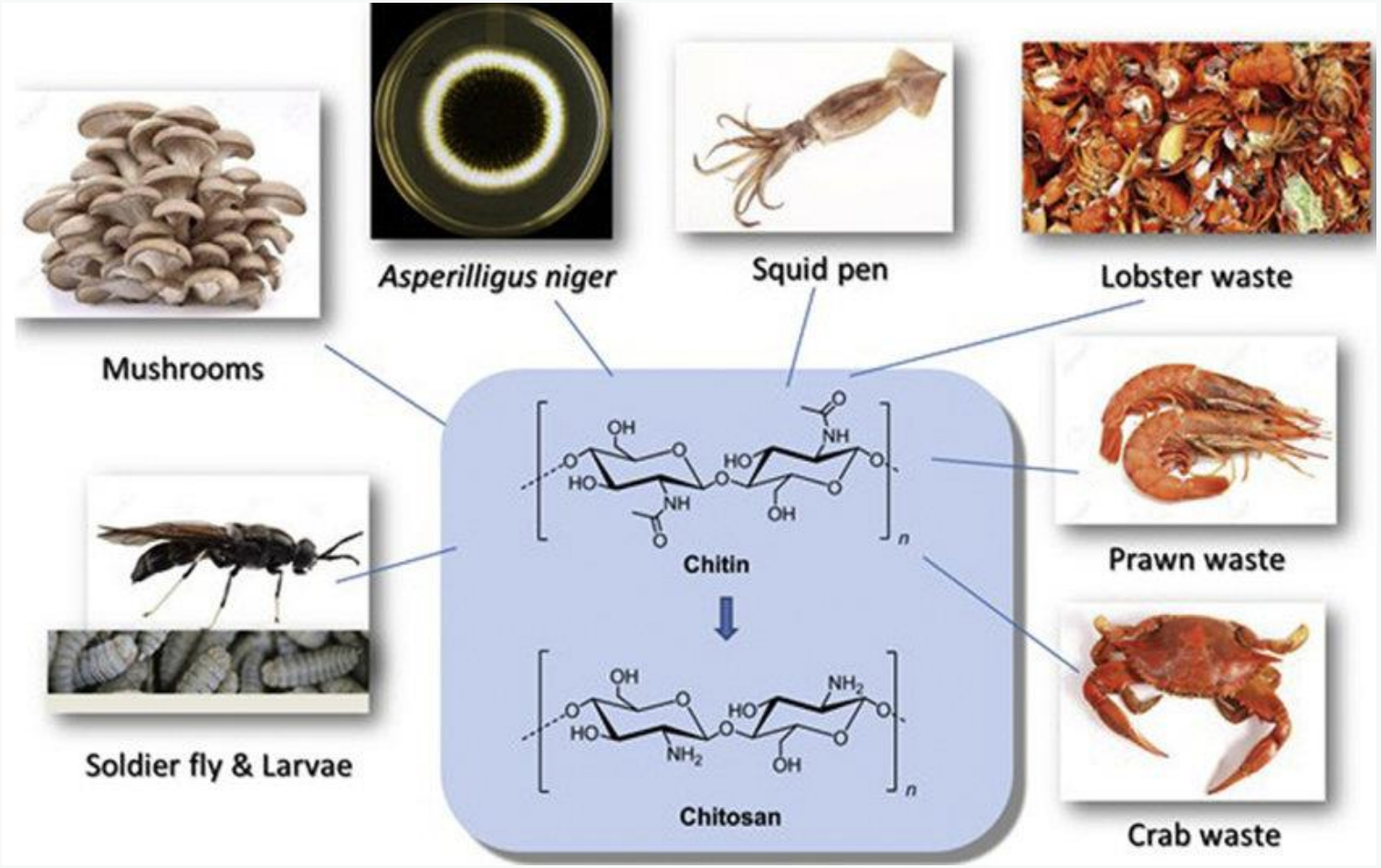
Sharifi-Rad *et al.,* 2021

Chitin (and consequently chitosan) is the second renewable carbon source after lignocellulosic biomass

Advantages of its use:

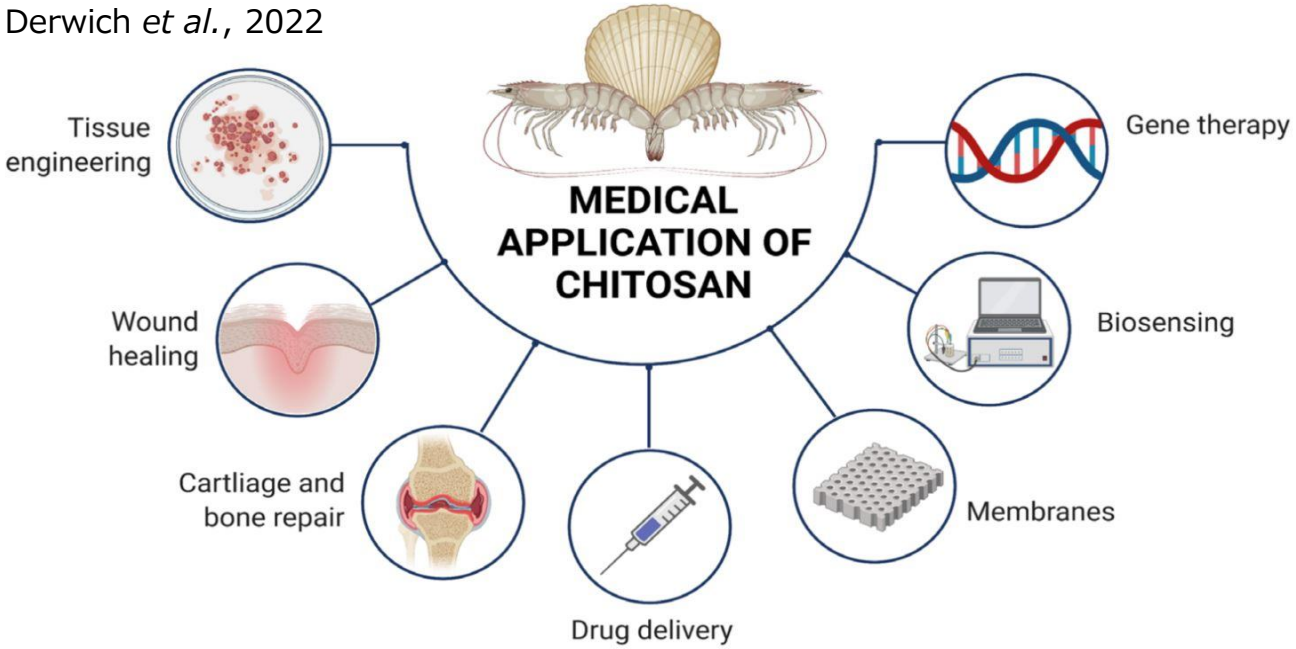
- Natural
- Safe (biocompatible, biodegradable, non-allergenic, low toxicity)
- Cheap
- **Several useful functions due to its heterogeneity (copolymer)**

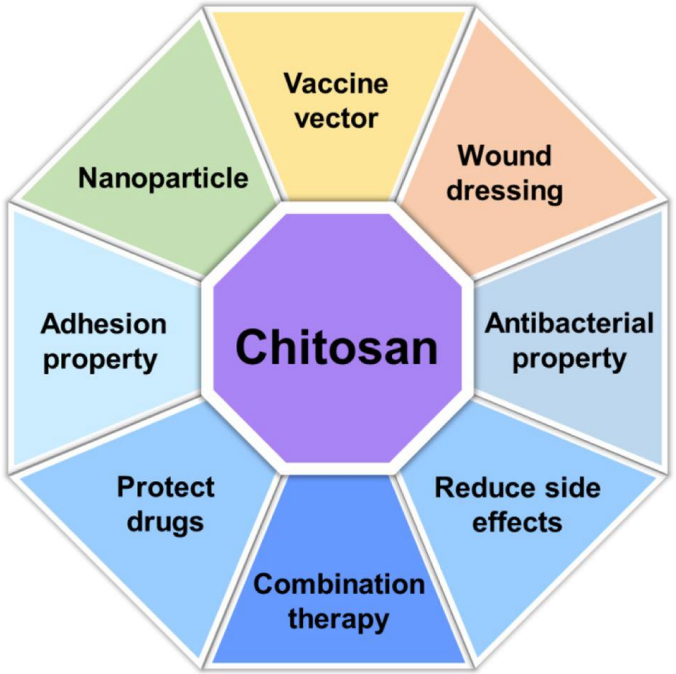
Malerba & Cerana, 2016



Sayed & Jardine, 2016

Derwich *et al.*, 2022





Meng *et al.*, 2021

Kashyap *et al*., 2015

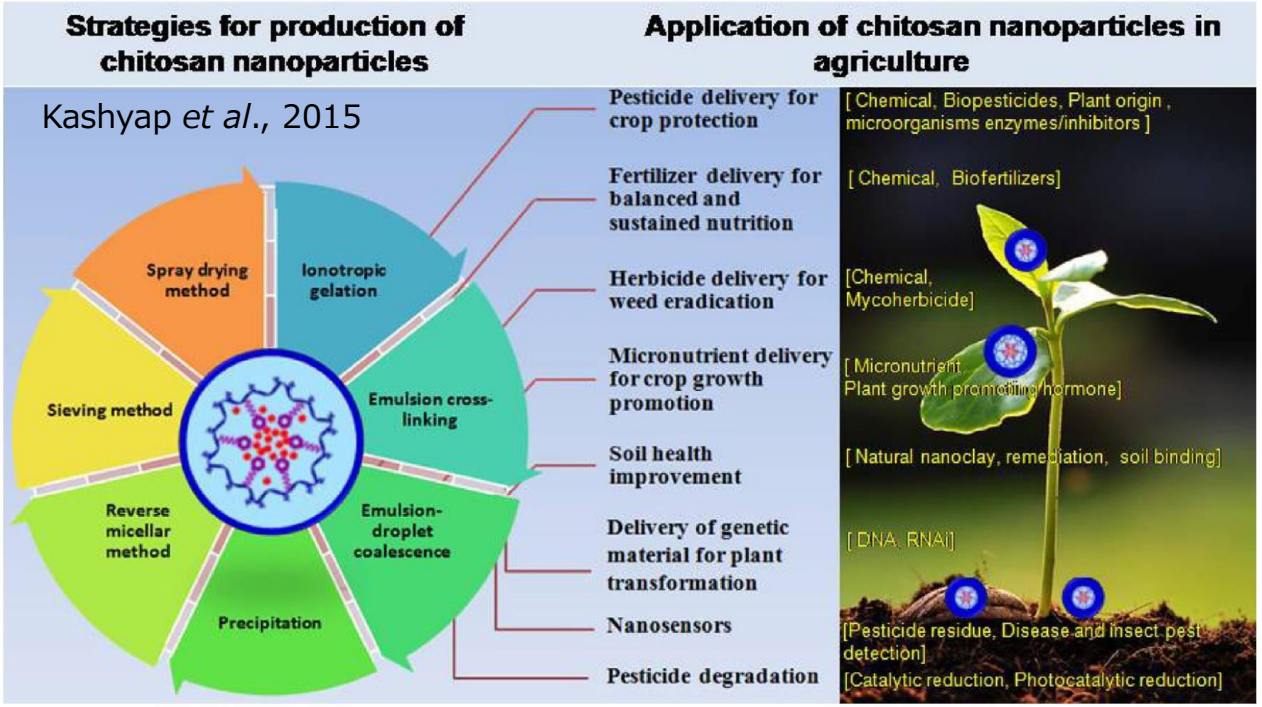
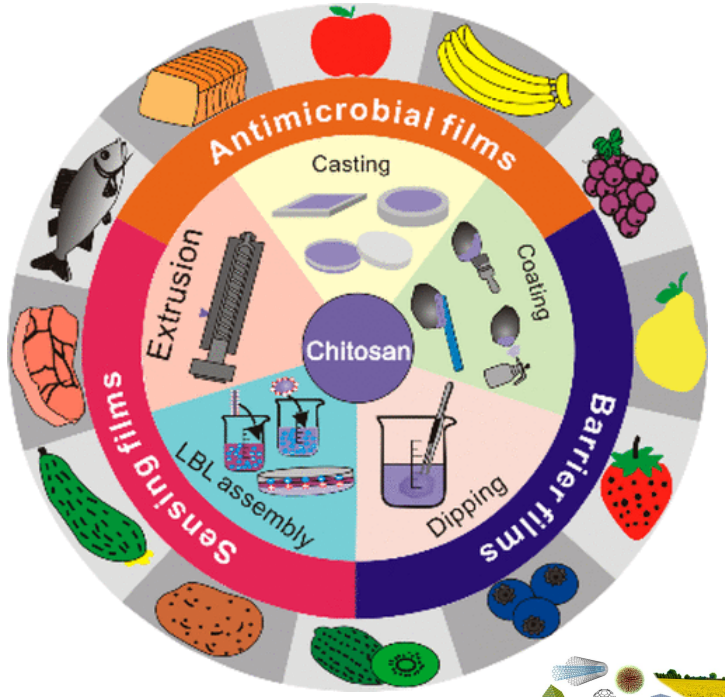


Fig. 1. Strategies for the production of chitosan naoparticles and their applications as a delivery system in agriculture.



