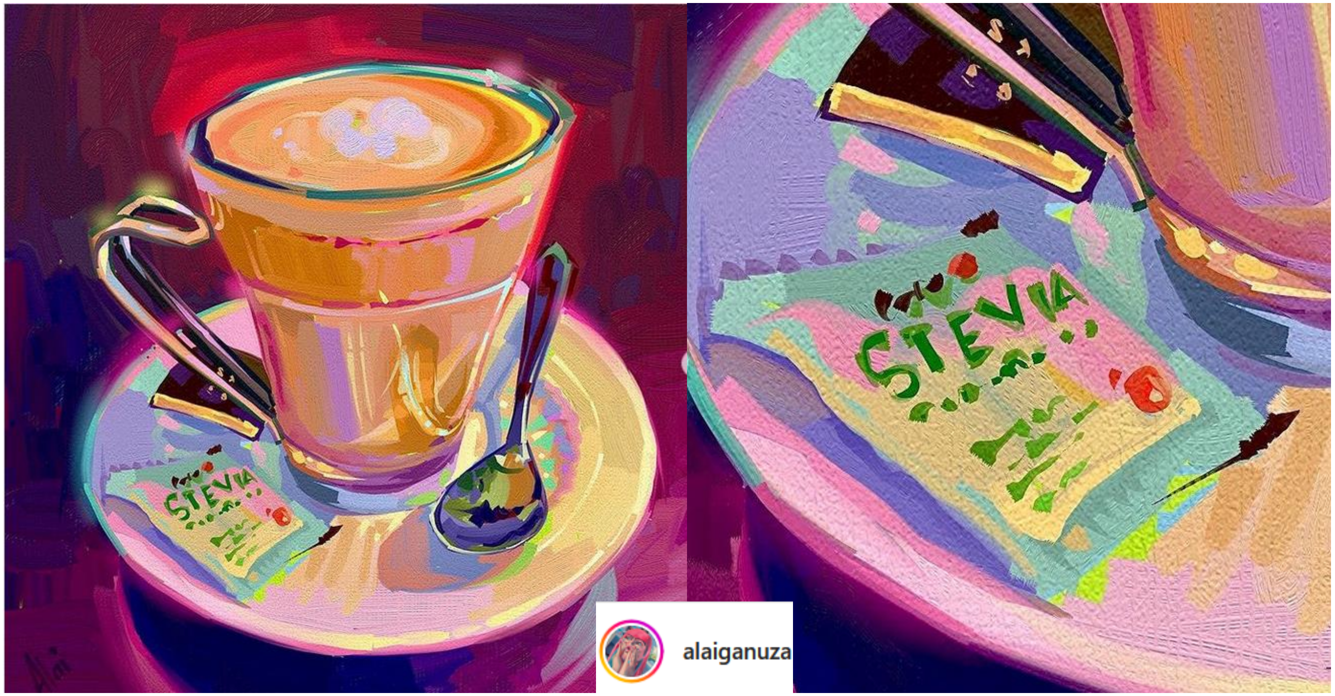
How to make sweet stevia sweeter: green technology of cold plasma



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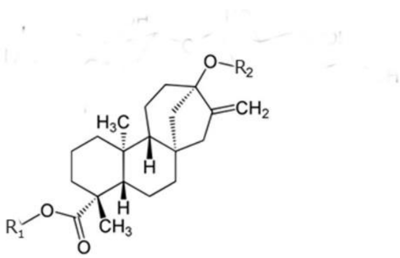
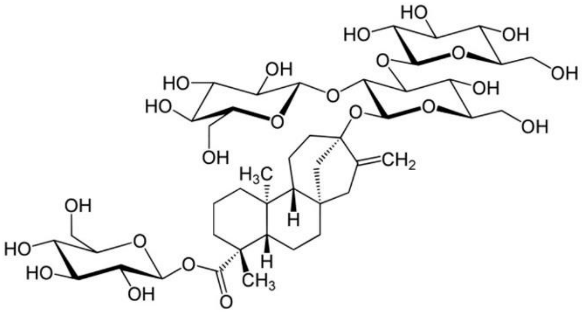
Steviol glycosides (SG) of *Stevia rebaudiana*

Stevioside (**Sevt**) and **rebaudioside A** (**RebA**) are the most abundant steviol glycosides (**SGs**) responsible for the sweetness of stevia.

As compared to **Stev**, **RebA** has an additional glucose monomer that gives it a higher sweetening potency and therefore is the most preferred component of the stevia leaf extracts. **RebA** also lacks the bitter aftertaste characteristic to **Stev**.

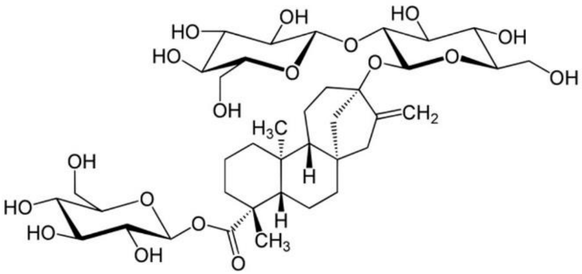


Backbone structure of steviol glycosides



Rebaudioside A

More sweet
No bitter aftertaste



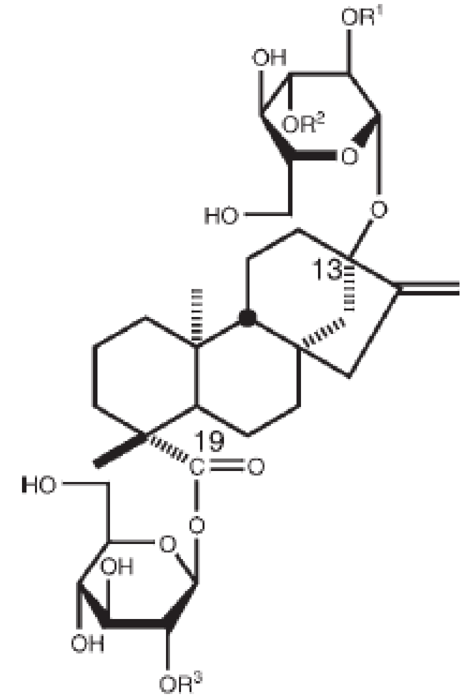
Stevioside

Sweetness of SG

	R^1	R^2	R^3	RS[a]	QT[b]
stevioside (**1**)	β–D–Glc	H	H	143	0
rebaudioside A (**2**)	β–D–Glc	β–D–Glc	H	242	+2
rebaudioside C (**3**)	α–L–Rha	β–D–Glc	H	nd	−1
rebaudioside D (**4**)	β–D–Glc	β–D–Glc	β–D–Glc	221	+3
rebaudioside E (**5**)	β–D–Glc	H	β–D–Glc	174	+1
dulcoside A (**6**)	α–L–Rha	H	H	nd	−2

[a]RS: relative sweetness to sucrose, nd: not determined

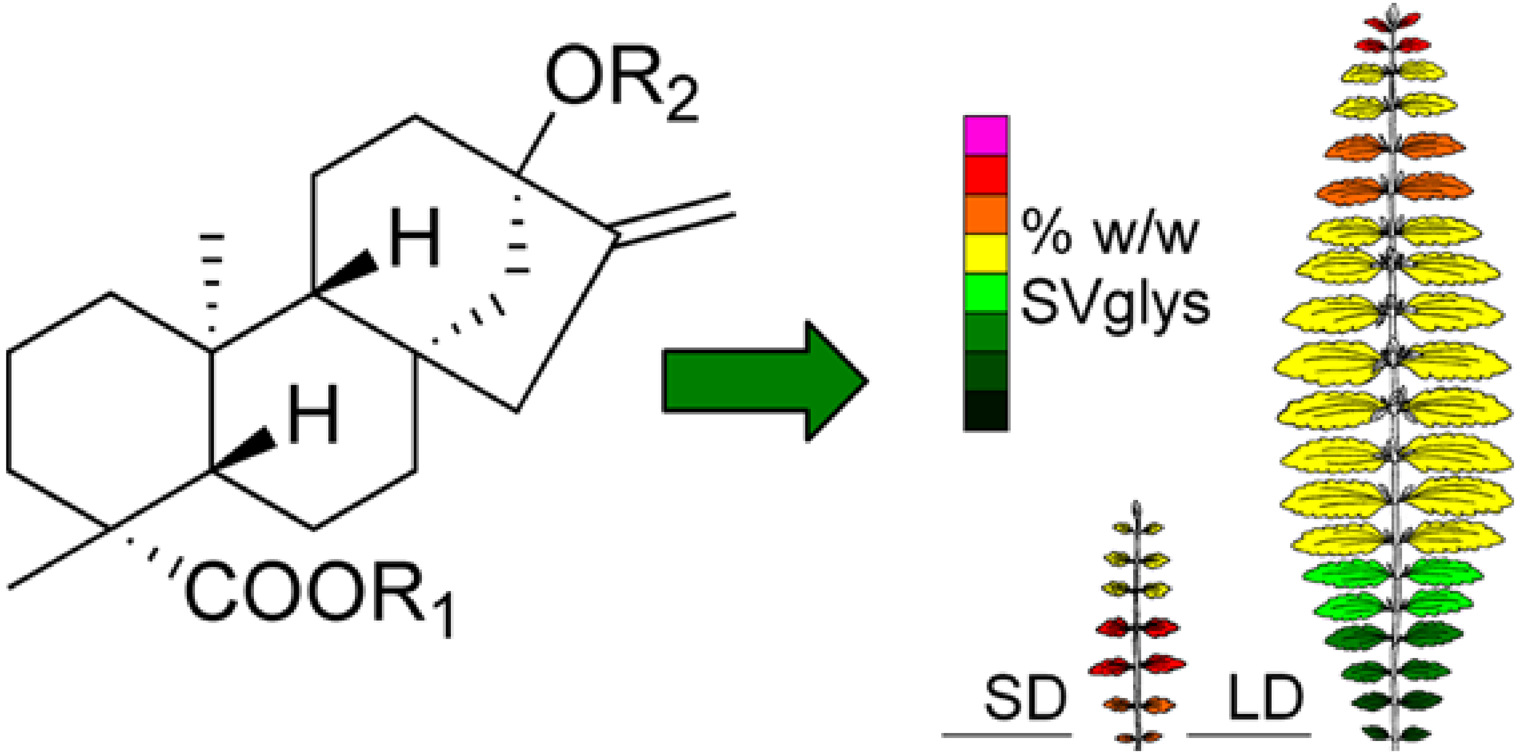
[b]QT: quality of taste, +: better, −: worse



There are at least 38 steviol glycosides identified in stevia to date (Libik-Konieczny et al, 2021).

Ohtani K, Yamasaki K. Methods to improve the taste of the sweet principles of *Stevia rebaudiana*. In „Stevia. The genus *Stevia*“. Ed. Kinghorn AD, 2002.

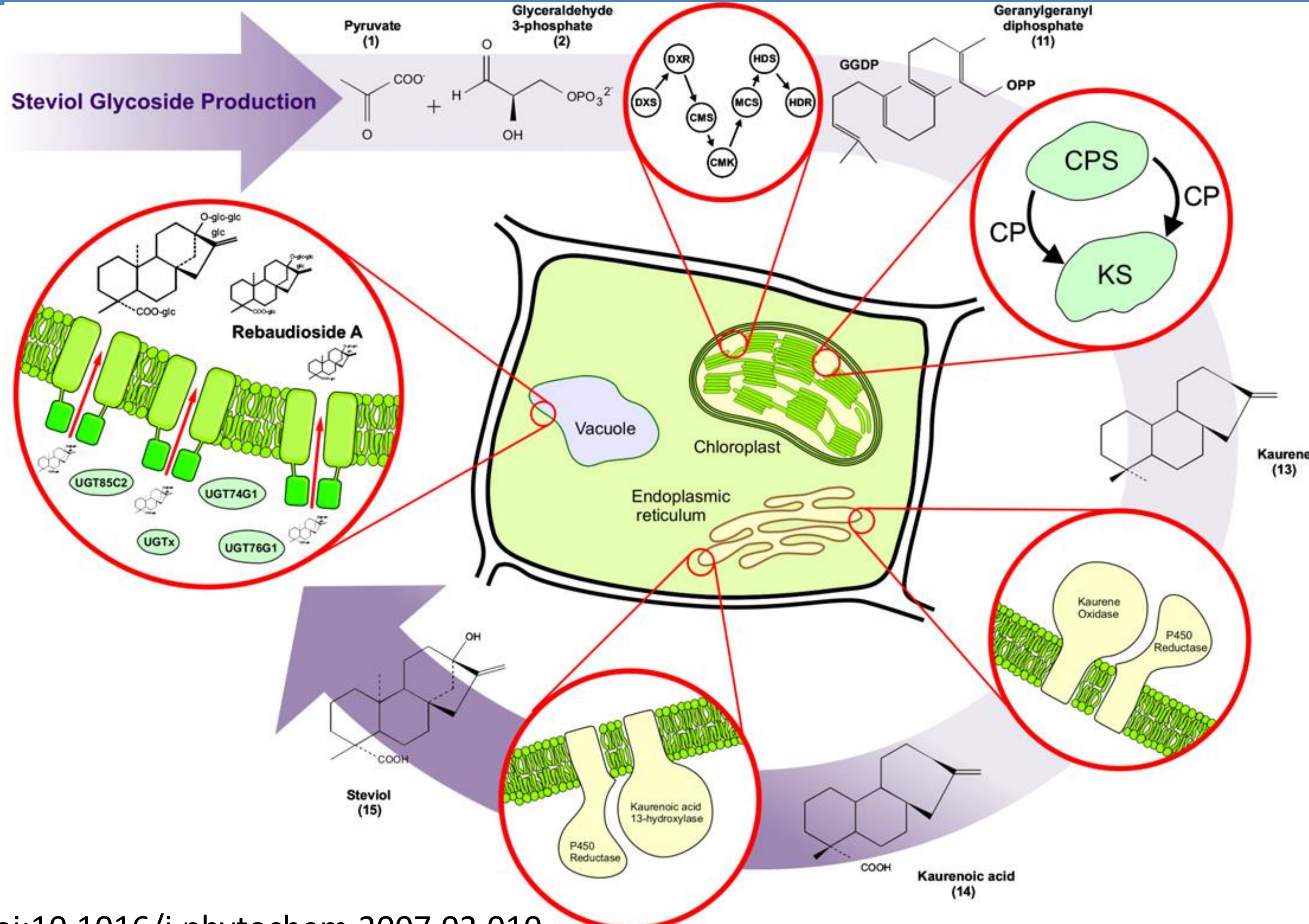
SG distribution in stevia



SD – short day (app. 8 h), LD – long day (app. 16 h)

Ceunen S, Geuns JMC. Steviol Glycosides: Chemical Diversity, Metabolism, and Function. J. Nat. Prod. 2013, 76, 1201−1228. dx.doi.org/10.1021/np400203b

Subcellular location SG biosynthesis in stevia



doi:10.1016/j.phytochem.2007.02.010

SGs functions: against plant pests and herbivores?