Wang *et al.*, 2017

Chitosan NPs are better than bulk chitosan

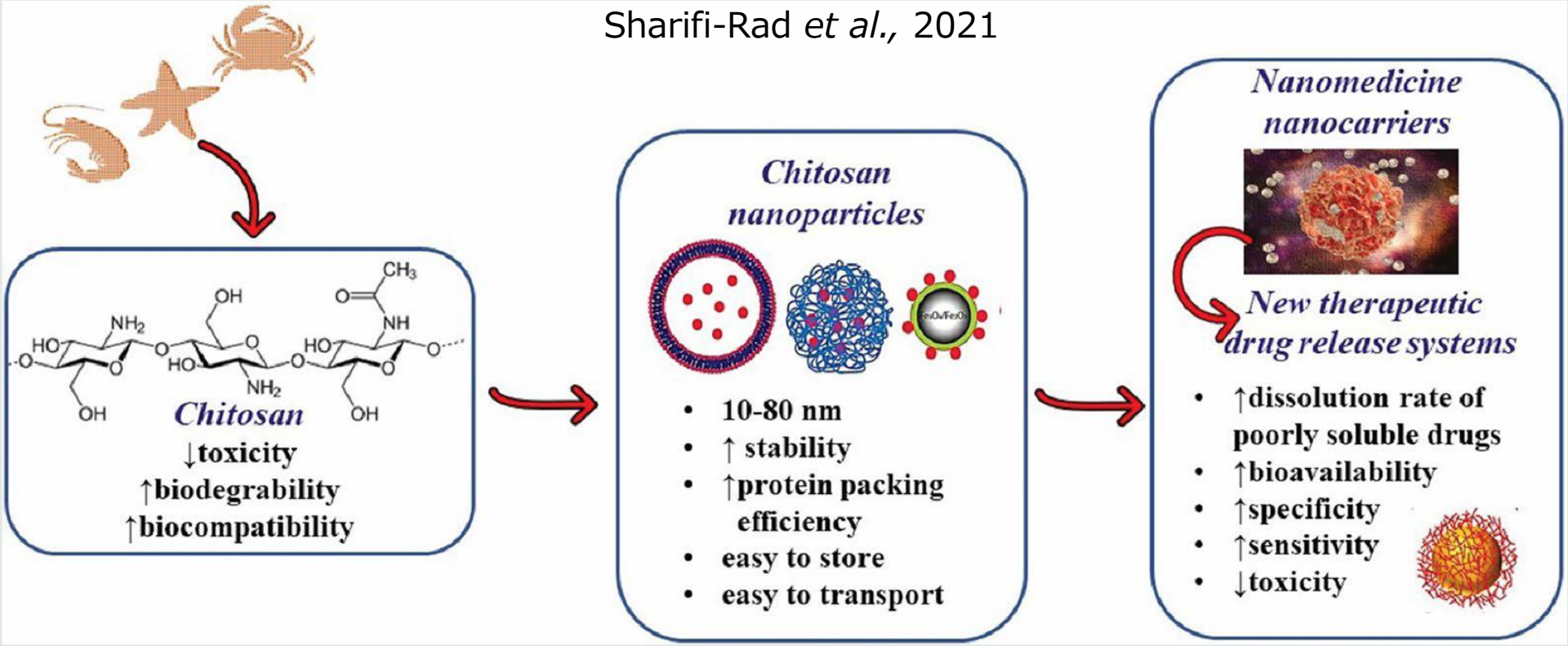
Properties on plants:

- **Boosting defense response** (cellular response)
- **Antimicrobial activity** (direct or indirect)
- **Plant growth and development** (enhances photosynthesis and other physiological responses, water use, nutrients uptake...)

Saharan & Pal, 2016

BUT...

Sharifi-Rad *et al.,* 2021



…Nanochitosan as compared to bulk chitosan has superior physico-chemical characteristics that provide enhanced biological activities:

high surface area, higher solubility, more affinity with outer membrane

Saharan *et al*. 2013; Van *et al*. 2013

Chitosan NPs can also:

- Block gene expression
- Induce signaling pathways linked to defense-related proteins
- Influence metabolism

Saharan & Pal, 2016

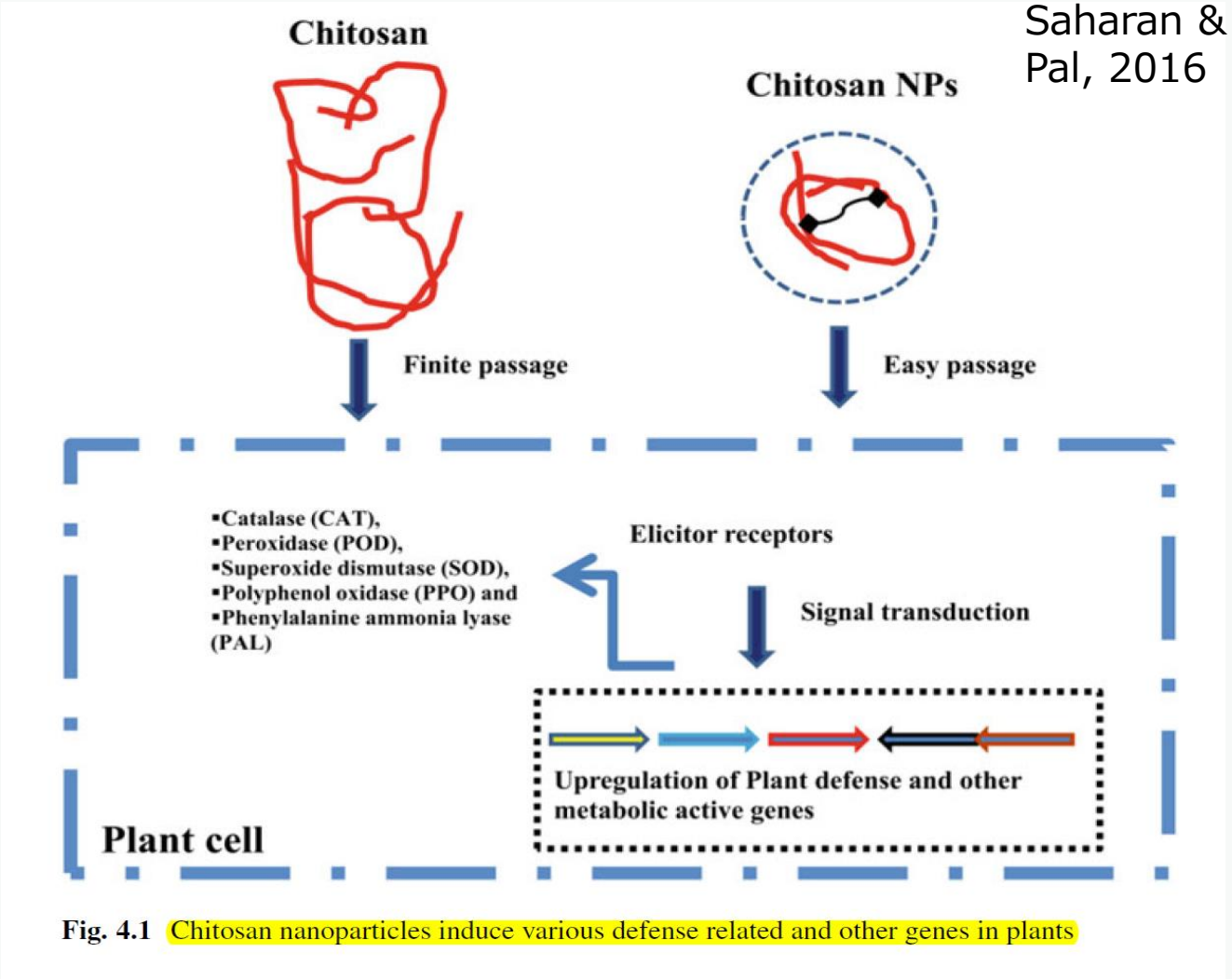


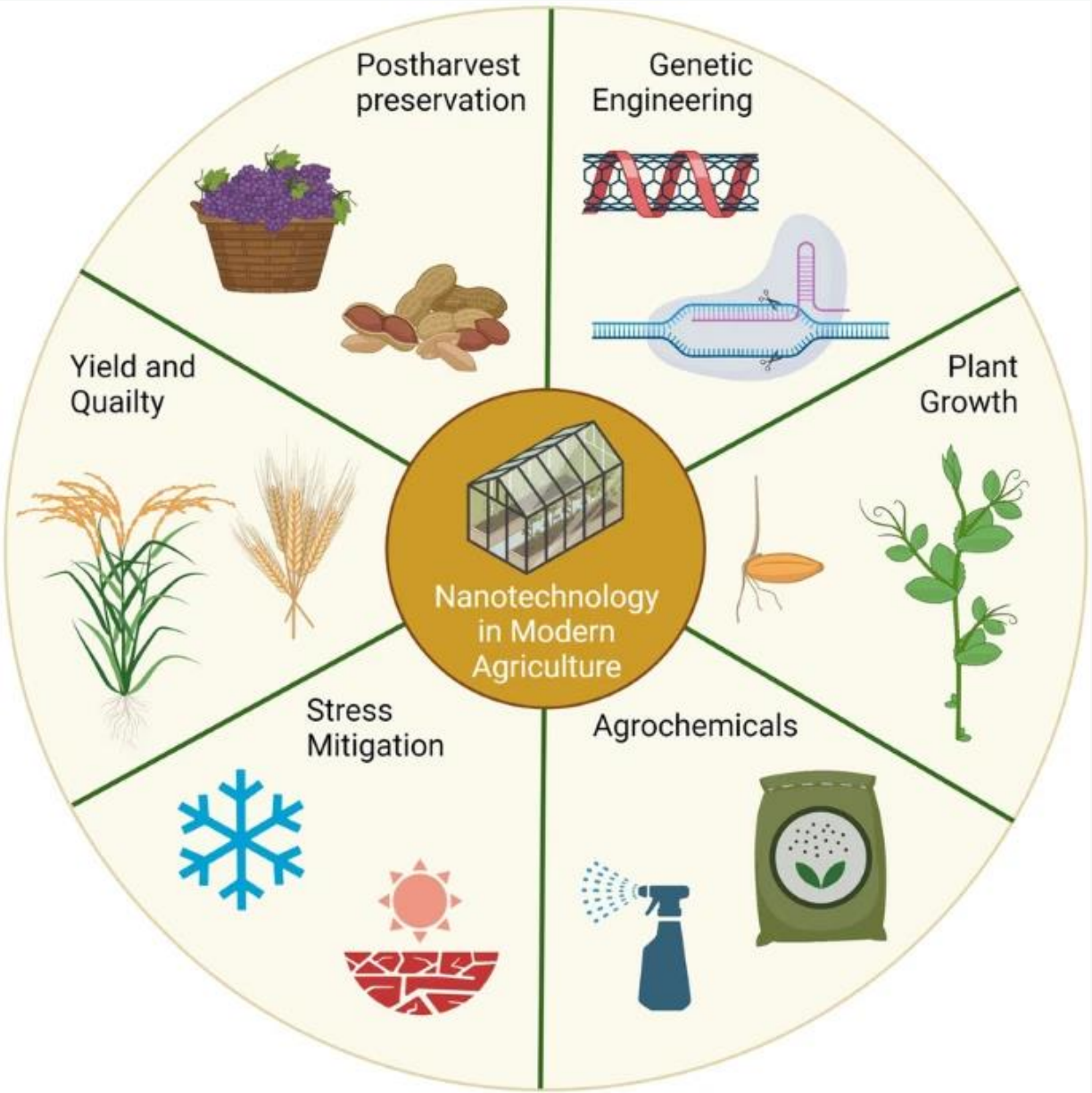
Fig. 4.1 Chitosan nanoparticles induce various defense related and other genes in plants