Grasshopper
Valanga irregularis

Red spider mite
Tetranychus urticae

Guinea pig
Cavia porcellus

Stevia rebaudiana

Feeding:

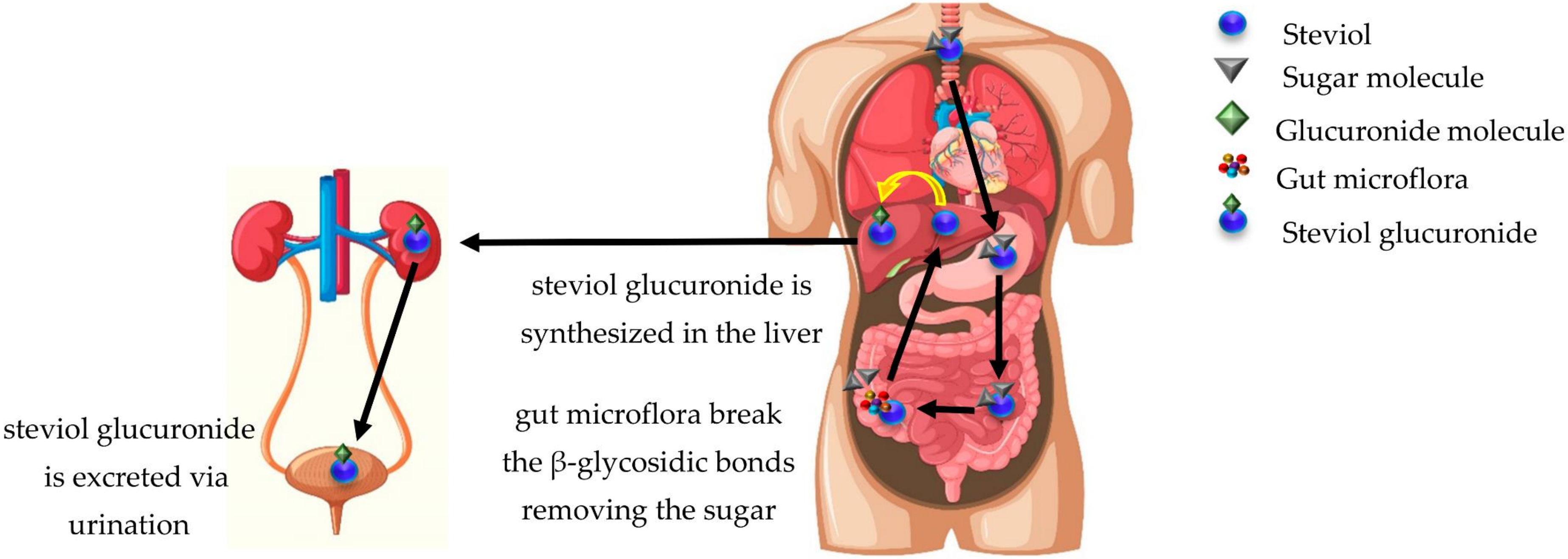
=Negative

=Neutral
(feeding mechanism that avoids chlorenchyma cells that contain SGs)

=Positive

DOI: 10.1021/acs.jnatprod.8b00958
J. Nat. Prod. 2019, 82, 1200–1206

SG metabolism in human body



Bacteroides species are primarily responsible for the hydrolysis of steviol glycosides (β-glycosidic bond) in the gut via their β-glucosidase activity.

The released sugar moieties are not absorbed and are most likely quickly utilized by the gut microbes as an energy source, thus making it a **zero-calorie sweetener.**

doi:10.3390/molecules27041362

More than a sweetener: SG as drug candidates

Antidiabetic action:

- In diabetic rats, Stev (0.2 g/kg IV) decreases glucose blood levels and increases insulin responses and reactions to an intravenous glucose tolerance test (IVGT);
- RebA increases insulin production in isolated murine islets of Langerhans depending on extracellular Ca^{2+} concentration;
- SGs increase the glucose intake in rat fibroblasts, etc.

Antihypertensive activity:

- Stev triggers vasorelaxation via inhibition of Ca^{2+} reflux into the blood vessel;
- Stev has selective antihypertensive effect - no evidence of a hypotensive effect in humans with normal arterial pressure levels, etc.

Anti-inflammatory property:

- Stev inhibits NF-κB, a transcription factor which controls expression of inflammatory cytokines;.
- Stev enhances the innate immune system, etc.

Reviewed in doi:10.3390/molecules28031258

More than a sweetener: SG as drug candidates

Antioxidant activity:

- Mixture of SGs augment the concentration and activity of CAT and SOD;
- Stev and RebA effectively control lipoperoxidation and protein carbonylation in a fish model;
- Stev prevents oxidative DNA damage in the livers and kidneys of a type 2 diabetes murine model.

Anticancer action:

- The activity of tumor promoter TPA is successfully inhibited with Stev in a murine skin-cancer model;
- Stev reduces mammary adenoma incidence in F344 rats, etc.

Antidiarrheal activity:

- Stev controls intestinal smooth muscle contraction;
- SGs demonstrates antibacterial action on *Escherichia coli;*
- SGs impede binding of rotavirus to host cells, etc.

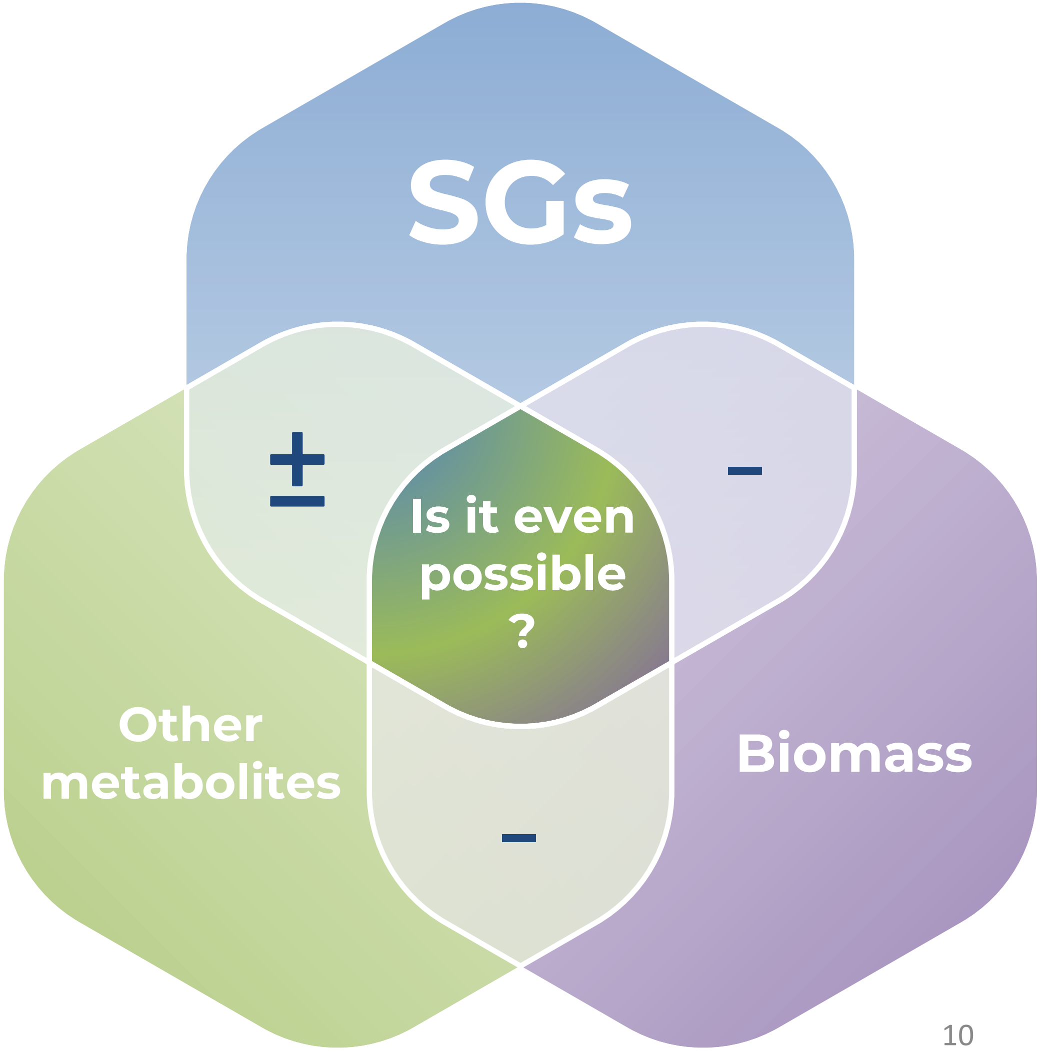
Effect on gut microbiota:

- *In vitro* and *in vivo* studies have shown no influence of SGs on gut microbiota growth.

Reviewed in doi:10.3390/molecules28031258

Goals of cold plasma application in stevia

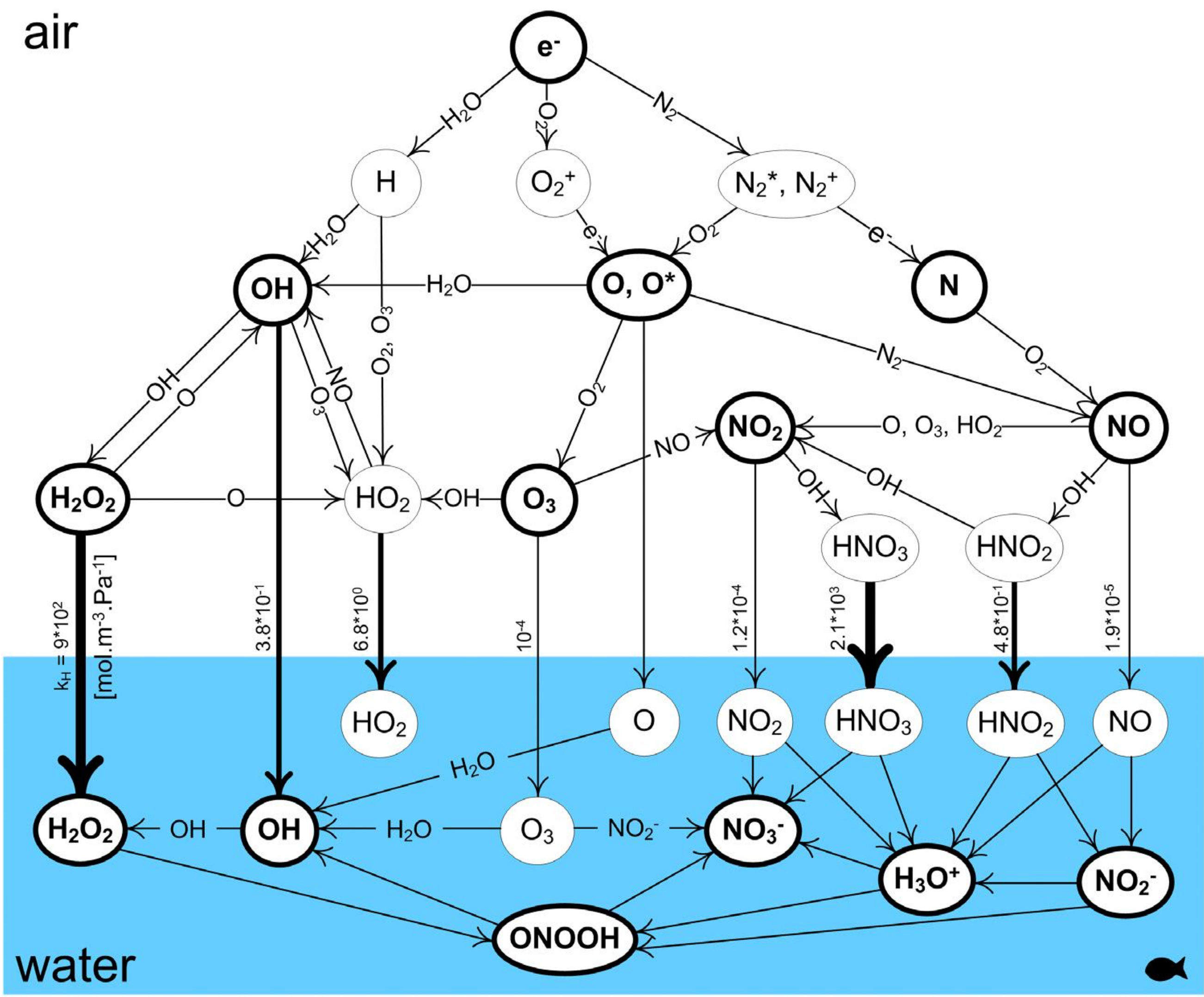
- **to increase biosynthesis of SGs**
- to increase SGs-enriched biomass
- to increase biosynthesis of other bioactive metabolites (i.e. antioxidants)
- To increase resistance to abiotic/biotic stressors and diseases



What is cold plasma (CP)?

Non-thermal plasma or cold plasma is a non-equilibrium gas discharge plasma, consisting of:

- charged particles, such as ions, free electrons,
- neutral particles, including gas molecules, free radicals,
- UV photons.



Machala *et al* 2019 *J. Phys. D: Appl. Phys.* **52** 034002

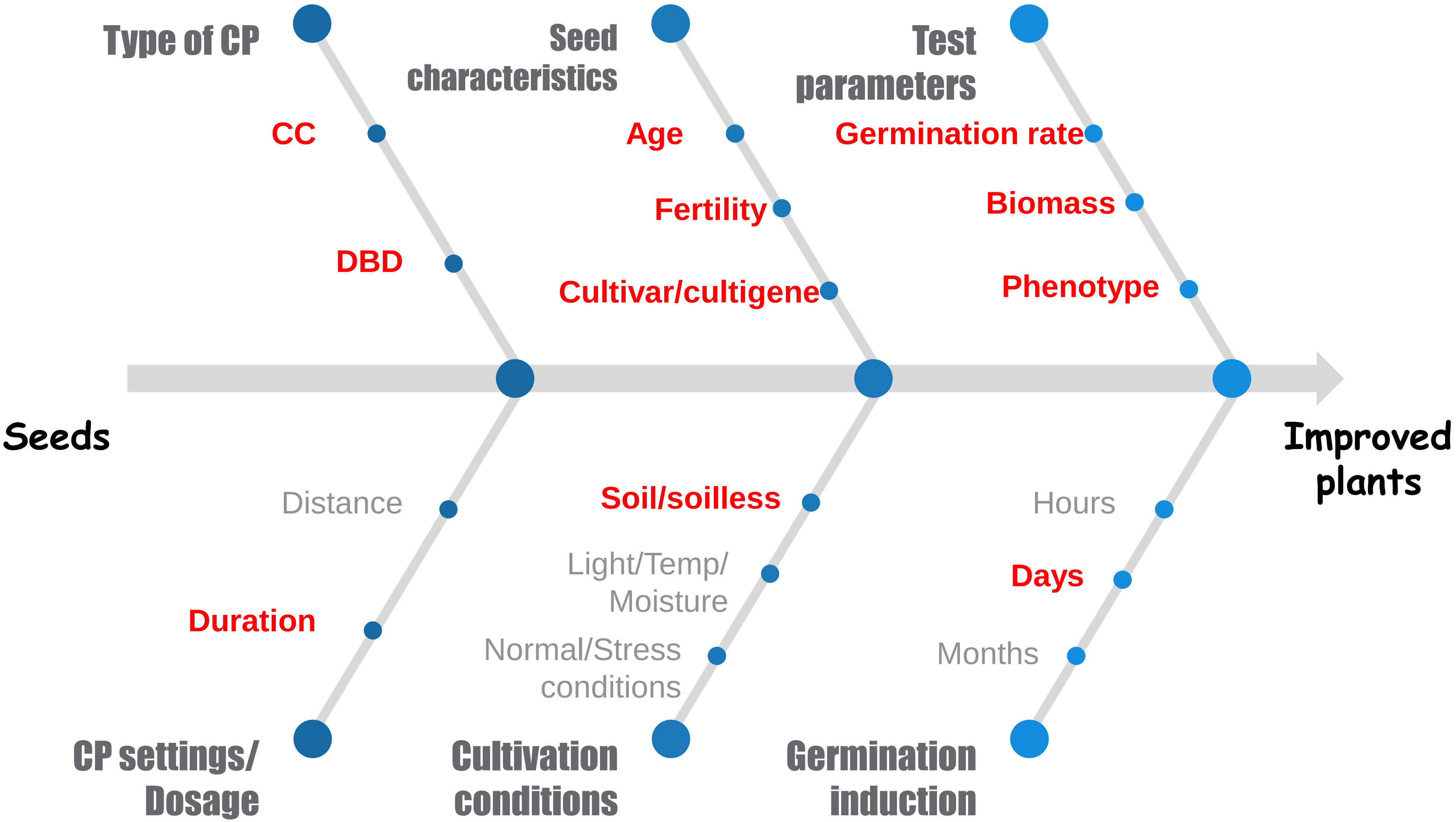
Conventional methods for SGs biosynthesis stimulation

- Breeding
- Fertilizers
- Biofertilizers/growth activators
- Phytohormones
- Nanoparticles
- Cultivation conditions (photoperiod, temperature, soil, etc.)

Post-harvest technologies:

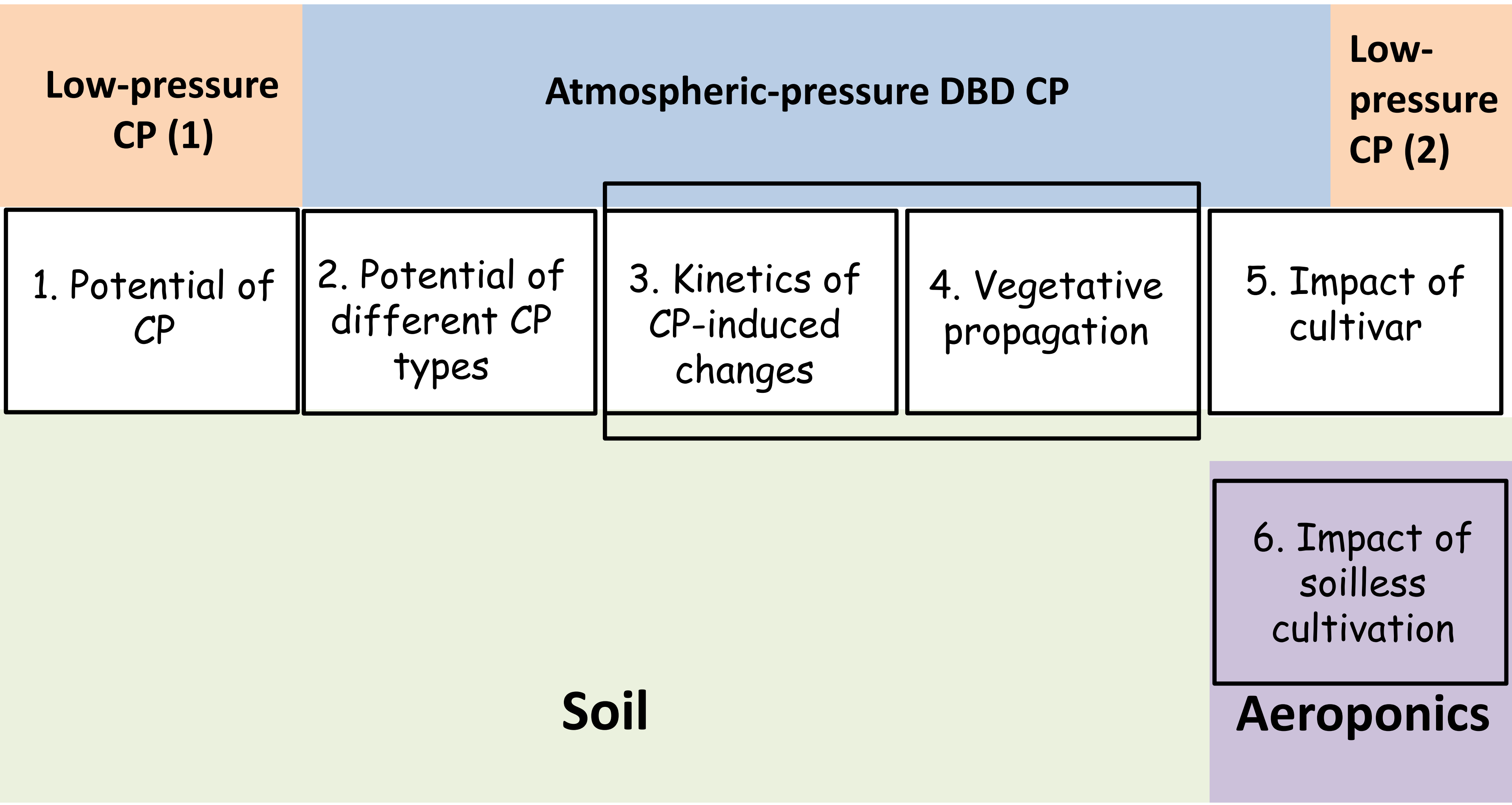
- Drying and extraction conditions
- Enzymatic conversion (i.e. Stev to RebA)

Factors contributing to CP-induced effects

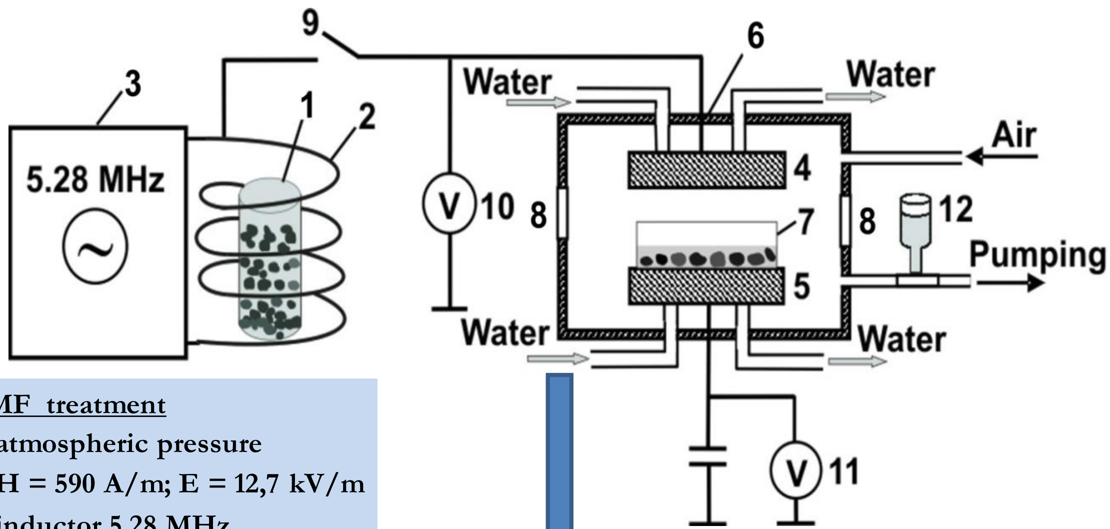


Stress + Object + Evaluation → Mechanisms

Experimental models



1. Experimental setup for seed treatment with CP

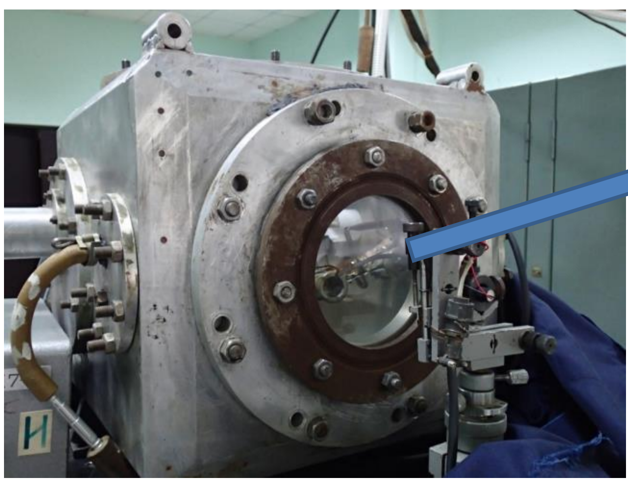


1 - dielectric container with seeds,
2 – inductor,
3 - RF generator,
4 - powered electrode,
5 - grounded electrode,
6 - vacuum chamber,
7 - Petri dish with seeds,
8 – window,
10, 11 - voltmeters,
12 - thermistor vacuum gauge.

<u>EMF treatment</u>

- atmospheric pressure
- H = 590 A/m; E = 12,7 kV/m
- inductor 5.28 MHz
- exposure – 5, 10, 15 min

Capacitively coupled plasma

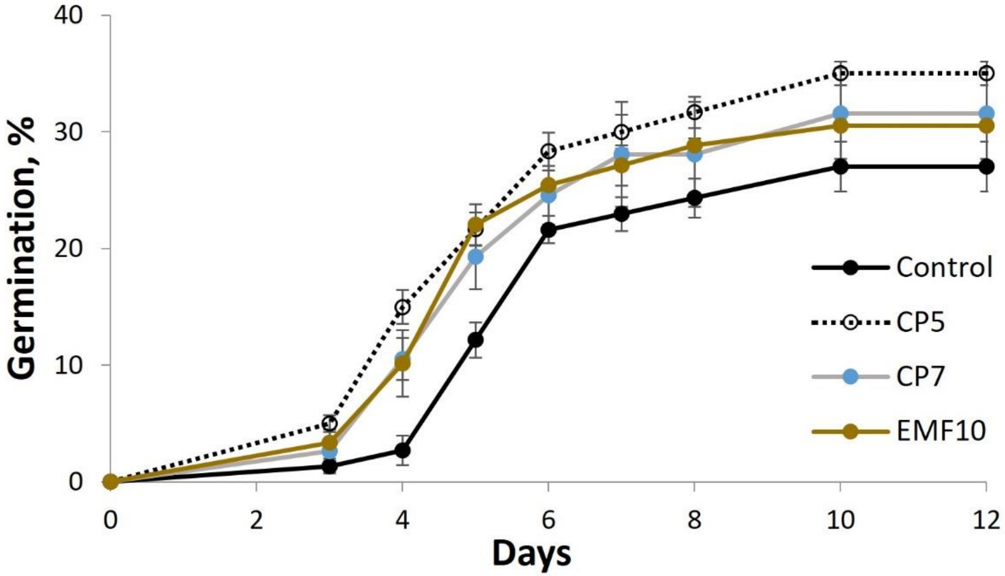


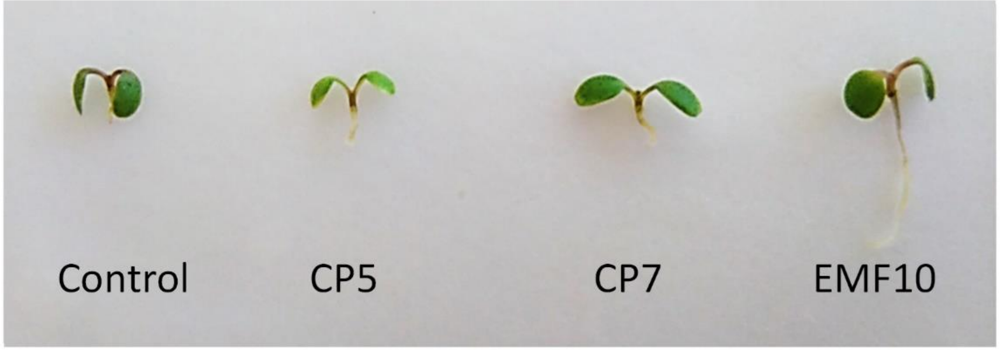
B. I. Stepanov Institute of Physics, National Academy of Sciences of Belarus