Saharan & Pal, 2016

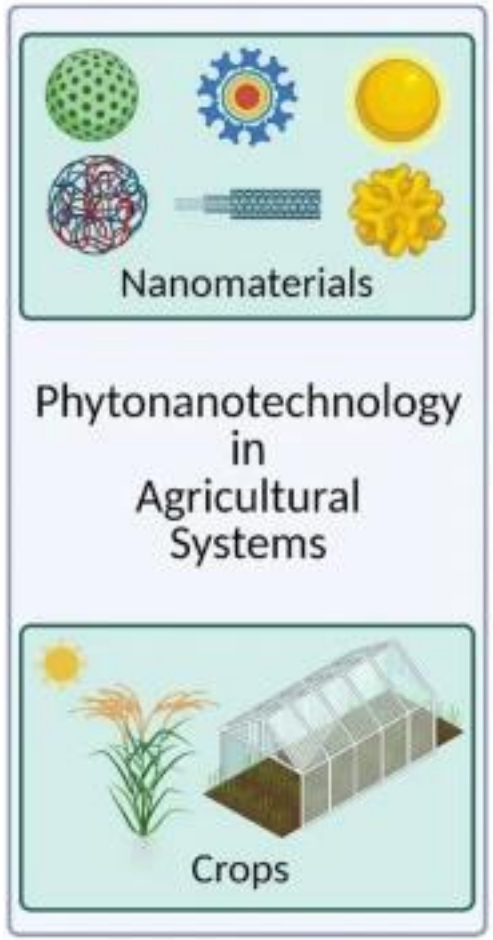
Chitosan NPs can also act as CARRIERS

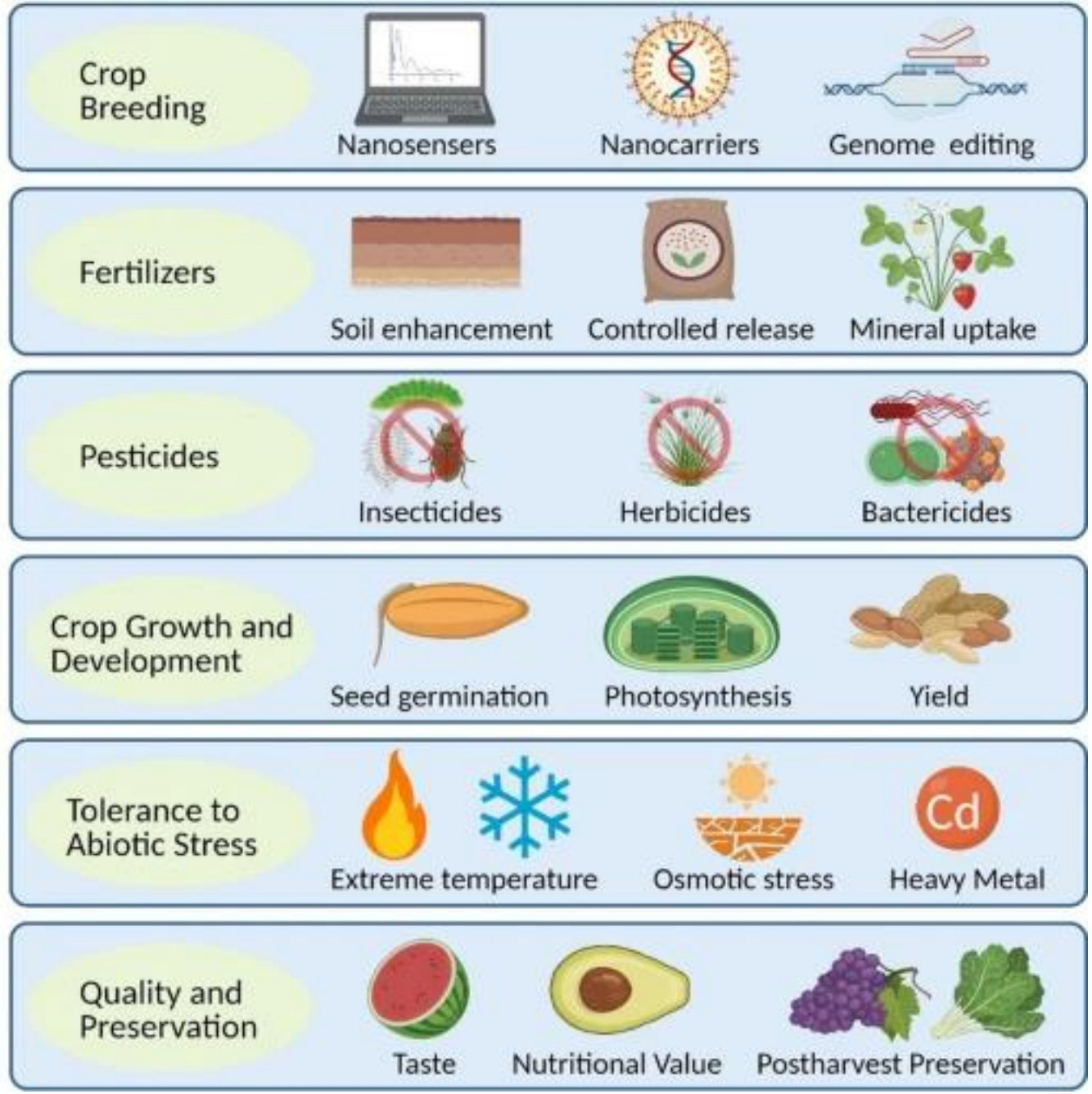
- Pesticide and herbicide delivery for crop protection
- Fertilizer & Micronutrient delivery for crop growth promotion
- Soil health improvement
- Delivery of genetic material

Kashyap *et al*., 2015



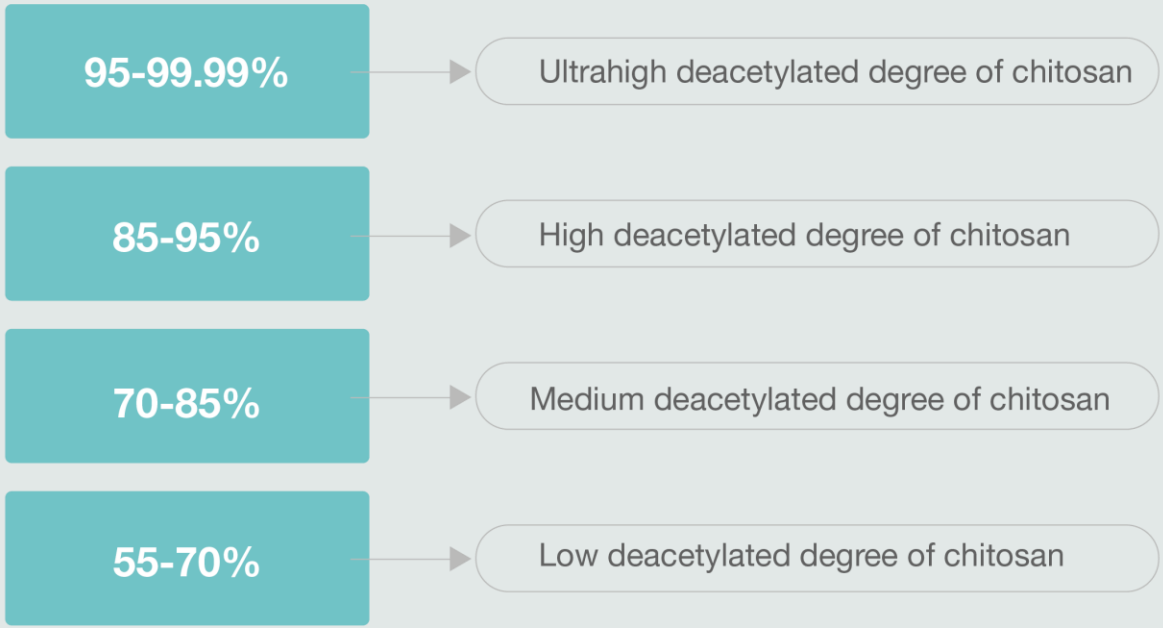
Jiang *et al*., 2021



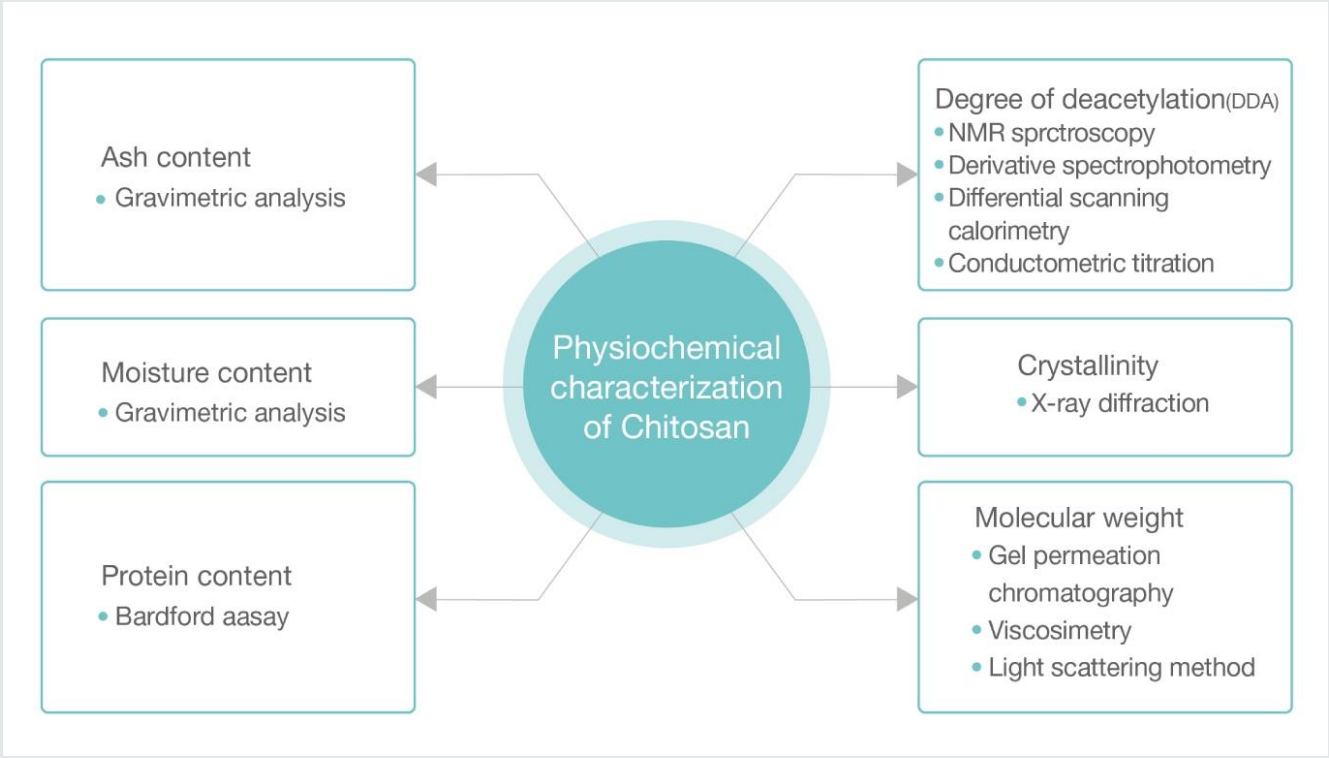


Jiang *et al*., 2021

Ok, that's interesting, but... HOW IS IT DONE IN PRACTICE?



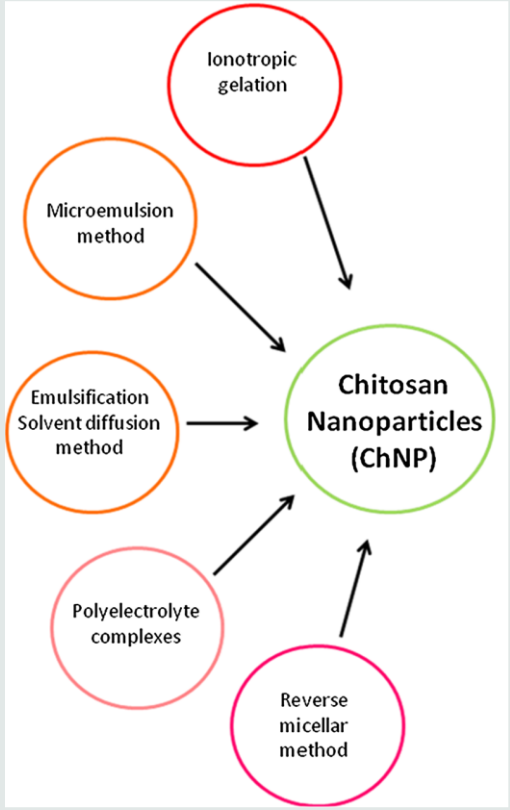
https://axiobio.com/chitosan/



THE CHOICE: Physicomechanical properties (solubility, toxicity, hydrophobicity) of chitosan are influenced by its **degree of deacetylation** and **molecular weight**, depending on the **source of chitin**.

Jhaveri *et al*., 2021

1. Emulsion cross-linking
2. Emulsion-droplet coalescence
3. **Ionotropic (ionic) gelation**
4. Precipitation
5. Reverse micelles
6. Seiving method
7. Spray drying



Grenha, 2012
Kashyap *et al*., 2015
Saharan & Pal, 2016
Divya & Jisha, 2018
Jhaveri *et al*., 2021
.....

Different synthesis methods

Ionotropic gelation

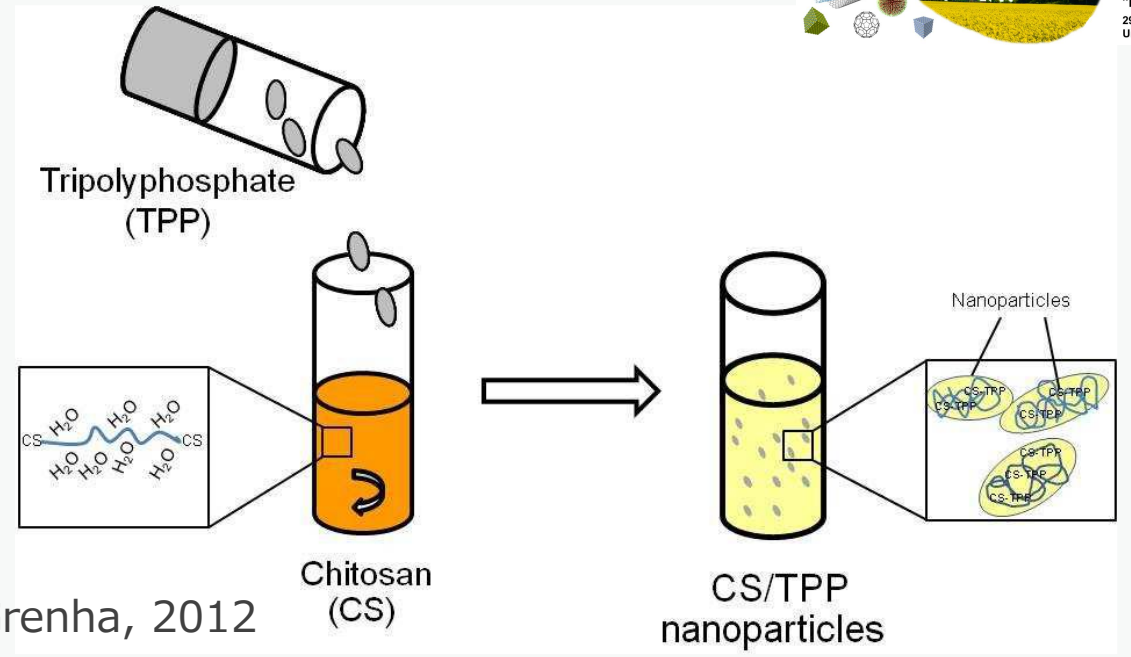
Most accepted method:

- Fast
- Stable
- Non-toxic
- Organic solvent - free

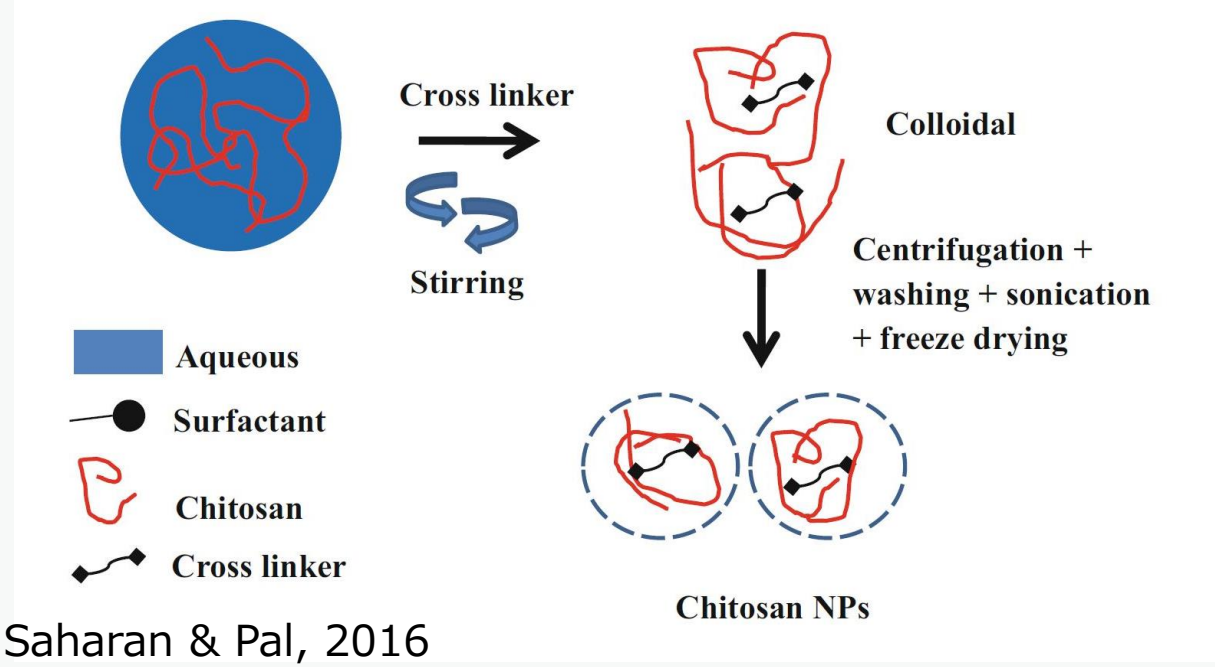
Easy procedure:

- Week acidic aqueous solution
- Add reagents while stirring constantly
- **Electrostatic interaction** between positively charged amino groups (NH_3+) of chitosan and negatively charged cross linkers

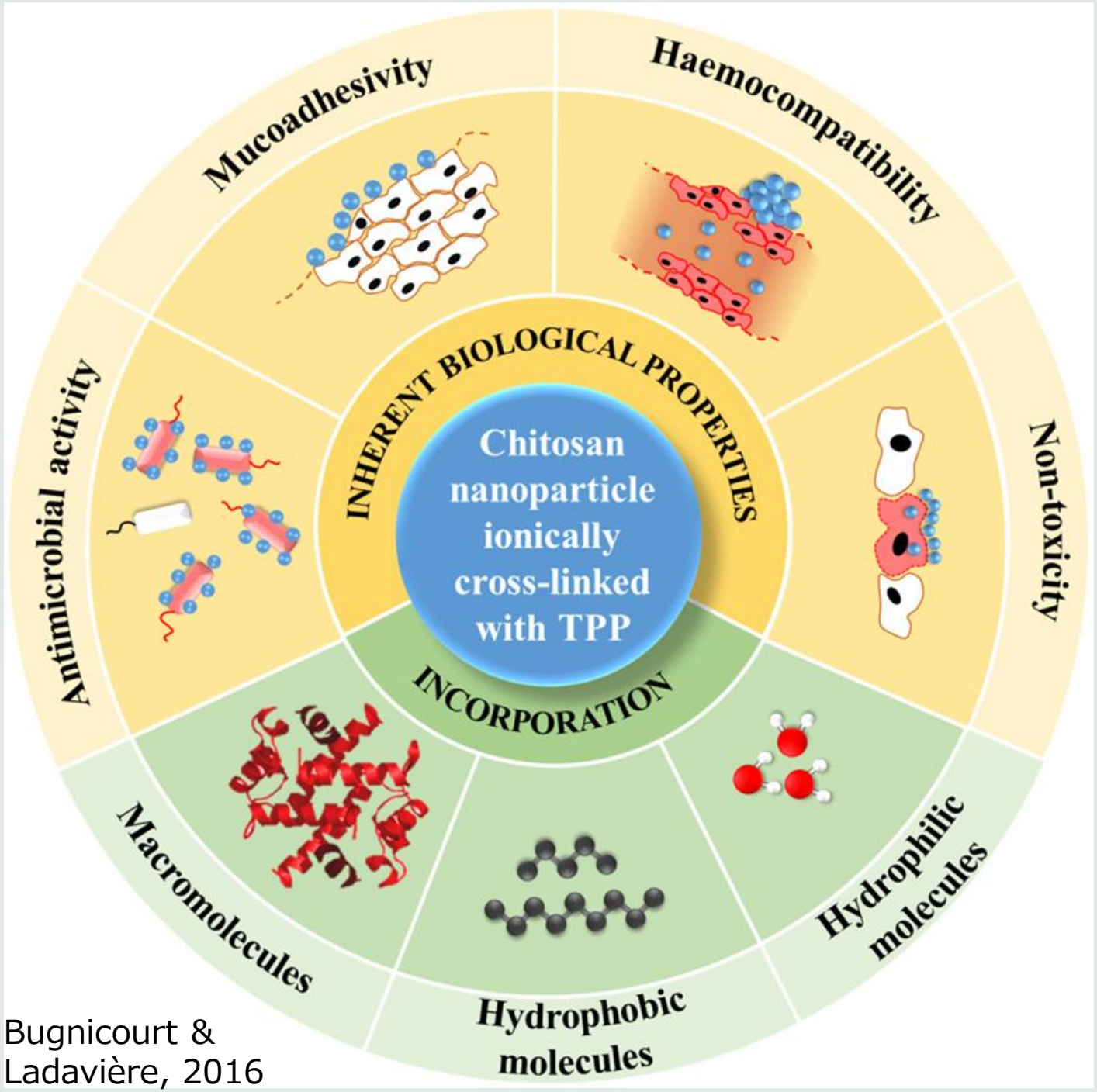
Saharan & Pal, 2016



Grenha, 2012



Saharan & Pal, 2016

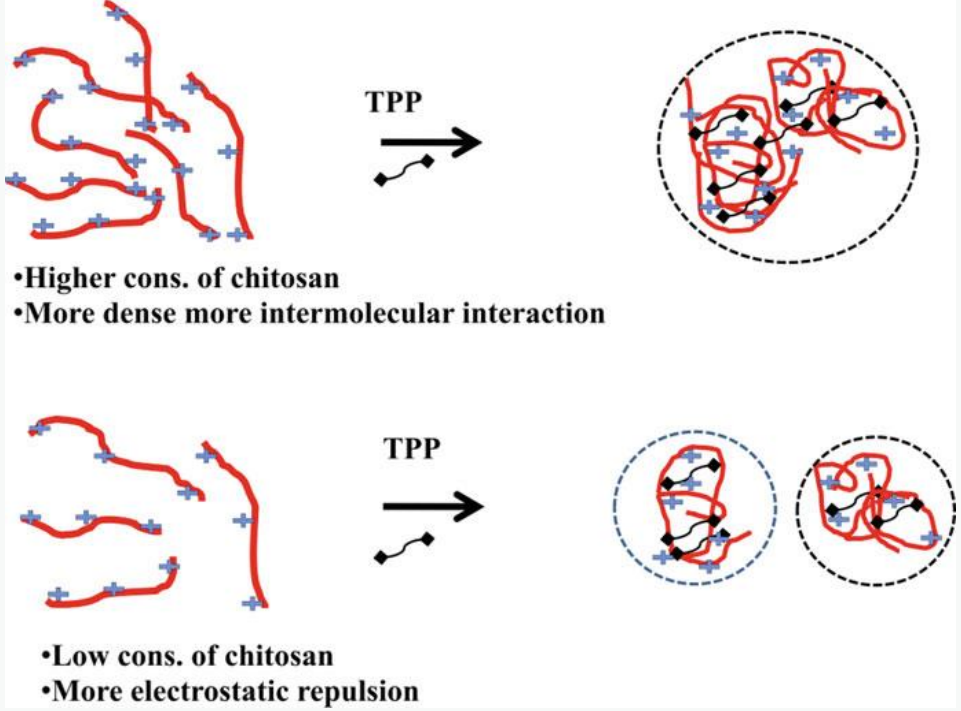


Bugnicourt & Ladavière, 2016

Chitosan NPs ionically cross-linked with TPP

- Tripolyphosphate (Na-salt)
- Polyanion
- Non – toxic
- No chemical cross-linking
- No additional steps for removing oil or surfactant

Saharan & Pal, 2016



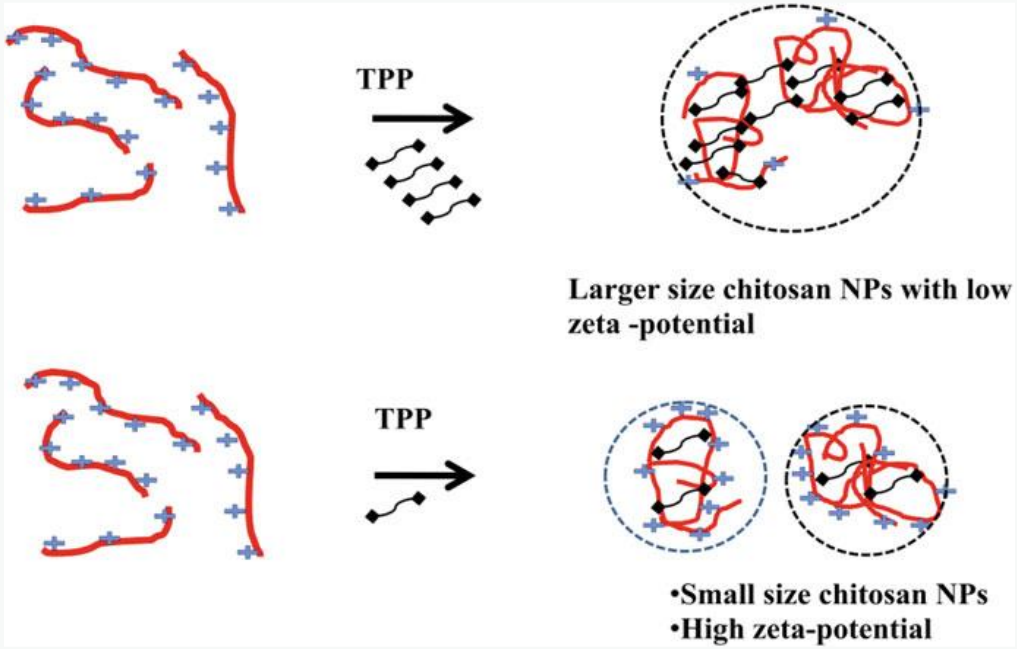
1.Chitosan concentration

Interchain hydrogen bonds Vs. electrostatic interactions

(Acidic solution: Amino groups get protonated - strong repulsion)

2.TPP concentration

Intermolecular cross-linking and occupation of positive amino groups



Saharan & Pal, 2016

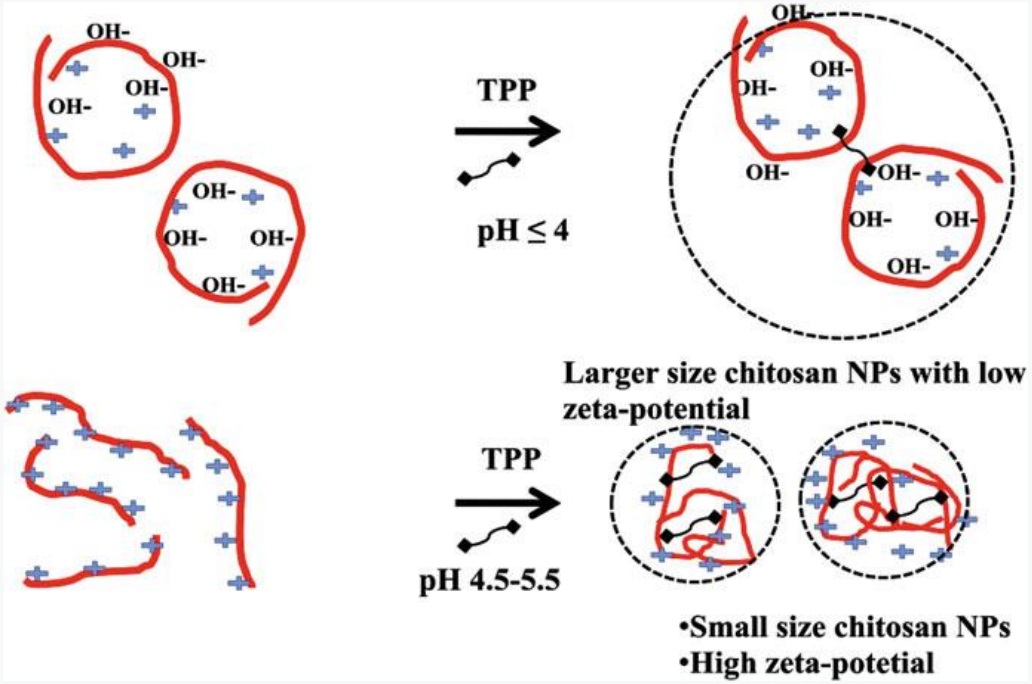
Factors affecting synthesis

Optimal values

- 2.0–1.5 mg/ml chitosan
- 1.0–0.5 mg/ml TPP
- Mass ratio chitosan:TPP from 2.5:1 to 5:1