<u>CP treatment</u>:

- 5.28 MHz air plasma,
- gas pressure – 40 P
- the specific RF power – 0.1 - 0.6 W/cm^3
- exposure – 2, 5, 7 min

CP (CC) effect on germination *in vitro, cv. "Criolla"*



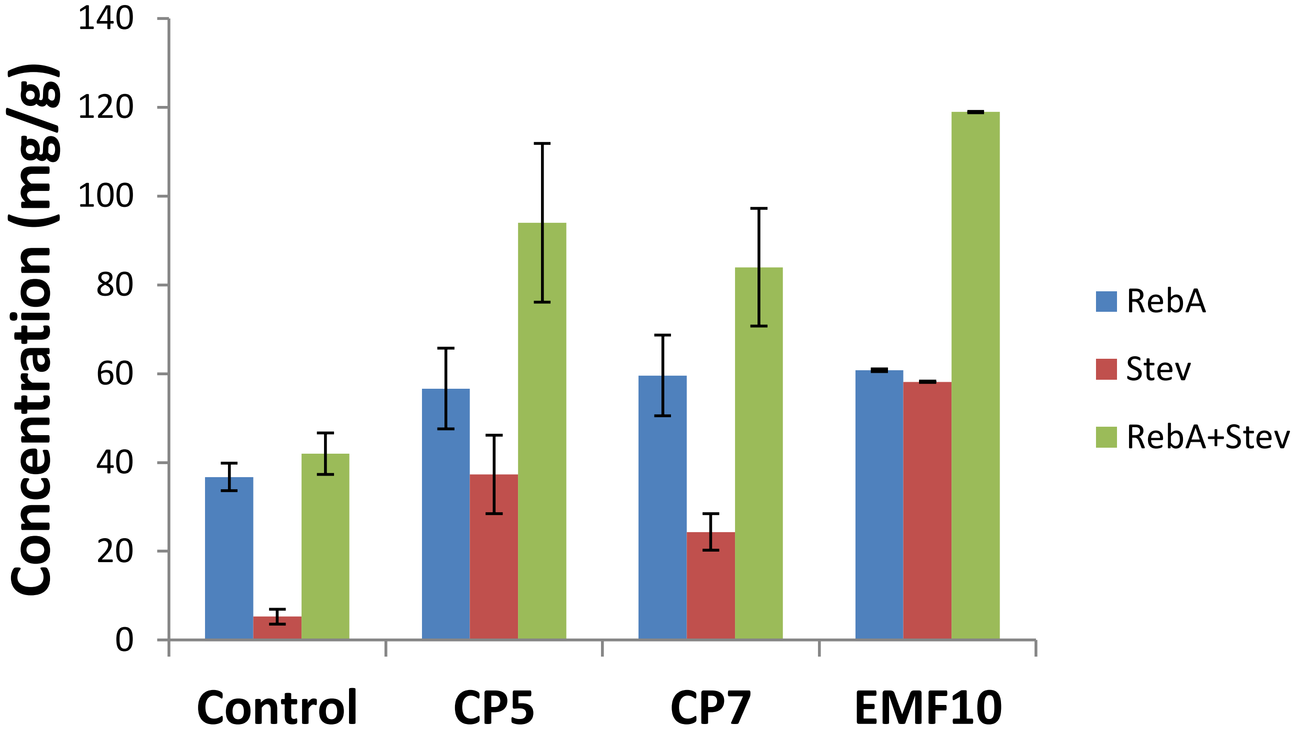


CP increased germination rate, yield and development of sprout roots

CP (CC) effect on Stev and RebA

Pre-sowing seed treatment with CP induces **increase** in the amount of **steviol glycosides** in *S. rebaudiana* cultivar ***"Criolla"*** **:**

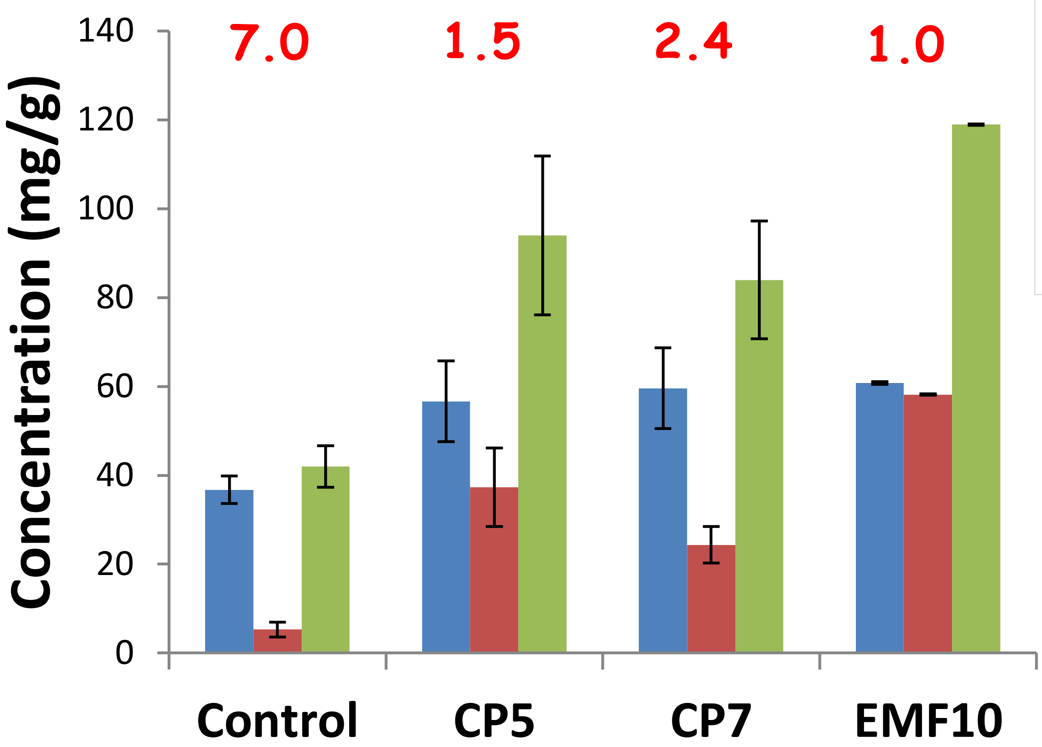
- **1.5-fold** increase in Reb A amount,
- up to **7-fold** increase in Stev amount (5min) (and up to 11-fold in EMF10).
- however, CP decrease RebA/Stev ratio.

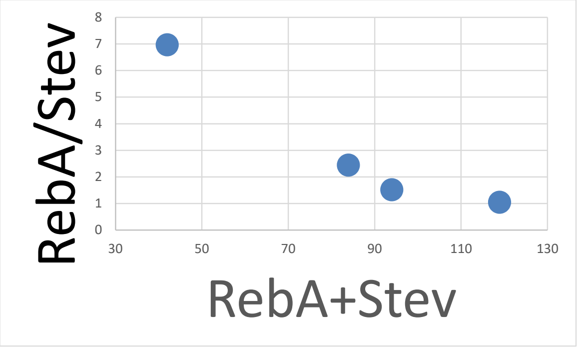


* - all changes are statistically significant as compared to control, $p < 0.05$

CP (CC) effect on RebA/Stev ratio

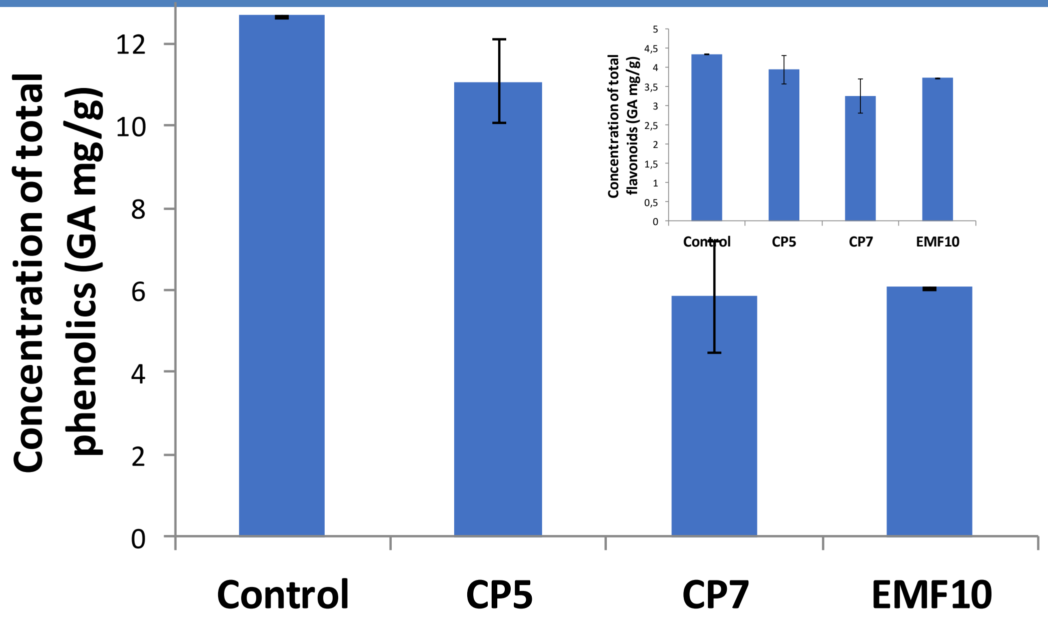
RebA/Stev ratio is important for taste properties when the mixture of SGs is used as a sweetener: the higher the ratio, the better the taste. 1 to 1 ratio is already acceptable.



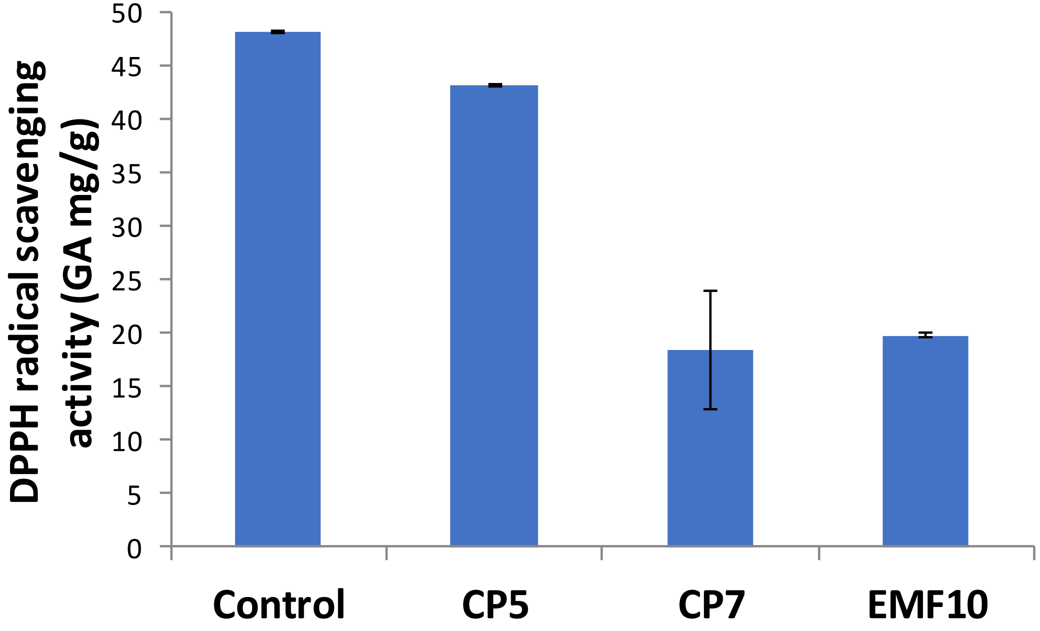


* - all changes are statistically significant as compared to control, $p < 0.05$

CP and EMF effect on total phenolics, flavonoids and antioxidant activity



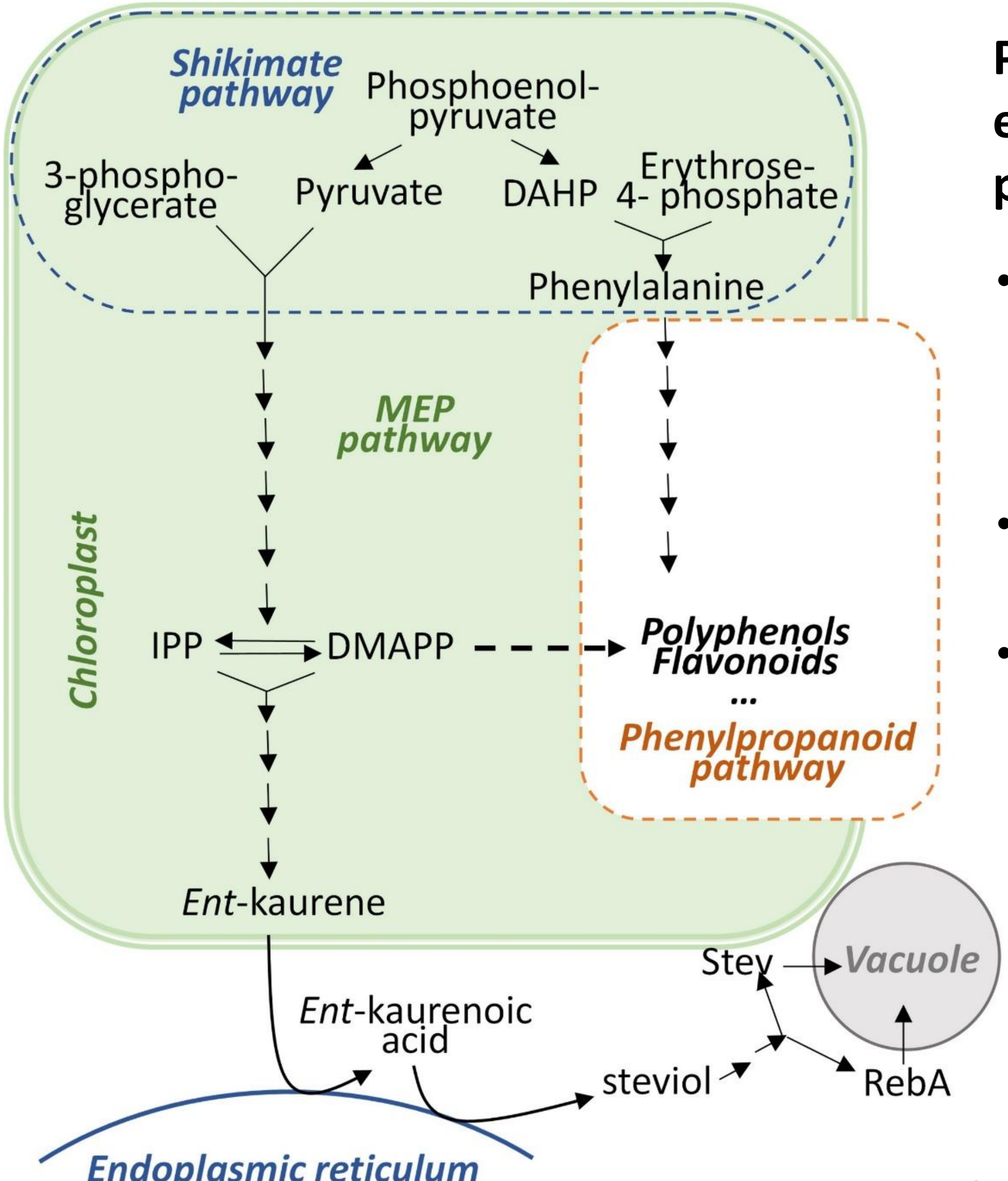
CP7 and EMF10 decreased total phenolics concentration 2.2 and 1.8-fold, respectively.



The amount of total phenolics strongly correlates with radical scavenging activity (R^2 = 0.9965).

* - all changes are statistically significant as compared to control, p <0.05

Biosynthetic pathways of terpenoid and flavonoid/polyphenols



Possible explanations of the opposite effects on biosynthesis of SGs and phenolics:

- Competition between MEP and phenylpropanoid pathways for the common precursor phosphoenolpyruvate
- DMAPP can be used for the synthesis of both flavonoids and terpenoids
- Certain transcription factors coordinate metabolic activities between the flavonoid and terpenoid biosynthetic pathways

DAHP, 3-deoxy-D-arabino-heptulosonate 7-phosphate; DMAPP, dimethylallyl diphosphate; MEP, methyl-erythritol phosphate.

Judickaite et al. Plants 2022, 11, 611.

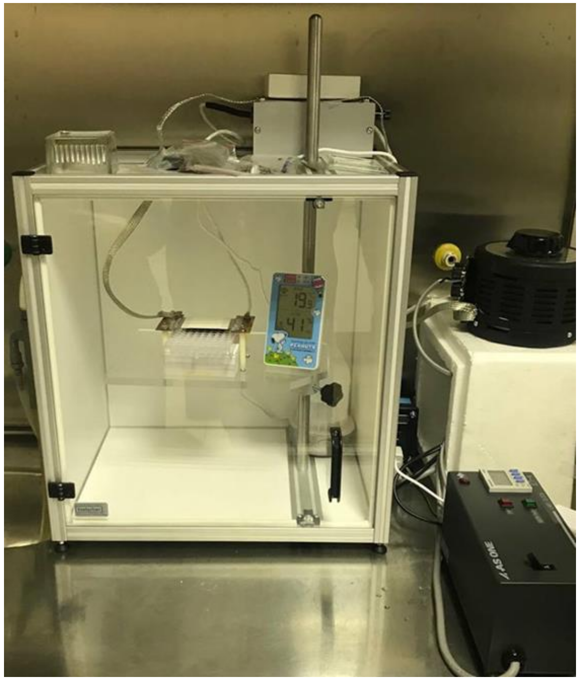
CP (CC) effects in *Stevia rebaudiana*

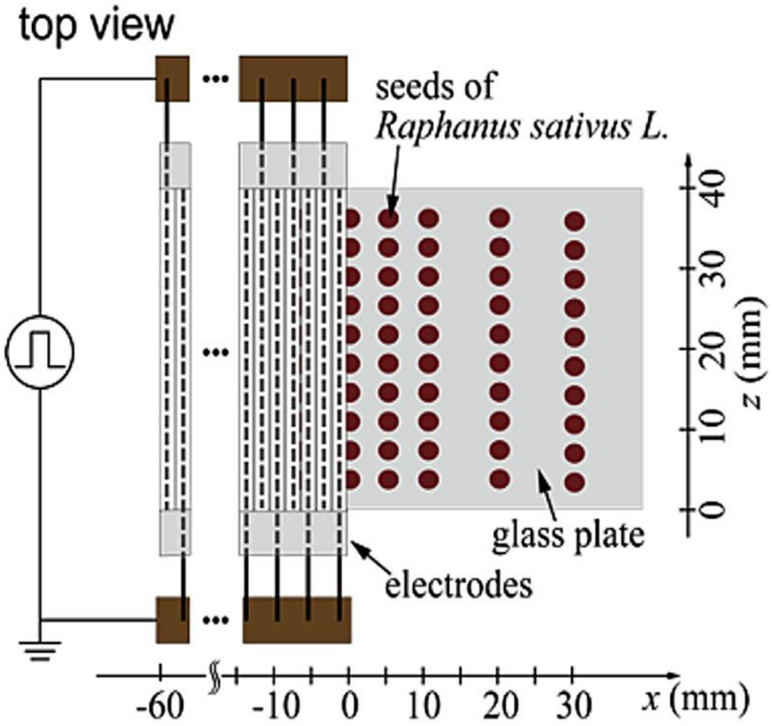
Judickaite et al. Plants 2022, 11, 611.

2. CP (AP-DBD) equipment

Experimental setup for seed treatment with CP (AP)

at Vytautas Magnus University, installed by prof. Shiratani M. and prof. Koga K. from Kyushu University, Japan.





<u>CP treatment</u>:

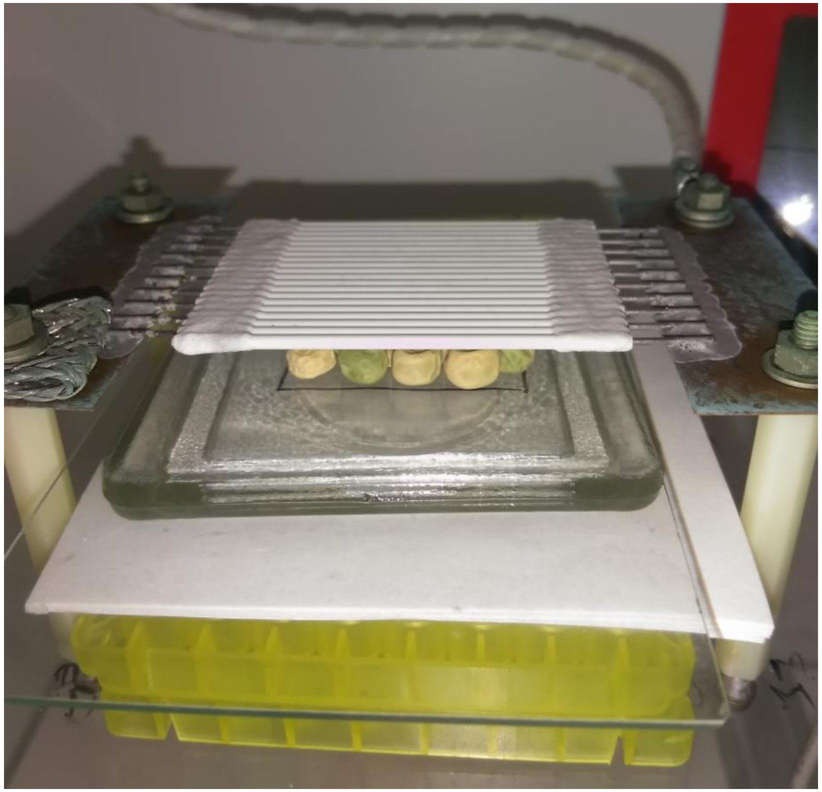
- Discharge voltage – 9.2 kV
- Discharge current – 0.2 A
- Discharge power density - 1.49 W/cm^2

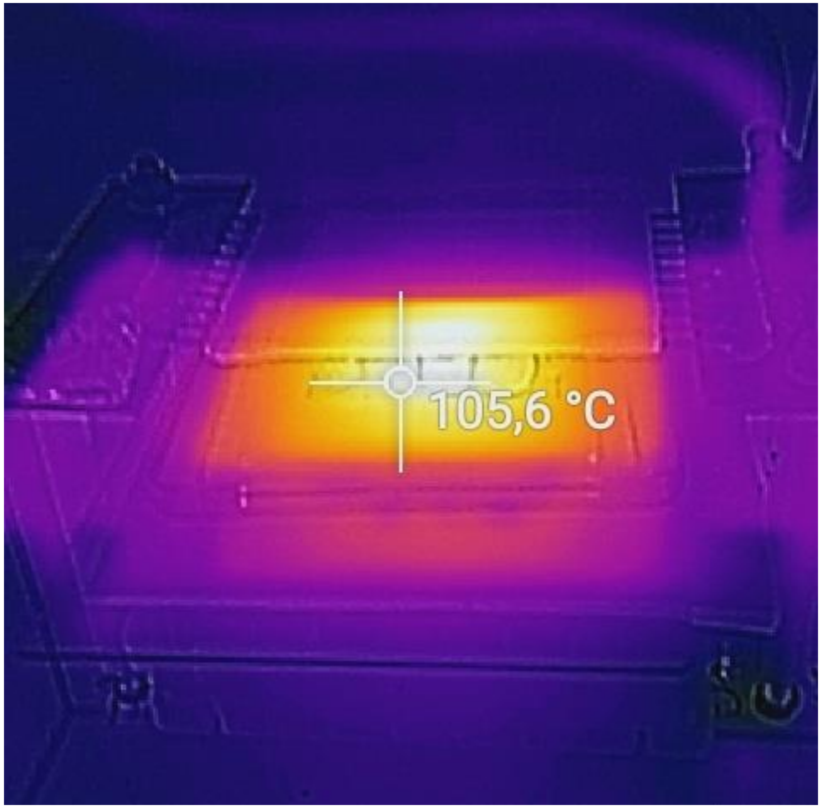
Dielectric-barrier discharge (DBD) plasma

Sarinont et al. Arch Bioch Biophy, 2016, 605, 129-140.

CP (AP-DBD) equipment

Temperature of electrodes must be controlled by choosing optimal treatment-break mode.