Saharan & Pal, 2016

Factors affecting synthesis

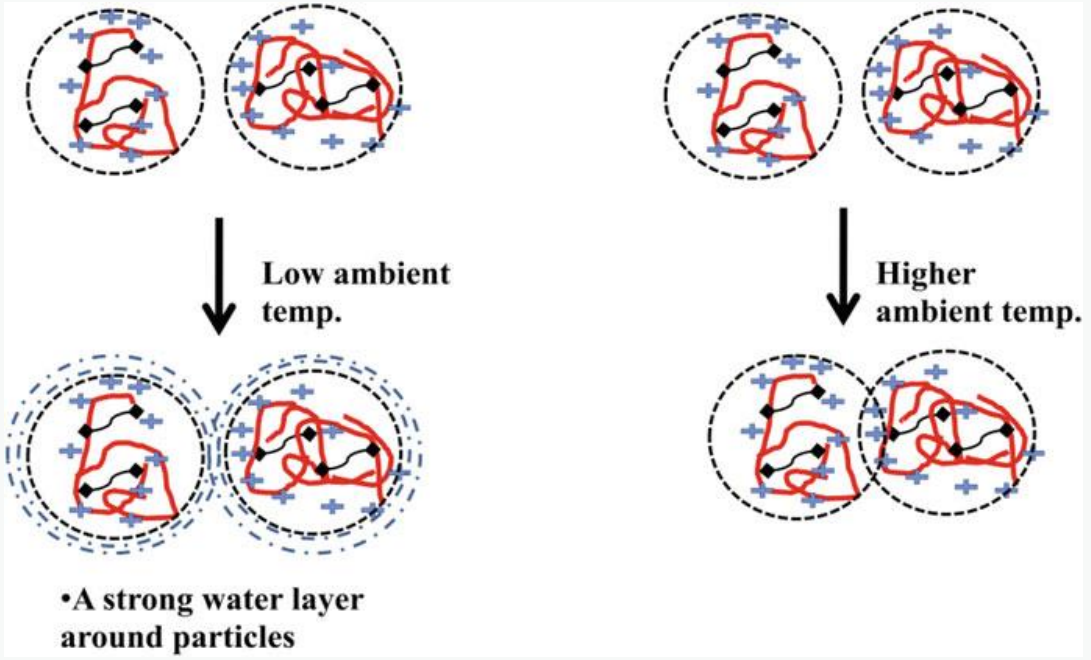


3. pH

Different degree of repulsion affects the compactness of NPs

4.Temperature

Influence of the hydration layer

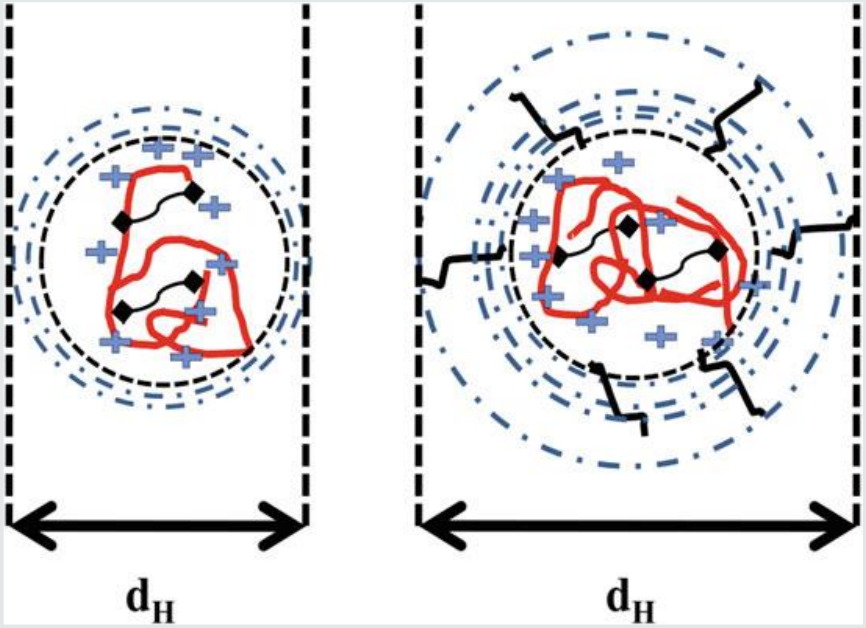


Saharan & Pal, 2016

To remember: Chitosan is insoluble in neutral or alkaline conditions. Acidic solution: amino groups get protonated.

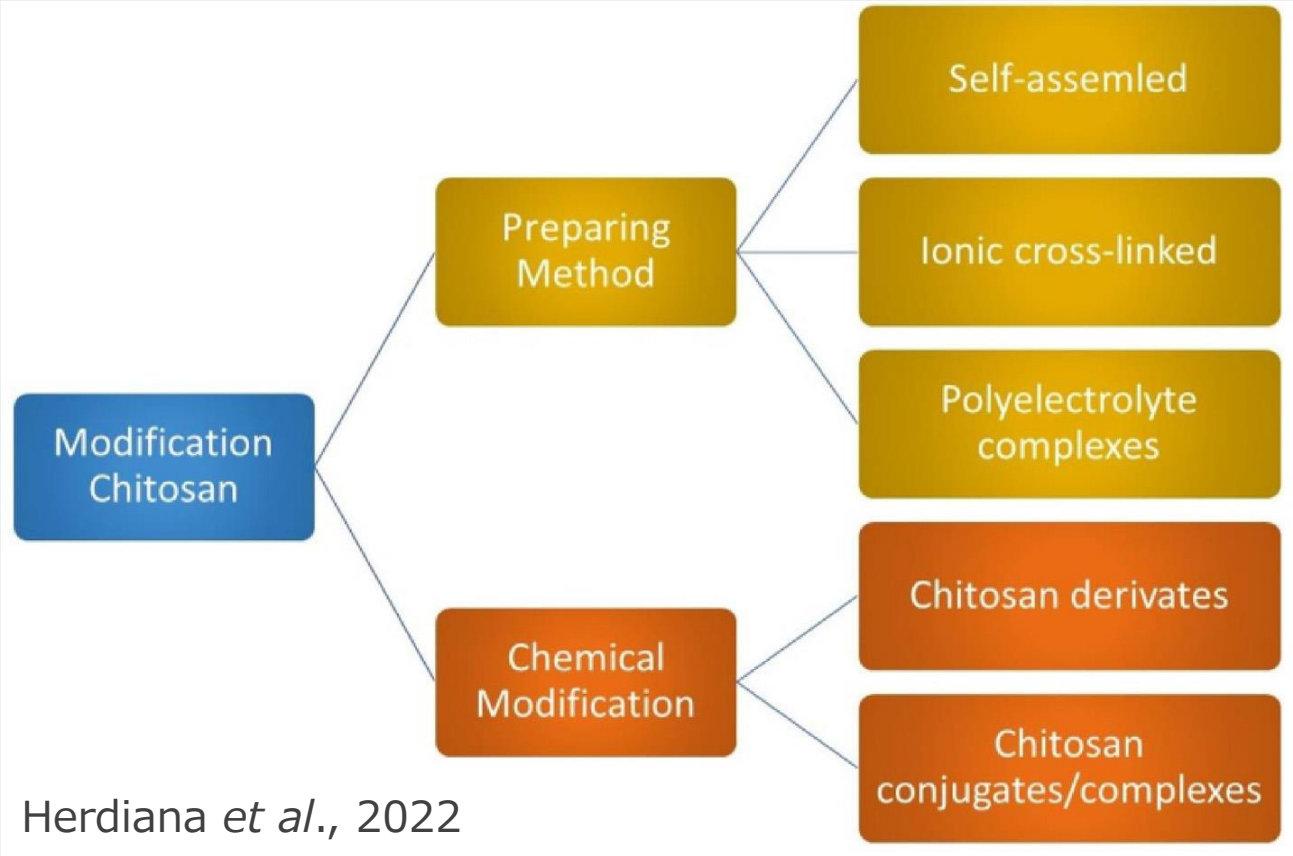
A rapid review of characterisation techniques

- **Dynamic Light Scattering – DLS** (hydrodynamic diameter)
- **Zeta potential** (surface charge)
- **Fourier Transformation Infrared Spectroscopy – FTIR**
- **Transmission and Scanning Electron Microscopy – TEM & SEM**
- **Atomic Force Microscopy – AFM**



Saharan & Pal, 2016

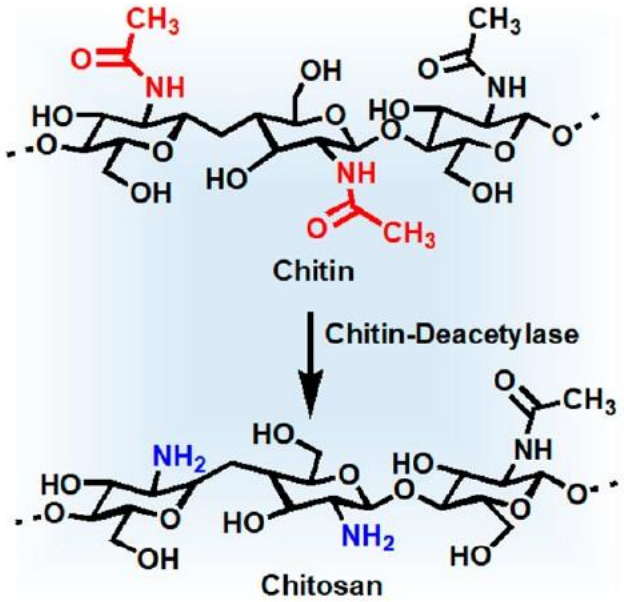
Saharan & Pal, 2016, Pereira *et al*., 2017