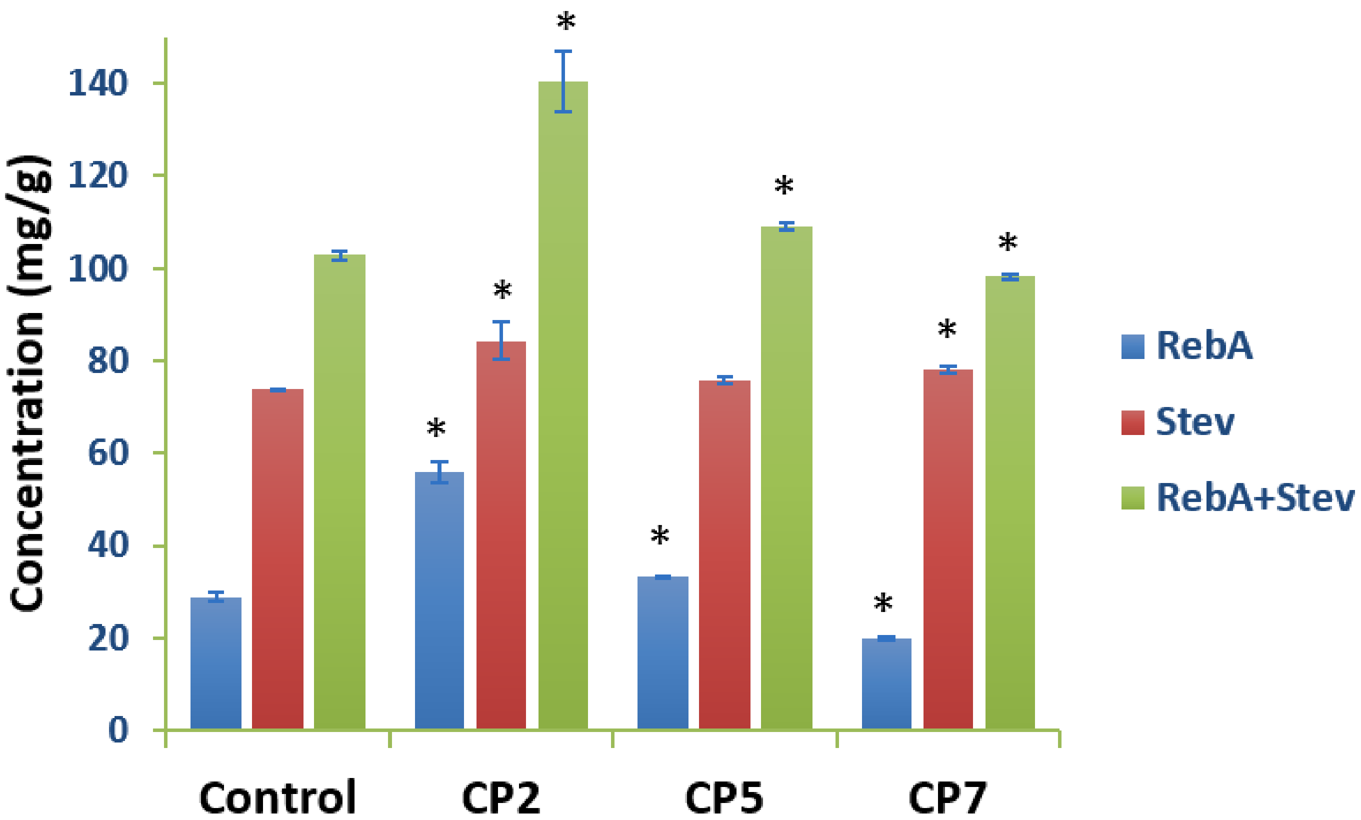




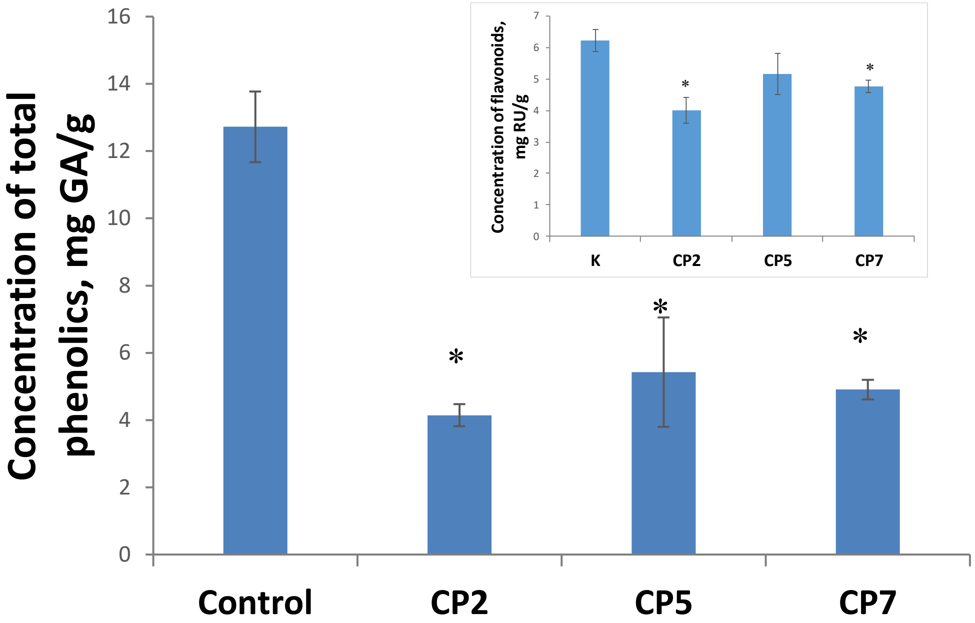
CP (DBD) effect on Stev and RebA amount

The optimal **2-min** pre-sowing seed treatment with CP (DBD) induces **increase** in the amount of **steviol glycosides** in *S. rebaudiana*:

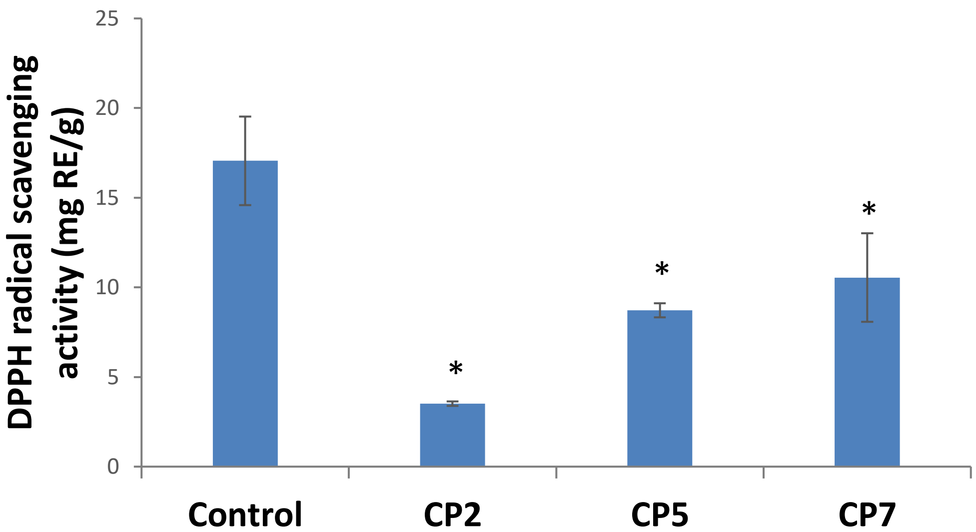
- **2-fold** increase in rebaudioside A amount,
- **14%** increase in stevioside amount, **37%** increase in RebA+Stev amount.
- **1.7-fold** increase in RebA/Stev ratio.



CP (DBD) effect on total phenolics, flavonoids and antioxidant activity

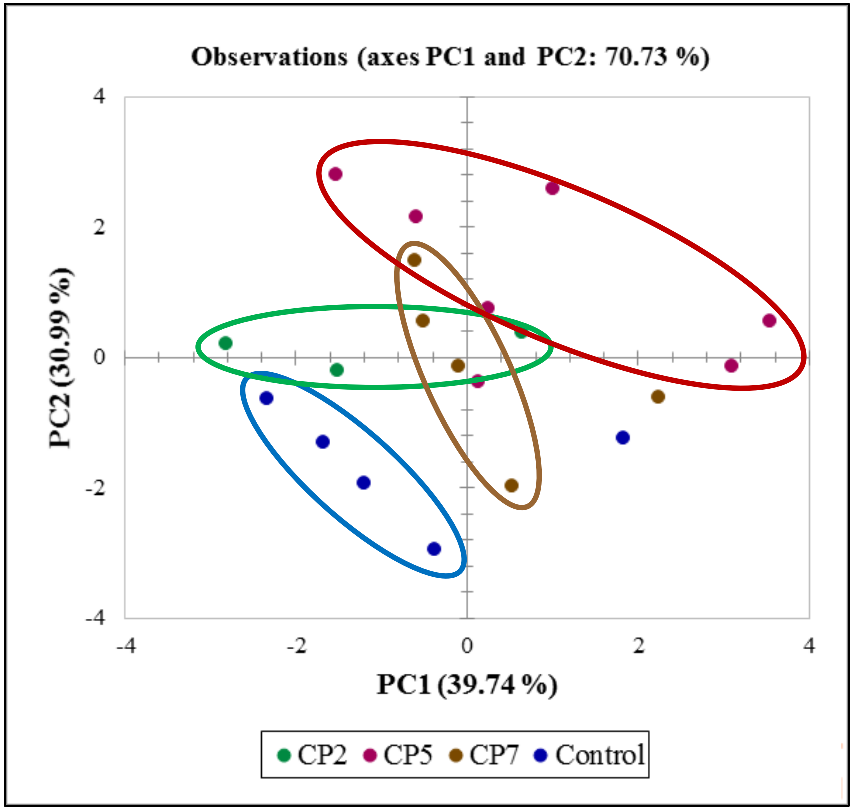


CP decreased total phenolics concentration 2.4-fold and it negatively correlates with increased Stev amount.

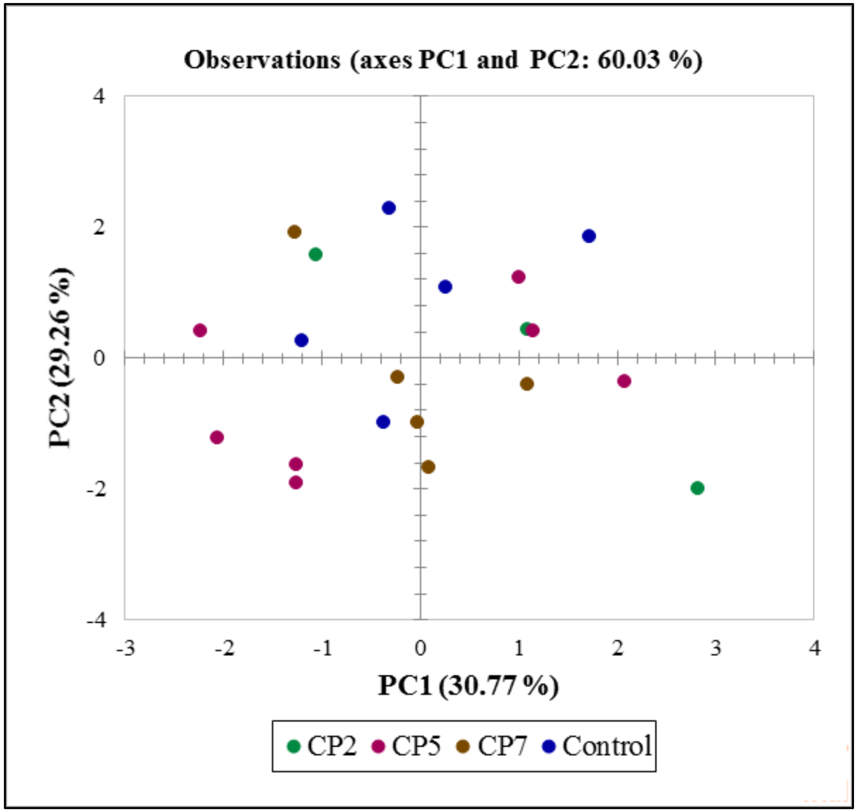


The amount of total phenolics correlates with radical scavenging activity.

Principle component analysis



Morphometric parameters (dry leaf mass, the number of leaves, plant height) and the concentration of **RebA** and **Stev**, **RebA/Stev**

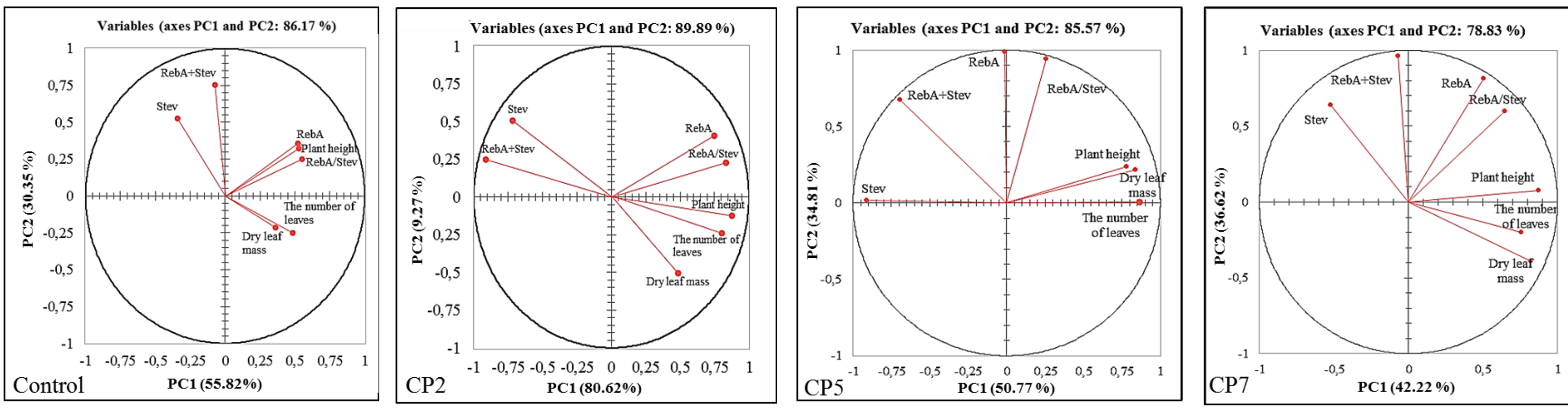


Morphometric parameters (dry leaf mass, the number of leaves, plant height) and biochemical variables (**TPC, TFC, AA**).

Correlation circles

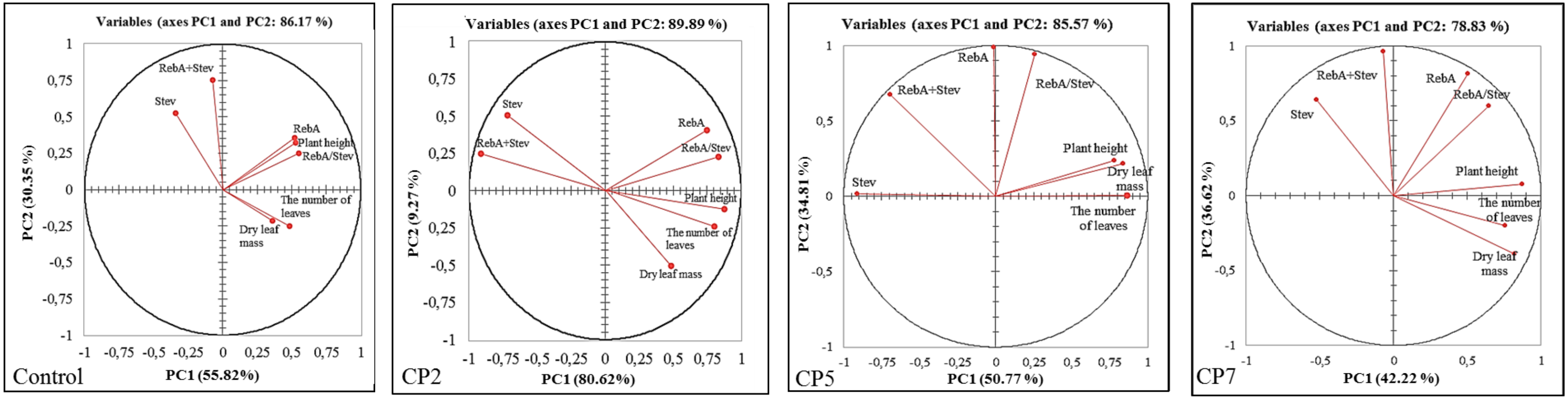
Each measured parameter was plotted against first two of the principal components (PC1 and PC2) and was shown as a vector, which signals the combined strength of the relationships between the variable and whether these associations are positive or negative:

- If they are pointing in **the same direction**, then they are **highly correlated**;
- If they are **orthogonal**, they are **unrelated**;
- If they are pointing in **opposite directions**, they are **negatively correlated**.