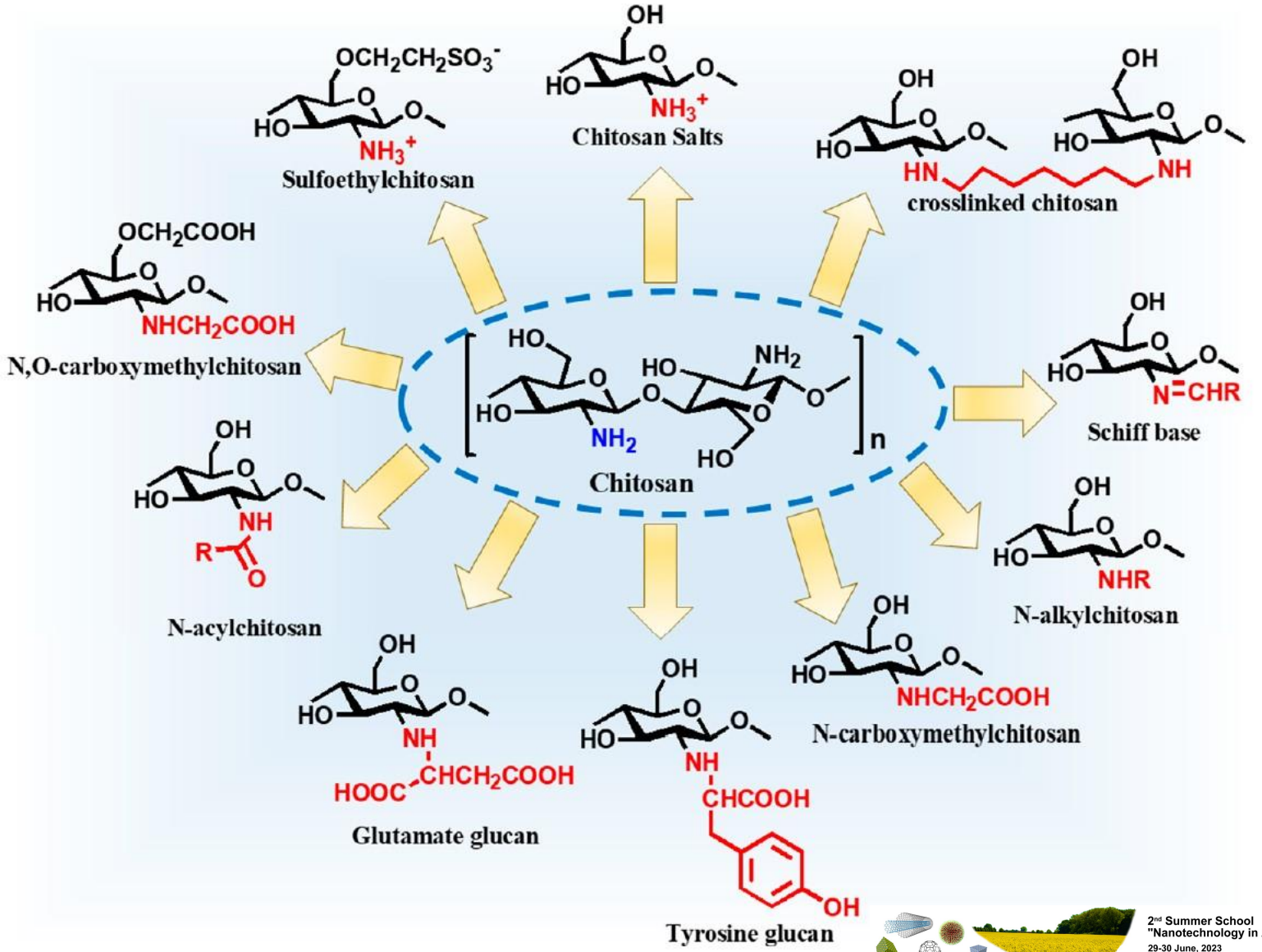


Herdiana *et al*., 2022

Modifications and combinations of multiple materials

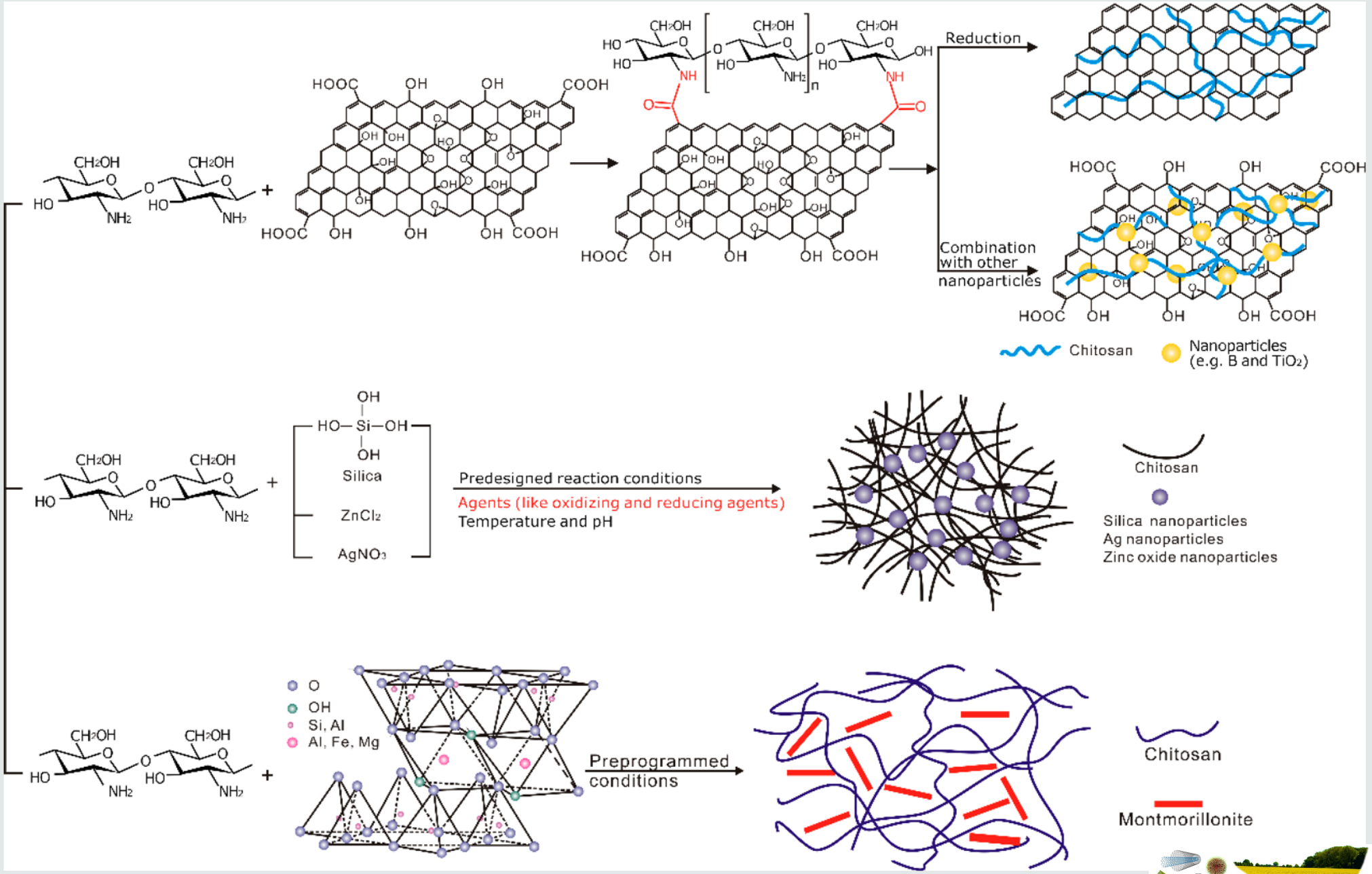
Modifications and combinations of multiple materials





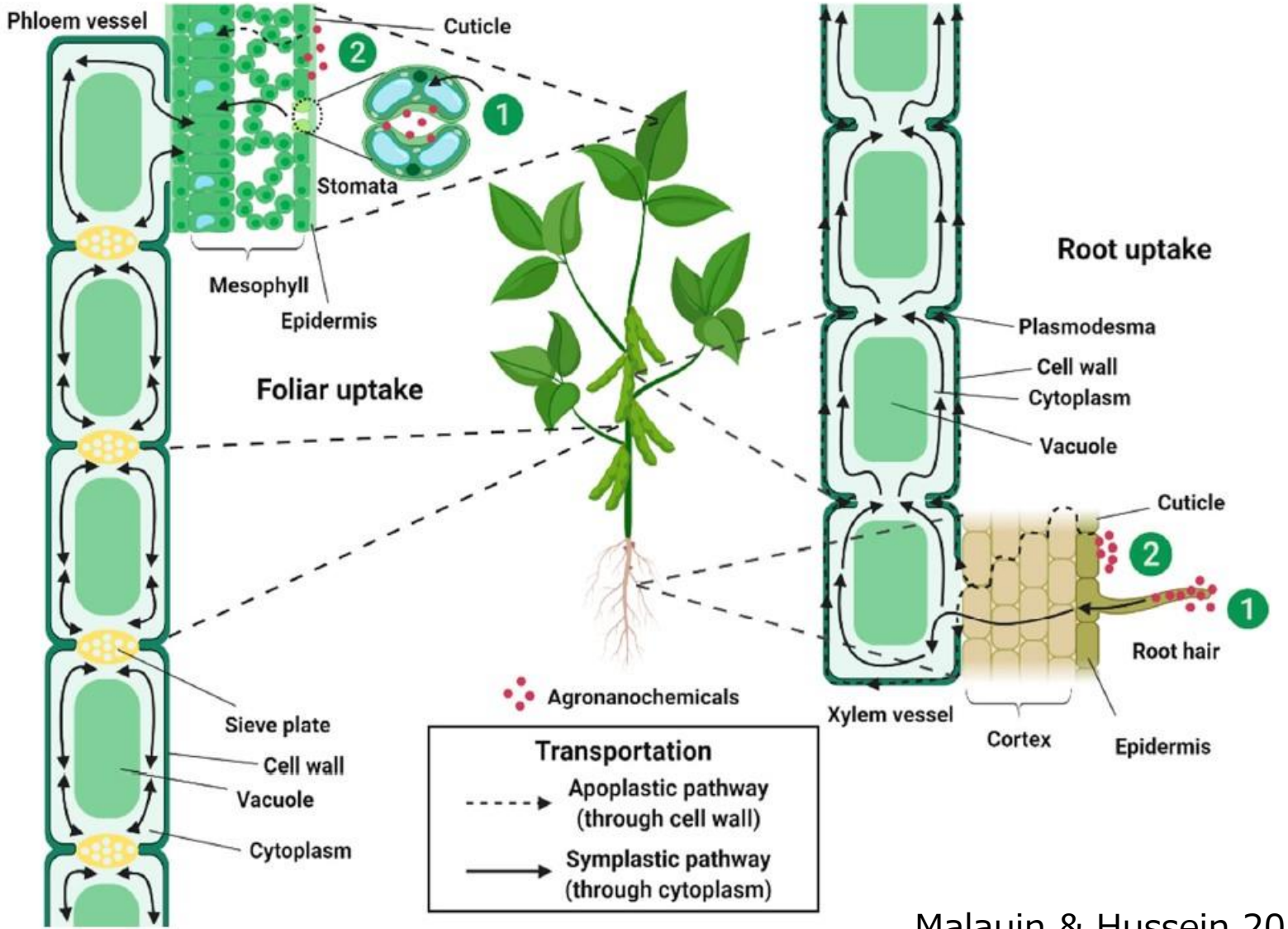
Jhaveri *et al*., 2021

Modifications and combinations of multiple materials



Wang *et al*., 2017

Plant Uptake

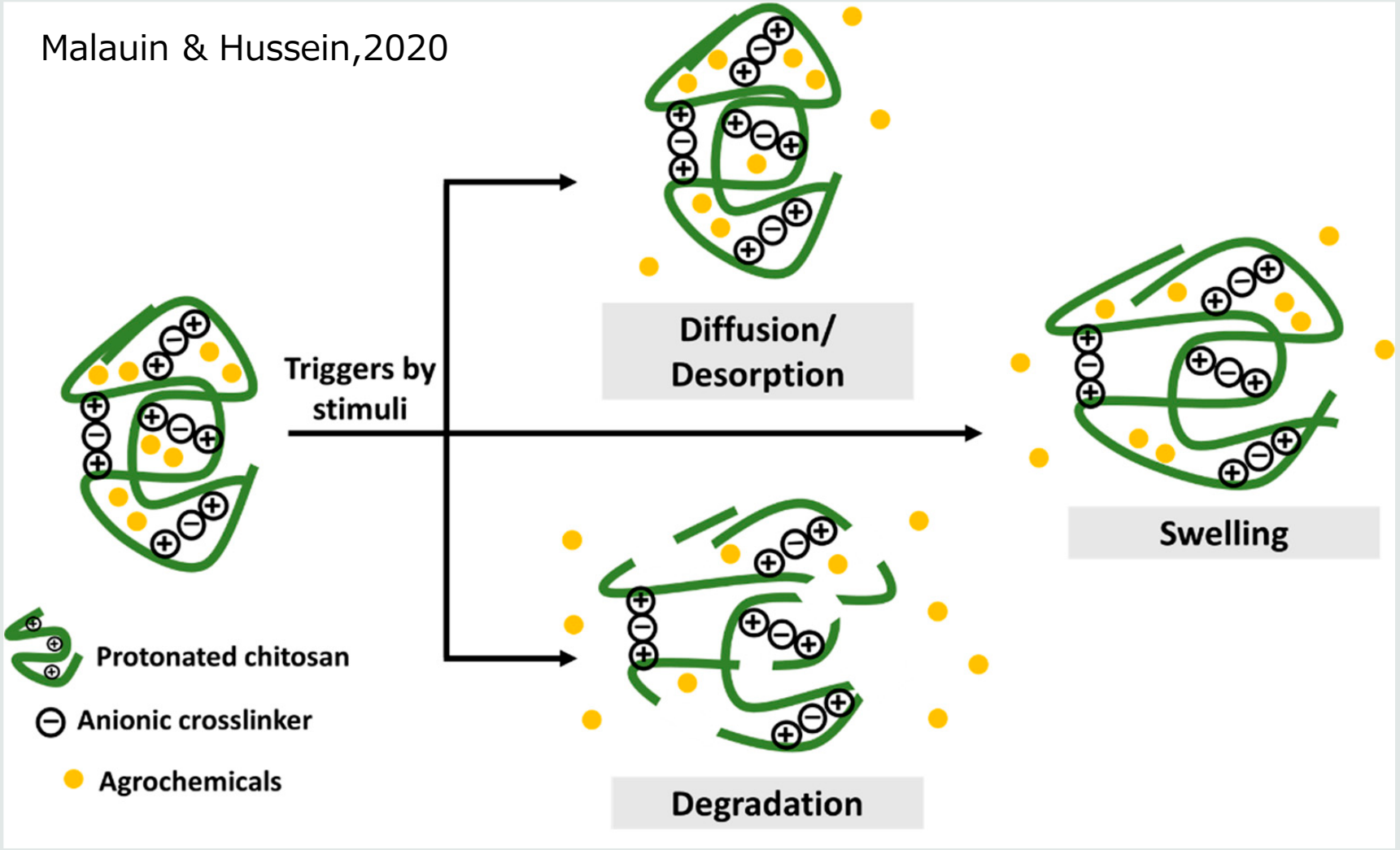


Malauin & Hussein,2020

Drug Release

- **Polymer erosion or degradation**
- **Diffusion/Desorption** (the drug permeates in the surrounding medium)
- **Swelling** (the polymer absorbs water until it dissolves and the chains detangle)

Jhaveri *et al*., 2021, Malauin & Hussein,2020



EXAMPLES OF PRACTICAL APPLICATION IN MY PhD THESIS

An overview on the subject

Agriculture and environment: more sustainable practices are needed

Nanotechnology for plant disease and nutrition management

My topic of study: nanoparticles functionalized with bioagents

In detail ...

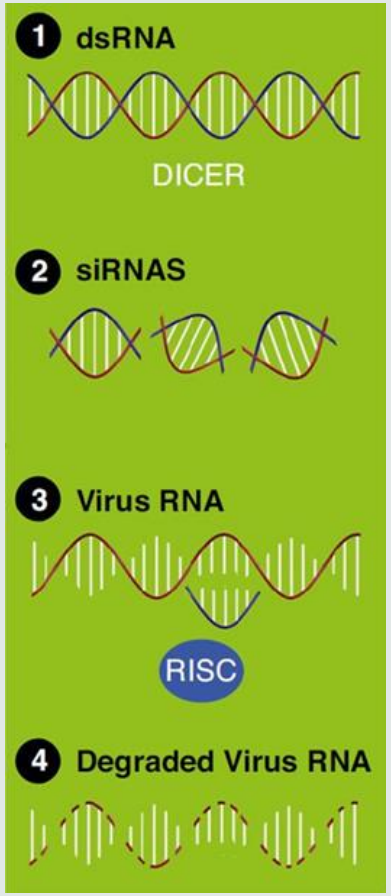
Chitosan nanoparticles (CHIT-NPs)

protective, carrier and biostimulant functions

Exogenous dsRNA

Against pathogen or weed

Induction of the RNA-interference (RNAi) mechanism



Mitter & Worrall, 2017

REFERENCES CHIT-NPS: Malerba & Cerana, 2016; Saharan & Pal, 2016; Kashyap *et al*., 2015.
RNAi: Mitter & Worrall, 2017; Nerva *et al*., 2020.

Aim of the project

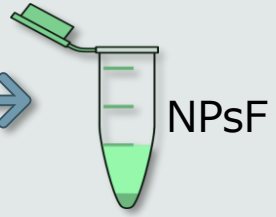
Evaluate whether the effect of dsRNA transported through chitosan NPs is improved compared to the application of naked dsRNa

Secondary purposes:

Biostimulation by CHIT and functionalizing agents

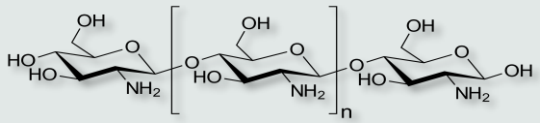
Interaction with plant surfaces and tissue entering pathways

A. Filtration of CHIT stock solution (0.2 µm filter)



NPsF

Chitosan (CHIT)



MW: 100 000 – 300 000 $gmol^{-1}$

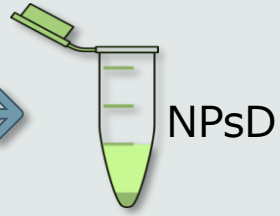
NPs synthesis & Functionalization 20 µg/mL dsRNAs

Evaluation of size, surface potential & retention capacity

Best choice

NPsF

B. Oxidative degradation treatment of CHIT stock solution (6% H_2O_2)



NPsD

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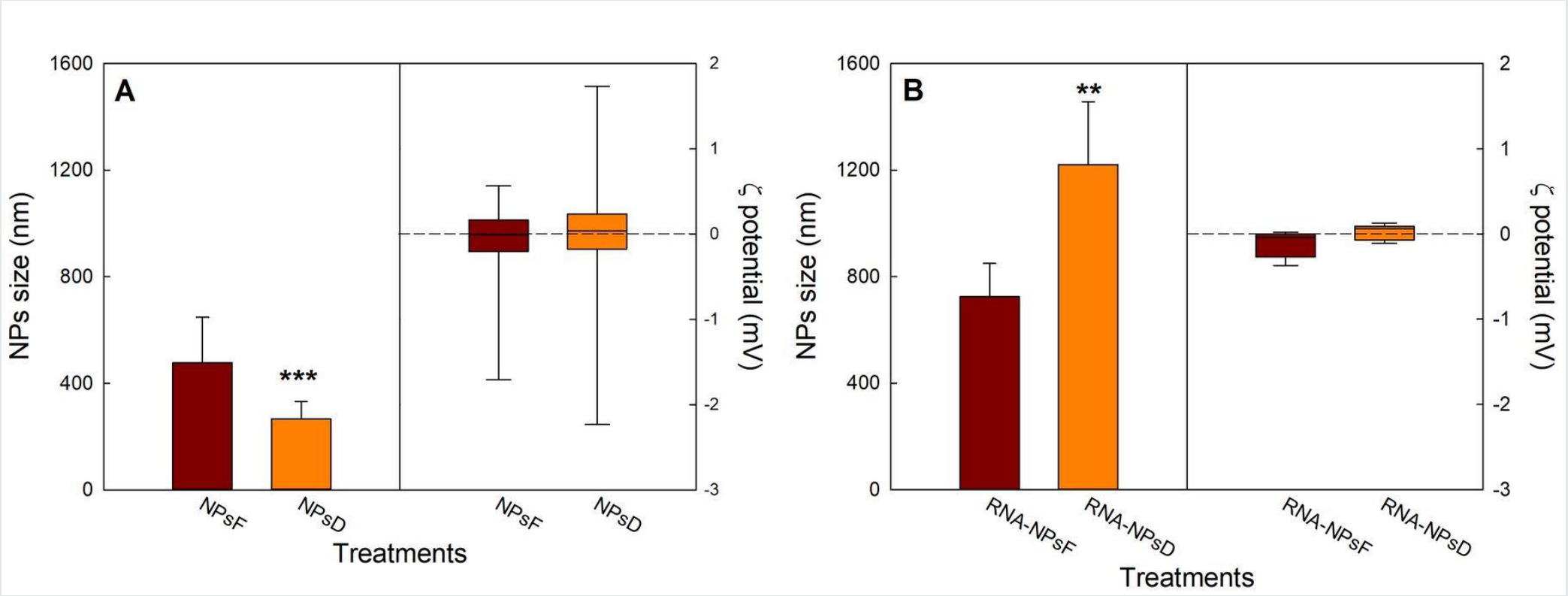
1st year activities: definition of the best protocol for synthesis and functionalization of nanoparticles

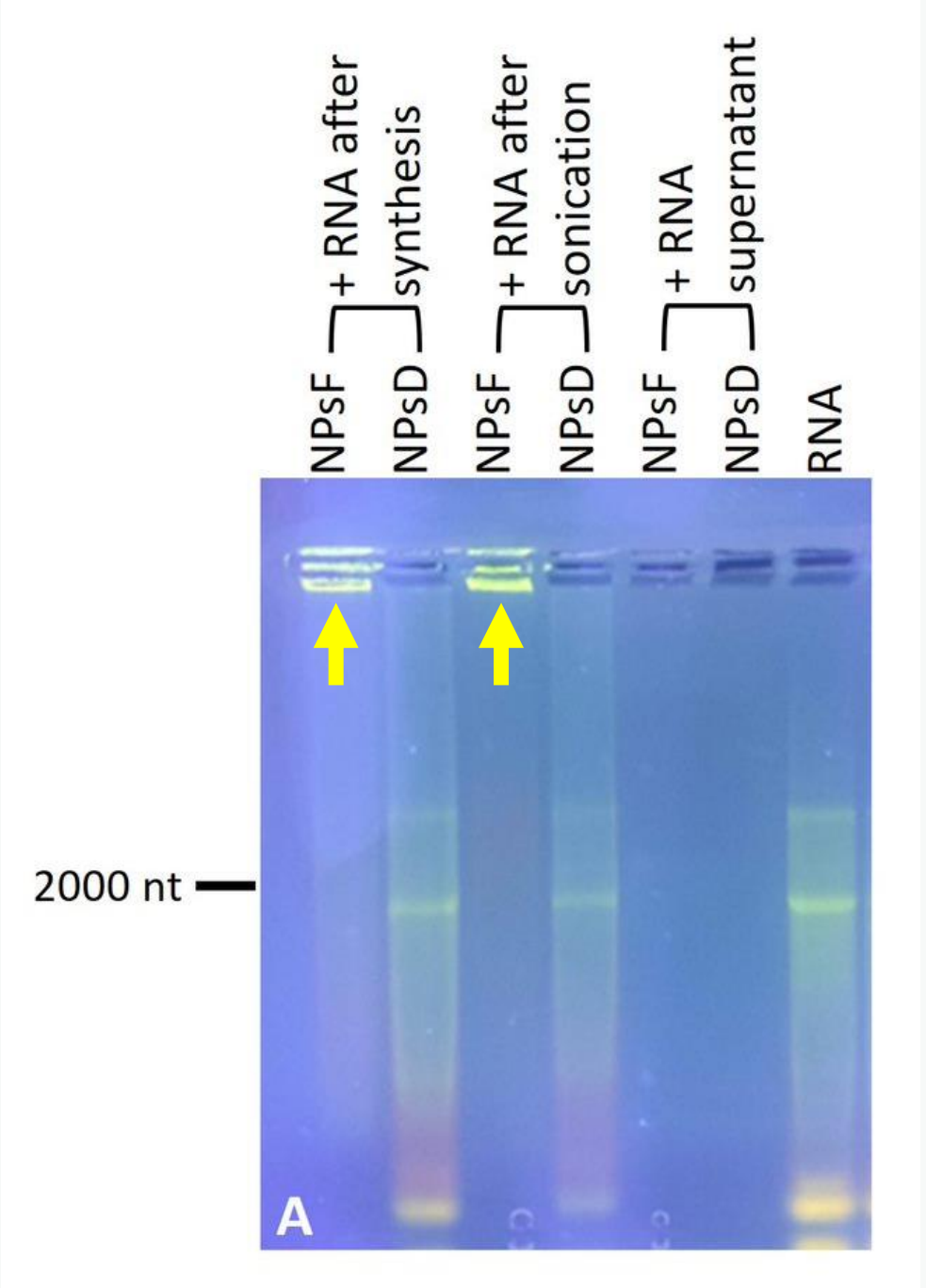
Aspect of CHIT NPs

NPs Size and Charge: Dynamic Light Scattering (DLS)

- **Particle size**: hydrodynamic diameter (nm)
- **Zeta (ζ) potential**: electrokinetic potential in colloidal systems (mV)



RNA-retaining nanoparticles prevent electrophoretic stroke



2nd year main tasks

1. Evaluation of the behavior of NPs after application on leaves of *Nicotiana benthamiana* Domin

NPs functionalization with Fluorescein Isothiocyanate (FITC).

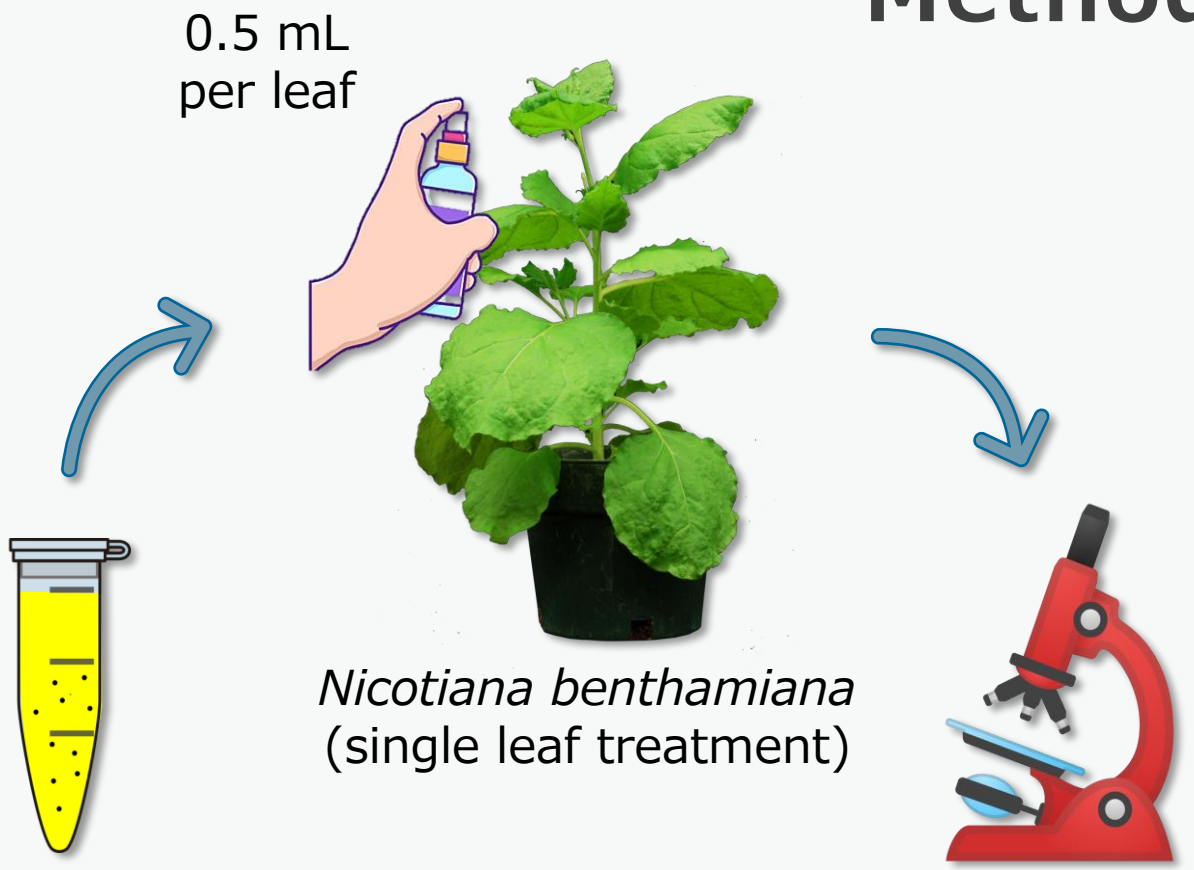
2. Assessment of the ability of NPs to protect dsRNAs from degradation

crea
Consiglio per la ricerca in agricoltura e l'analisi dell'economia agraria

Total RNA from a transformed *E. coli* strain able to synthetize the dsRNAs of Green Fluorescent Protein (***GFP*-dsRNA**) (Nerva *et al.*, 2020)

Methods

1. Evaluation of the behavior of NPs after application on leaves



Nicotiana benthamiana
(single leaf treatment)

NPs functionalized with FITC
(fluorescein-isothiocyanate)
diluted in 0.1% EIA
(Ethoxylated isodecyl alcohol)

Confocal microscopy analysis
Leica TCS SP8

2nd Summer School
"Nanotechnology in Agriculture"
29-30 June, 2023
University of Udine