The correlation circles of the **morphometric parameters** (dry leaf mass, the number of leaves, plant height) and the concentration of **RebA** and **Stev**, **RebA/Stev**

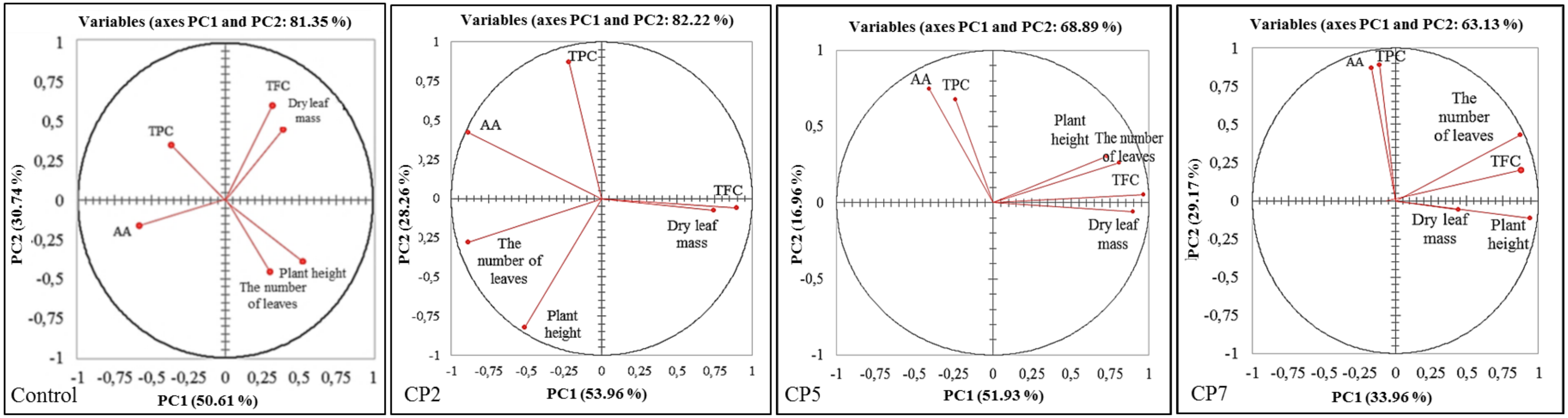
Correlation circles (morphometry&SGs)



The correlation circles of the **morphometric parameters** (dry leaf mass, the number of leaves, plant height) and the concentration of **RebA** and **Stev**, **RebA/Stev**

Control	strong positive correlation plant height-RebA, RebA/Stev
	strong positive correlation number of leaves-leaf mass
CP	weaker positive correlation plant height-RebA, RebA/Stev
	weaker positive correlation number of leaves-leaf mass
CP2	negative correlation plant height- RebA+Stev
Not affected	strong positive correlation RebA-RebA/Stev
	strong opposite correlation leaf mass-Stev

Correlation circles (morphometry&TPC, TFC, AA)



The correlation circles of the **morphometric parameters** (dry leaf mass, the number of leaves, plant height) and biochemical variables (**TPC, TFC, AA**).

Control strong opposite correlation plant height&number of leaves-TPC

CP5, CP7 diminish the correlation plant height&number of leaves-TPC
strong positive correlation TPC-AA

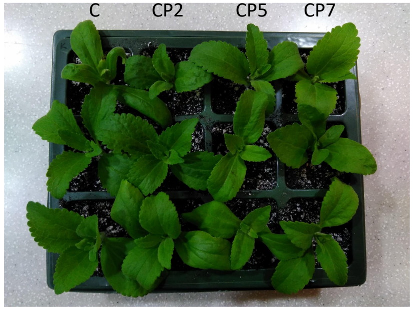
Not affected strong positive correlation leaf mass-TFC

3-4. Experimental scheme of CP (DBD) effect kinetics study

Seed treatment

- Day 6 — Sowing in soil
- Week 2 — Full germination
- Week 8 — Sampling
- Week 12 — Sampling
- Week 14 — Sampling &cutting shoots
- Week 20 — Final harvesting

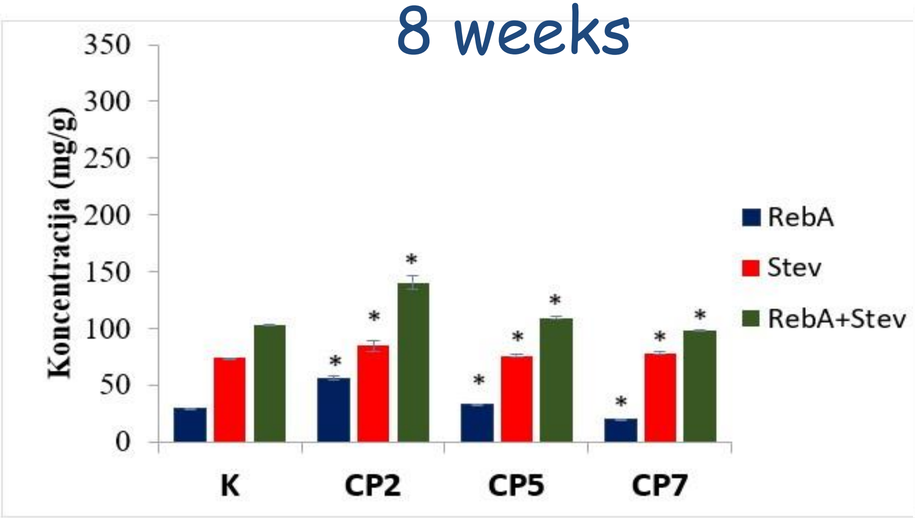
Vegetative propagation



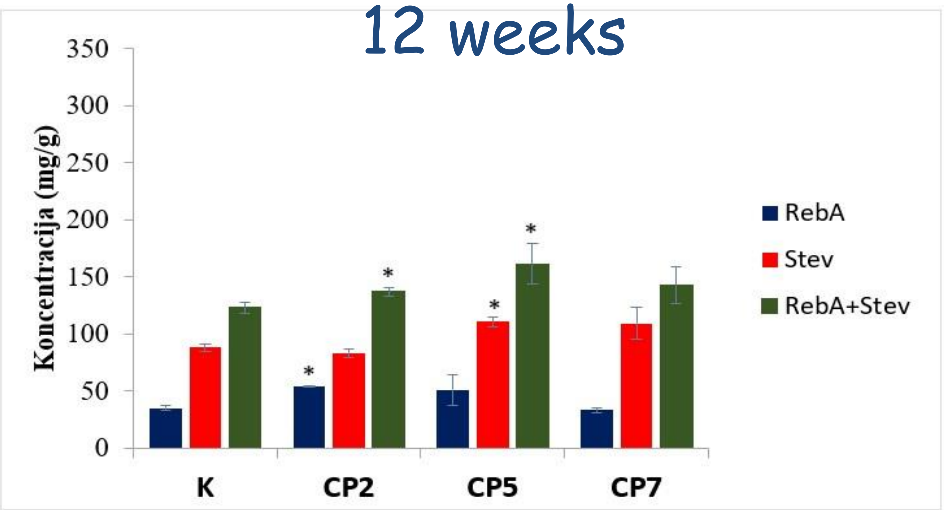
- Week 8 — Sampling
- Week 20 — Final harvesting

CP (DBD) effect on SG concentration kinetics

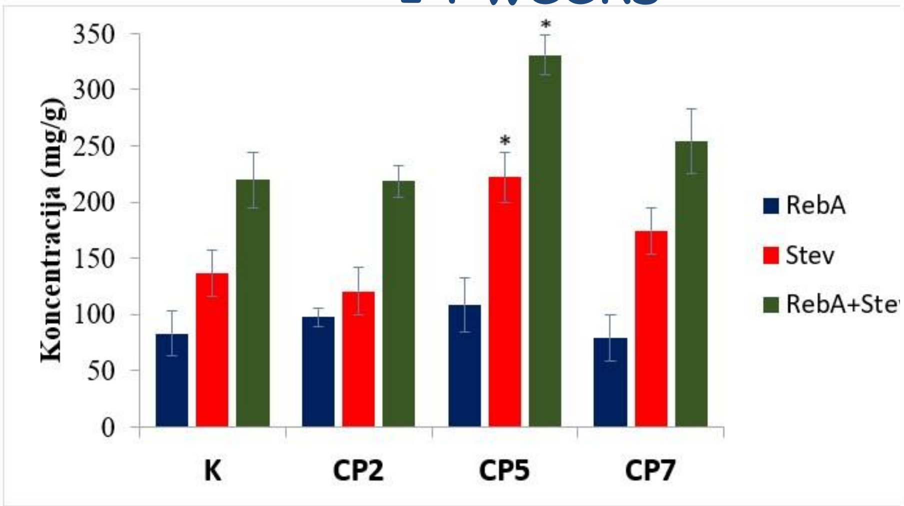
8 weeks



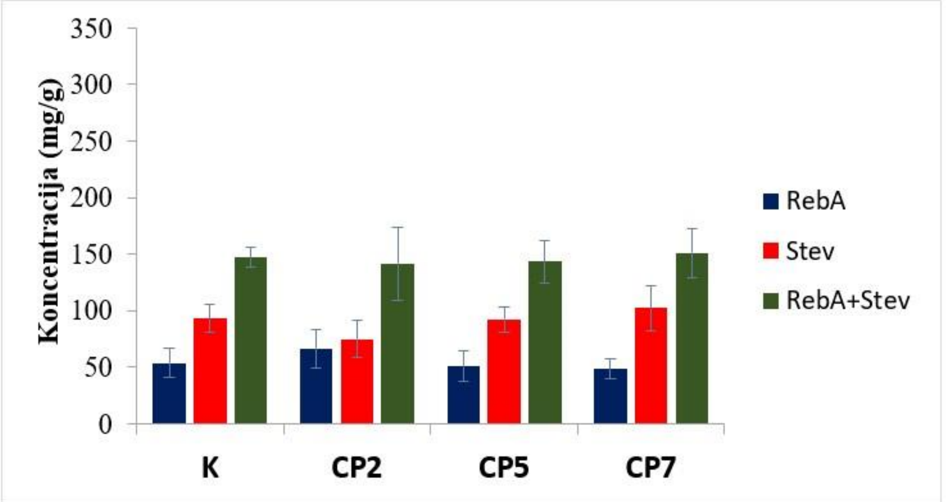
12 weeks



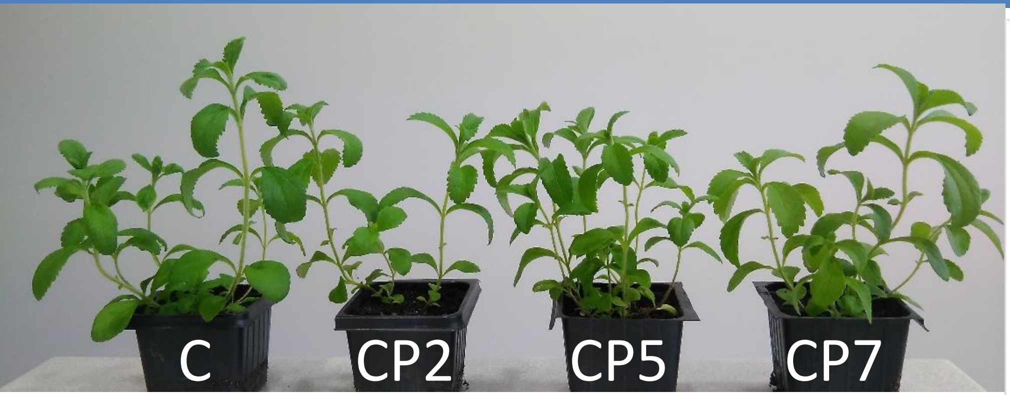
14 weeks

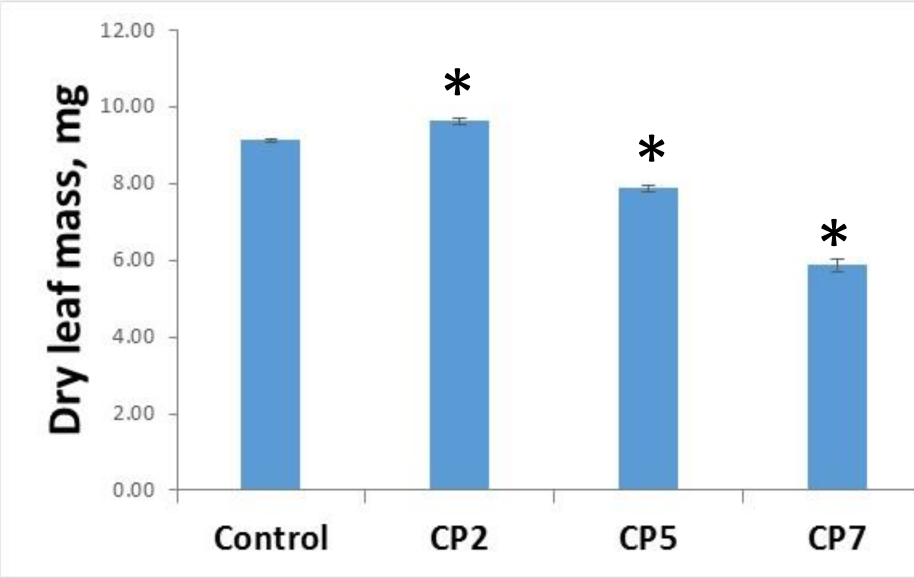


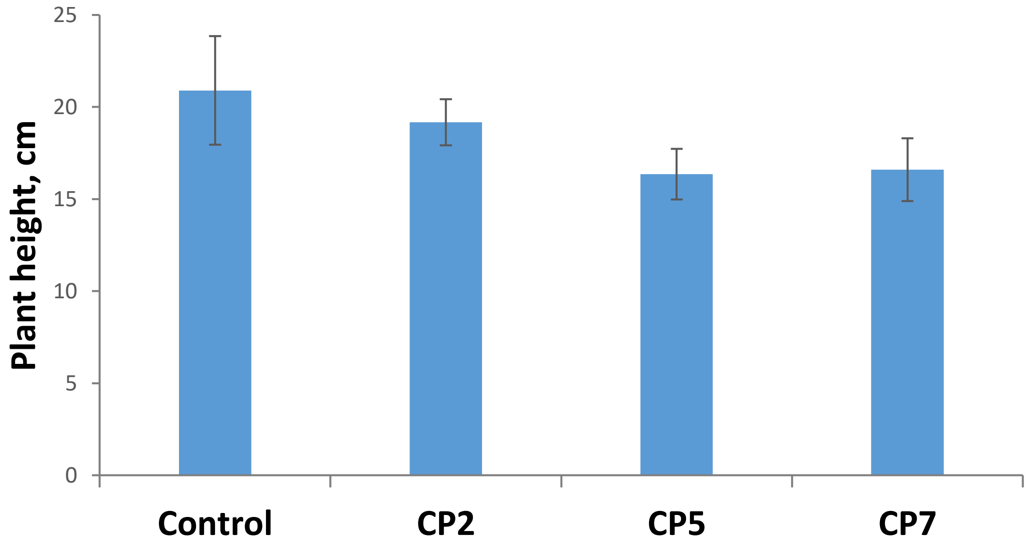
20 weeks



CP (DBD) effect on stevia morphology (8 weeks)