Empty NPs

FITC-NPs 1:20

FITC-NPs 1:5

FITC-NPs 1:1

FITC-NPs

Chlorophyll autofluorescence

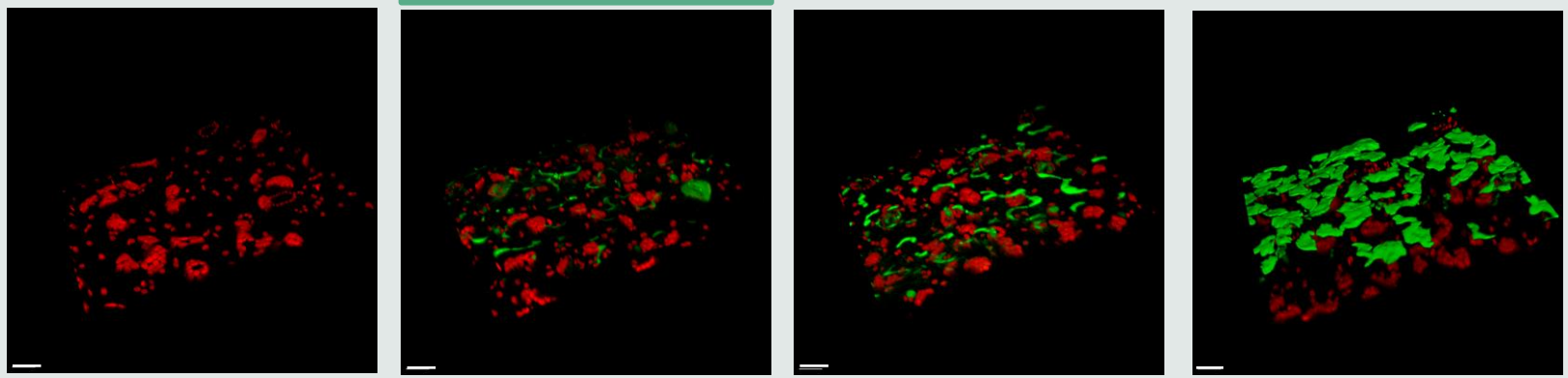
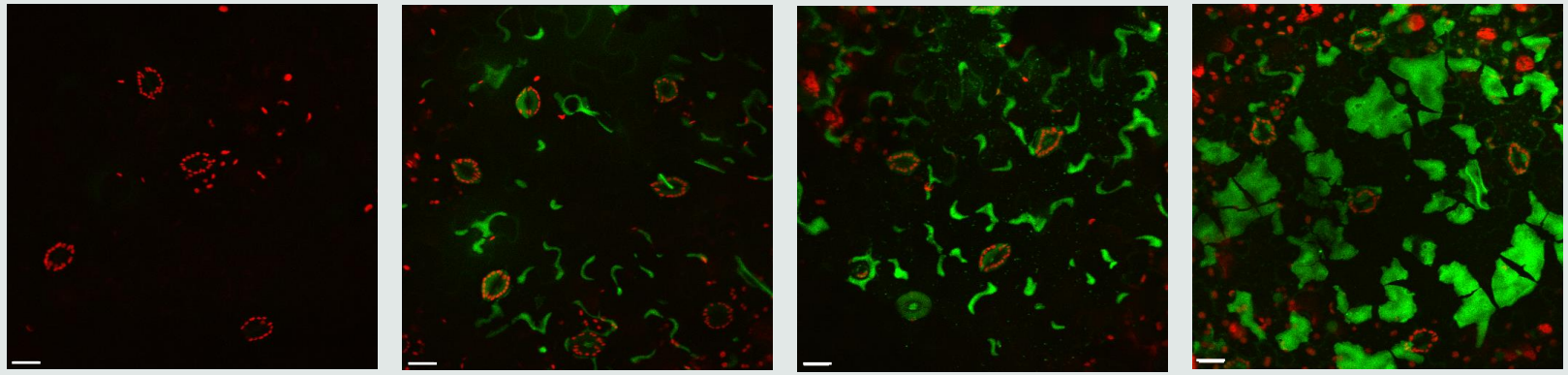
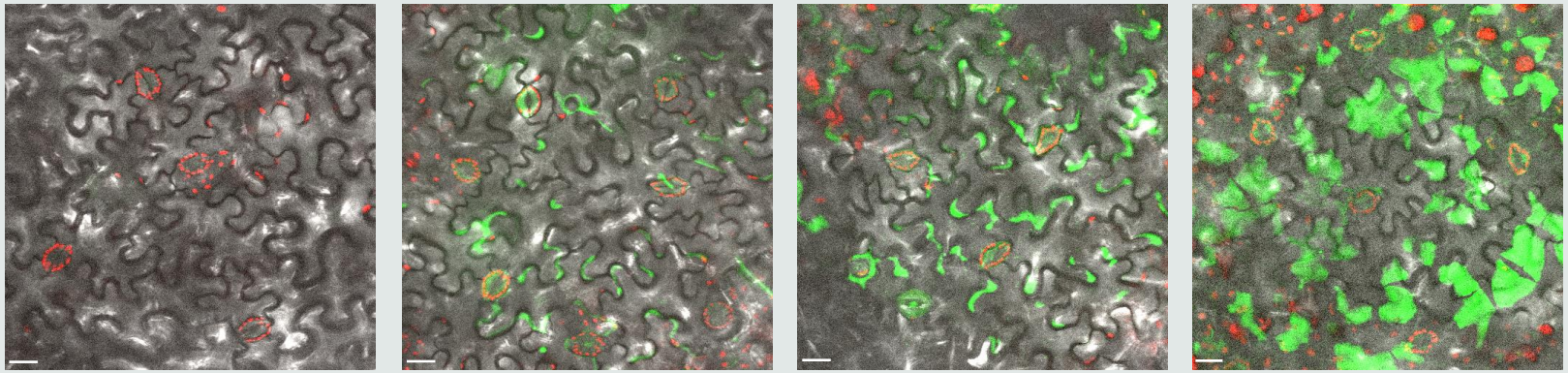
Free FITC 1:1000

Supernatant FITC-NPs 1:20

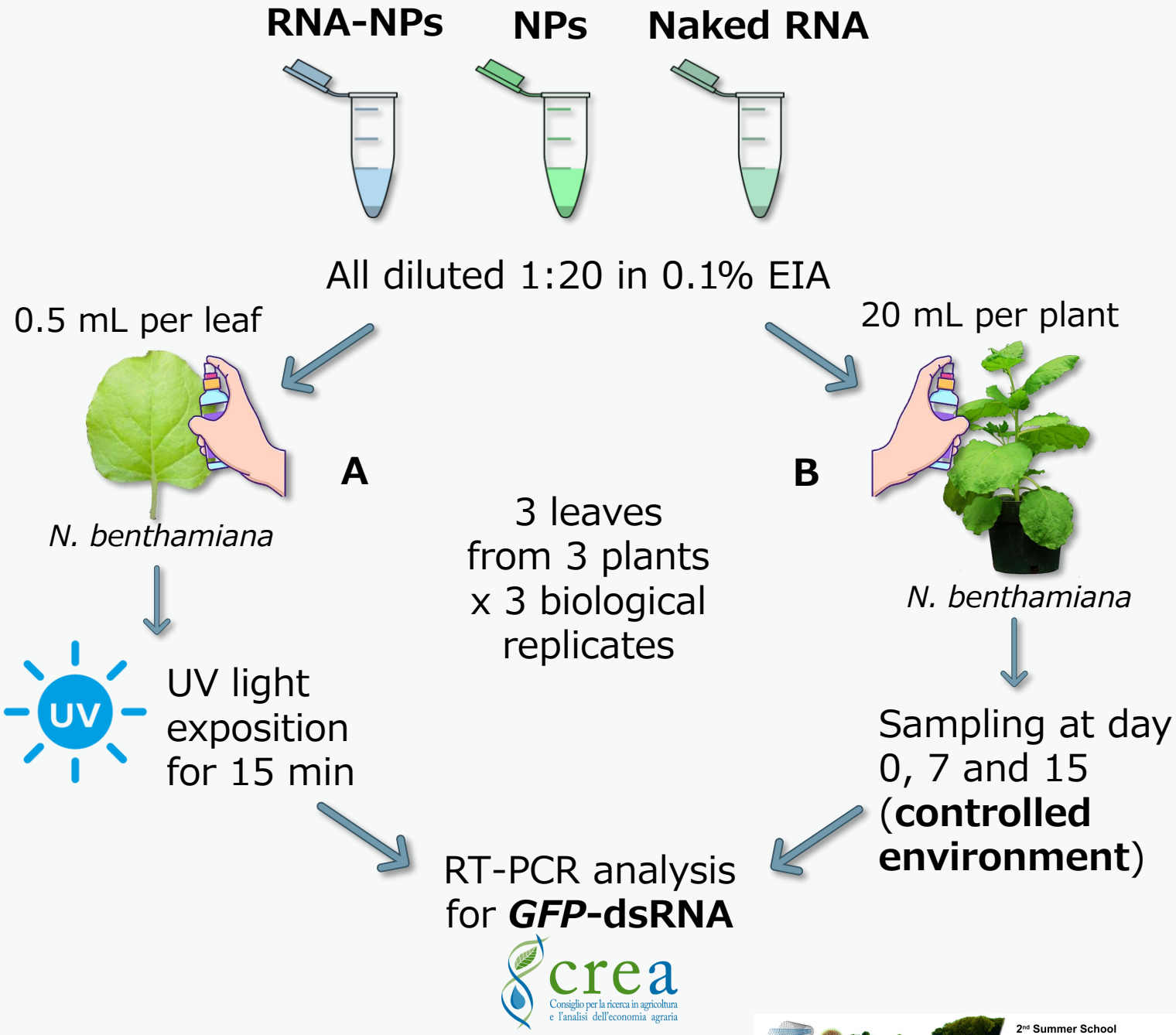
3D

Max. intensity projection

Overlay with brightfield

2.Assessment of the ability of NPs to protect dsRNAs from degradation



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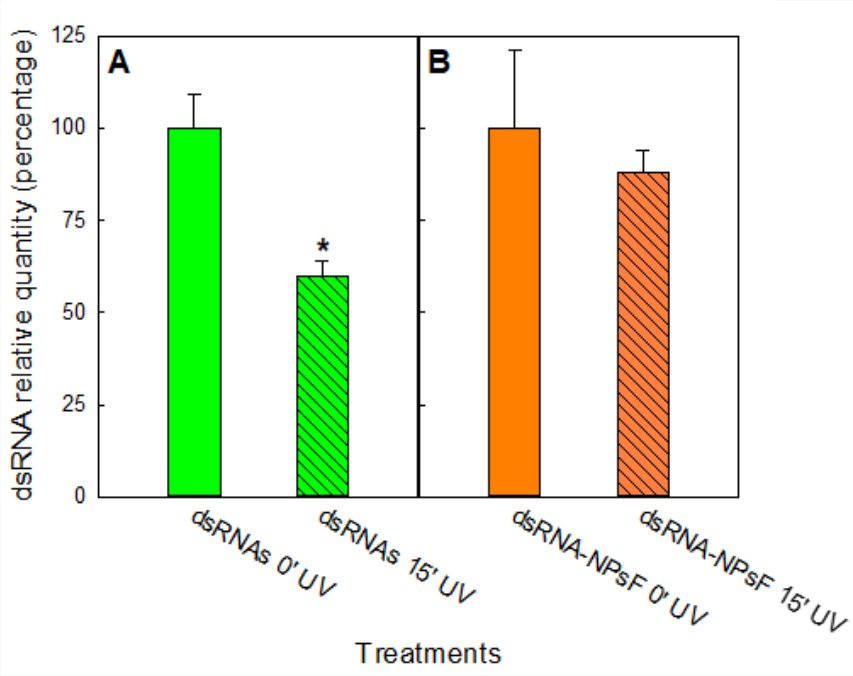
Results

...tiredness is also a result!

A



NPs protect *GFP*-dsRNA from photo-degradation

B

Days after treatment	Treatment	GFP-dsRNA RQ
7	NPs	0.02 ± 0.005
7	Naked RNA	**0.93** ± 0.203
7	NPs-RNA	**0.96** ± 0.178
15	NPs	0.02 ± 0.004
15	Naked RNA	**0.79** ± 0.140
15	NPs-RNA	**0.94** ± 0.205

No statistical significance, but a trend is visible

Conclusions

1

The uniform distribution of NPs on leaf surface depends on:

- Leaf morphology
- NPs concentration, size and zeta potential

Do NPs enter the plant? Work in progress...

2

NPs are presumably able to protect dsRNA

Actually, it is necessary to study their performance in an open-air environment.

How are we going on?

Inhibition experiments on ***Botrytis cinerea*** using NPs functionalized with a specific dsRNA sequence (Nerva *et al.*, 2020) with interference activity

B. cinerea + NPs-dsRNA

B. cinerea

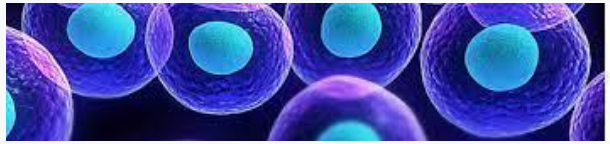
...That's it!

Special thanks to:

DI4A

My Supervisors: Prof. Enrico Braidot & Dr. Elisa Petrussa

All the Plant Biology research group

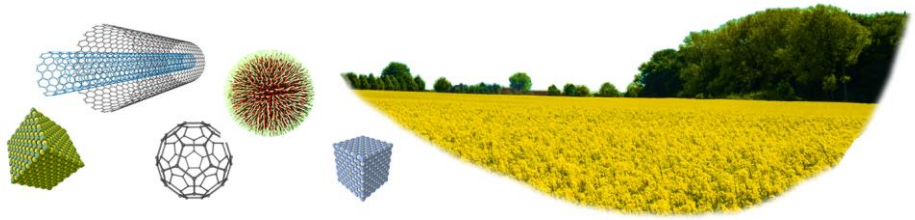


DAME

Dr. Francesca D'Este (Department of Medicine) – Confocal Microscopy

crea
Consiglio per la ricerca in agricoltura e l'analisi dell'economia agraria

Dr. Walter Chitarra, Dr. Luca Nerva and all collaborators of CREA in Conegliano (TV)



2nd Summer School
"Nanotechnology in Agriculture"
29-30 June, 2023
University of Udine

Thank you all very much for your participation and have a good job!