No statistically significant difference in plant height after 8 and 12 week-growth, but leaf mass was slightly increased in CP2 and decreased in CP5 and CP7 groups

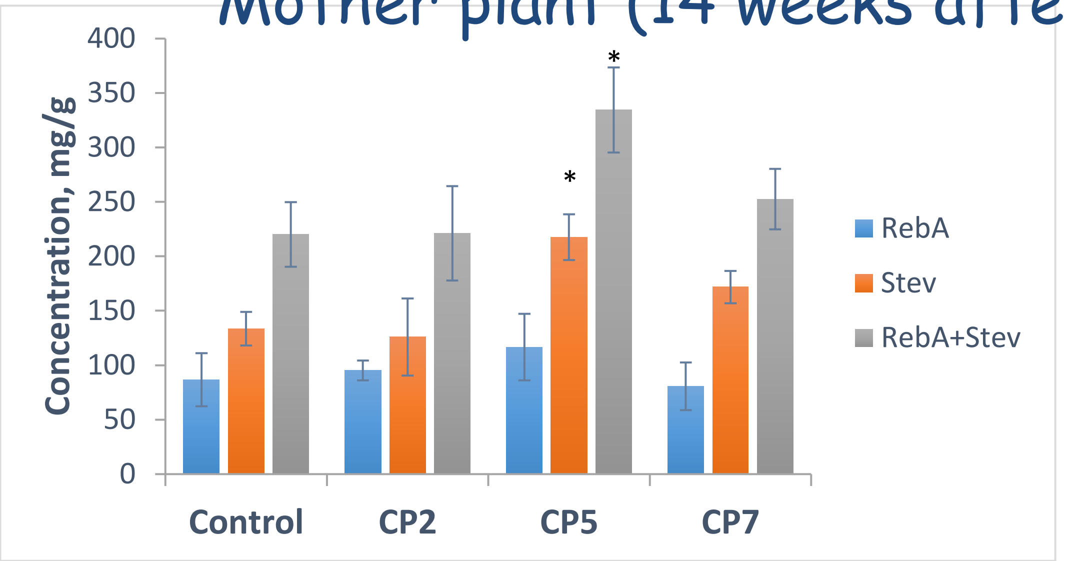
Vegetatively propagated stevia

Vegetatively propagated stevia (8 weeks)

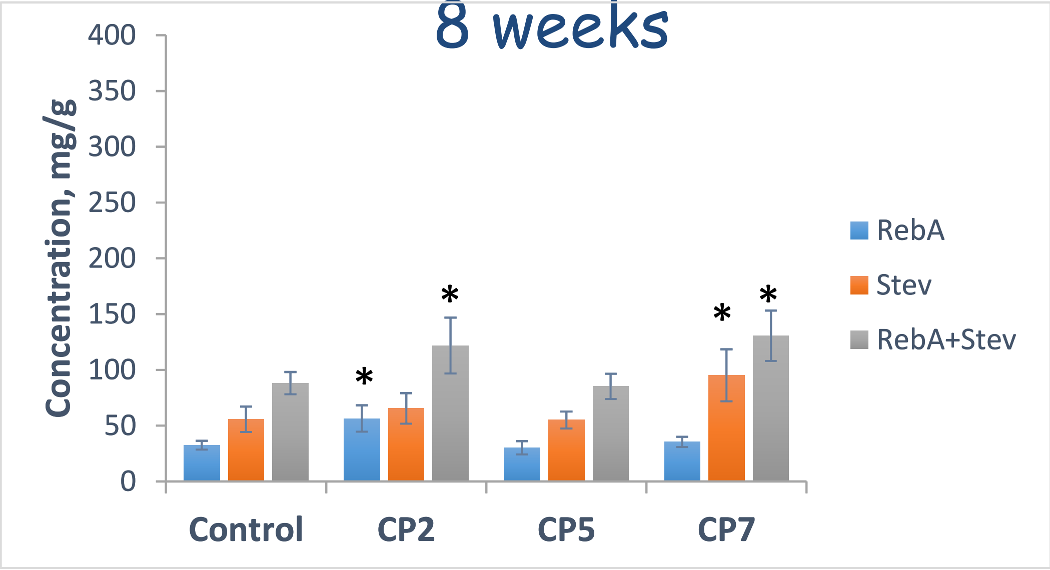
Too short day (light) time?
Too late cutting time/ vegetation state?

CP (DBD) effect in vegetatably propagated stevia

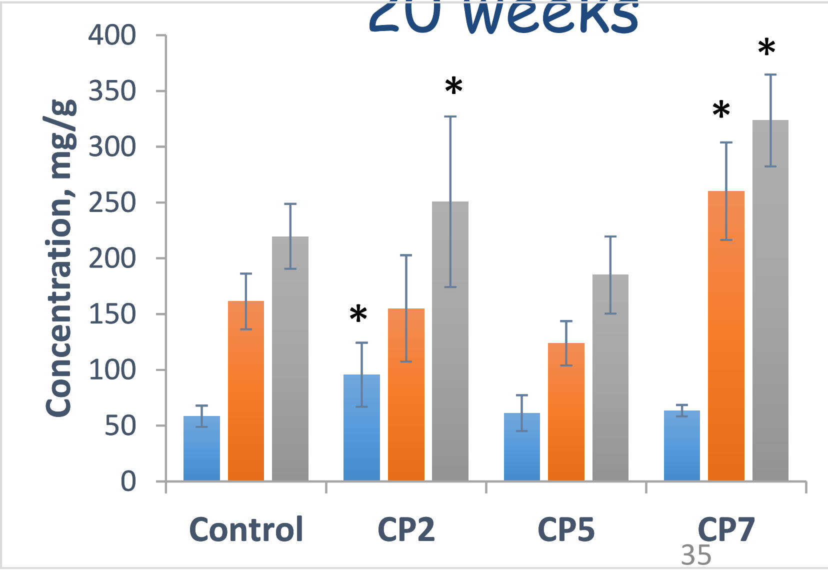
Mother plant (14 weeks after sowing)



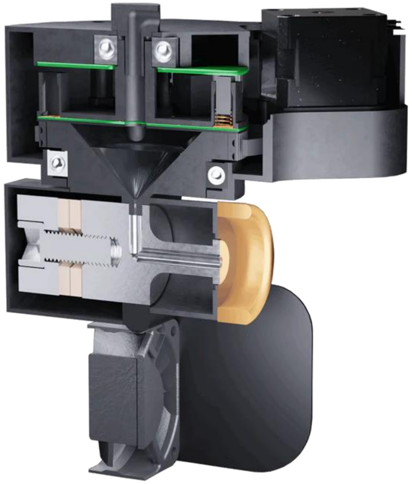
8 weeks



20 weeks



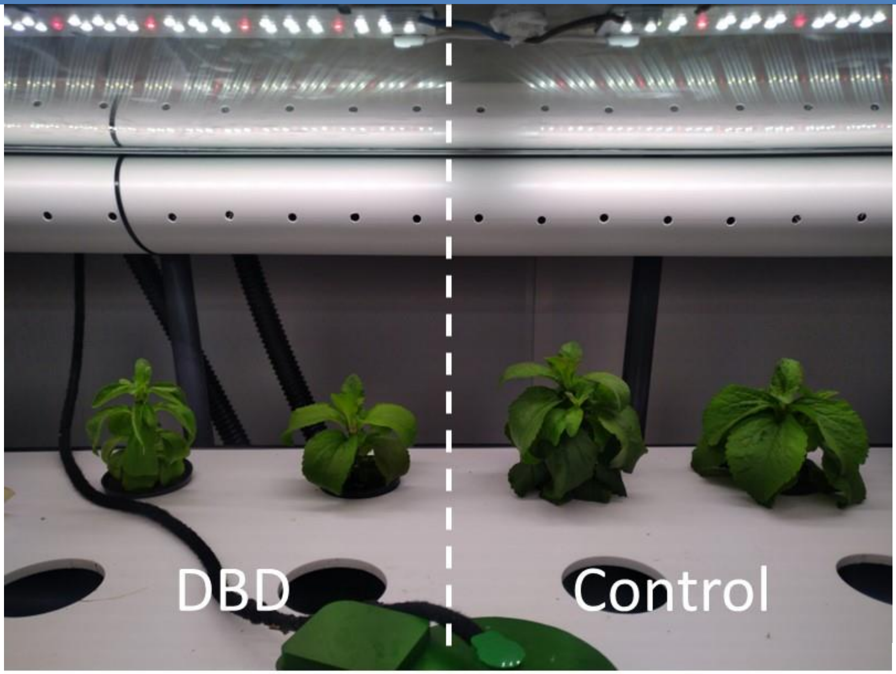
5. Aeroponics

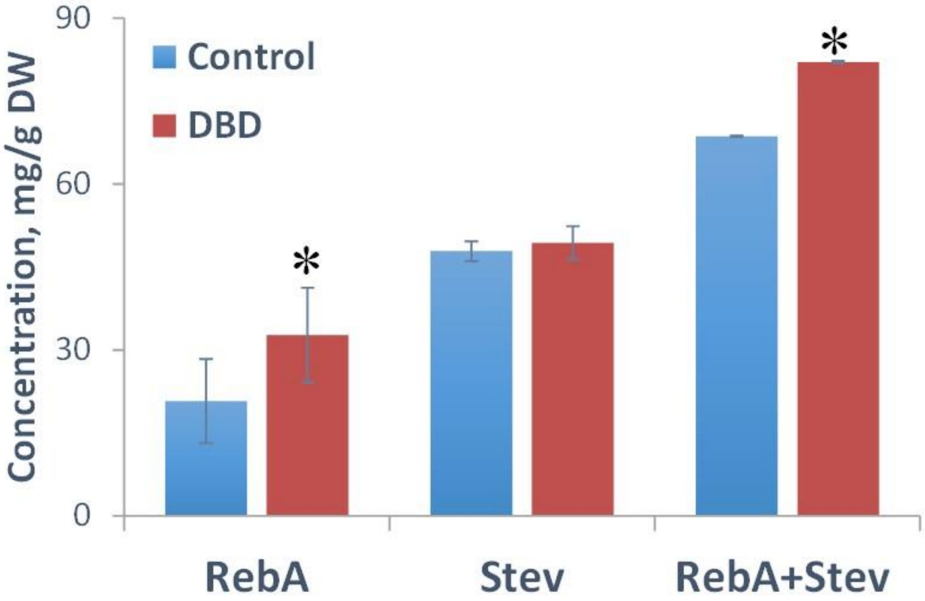


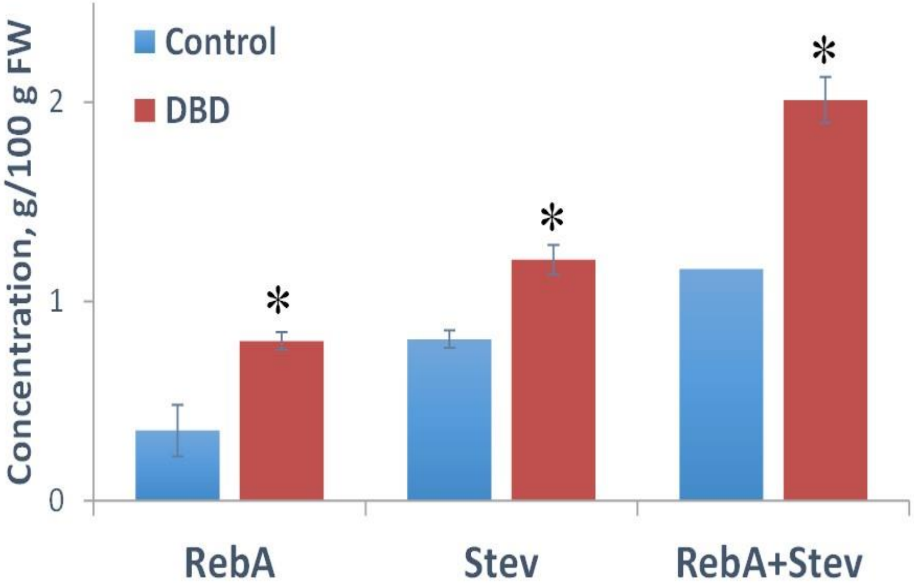
Baltic Freya

https://balticfreya.com/

DBD5+Aeroponics (pilot)



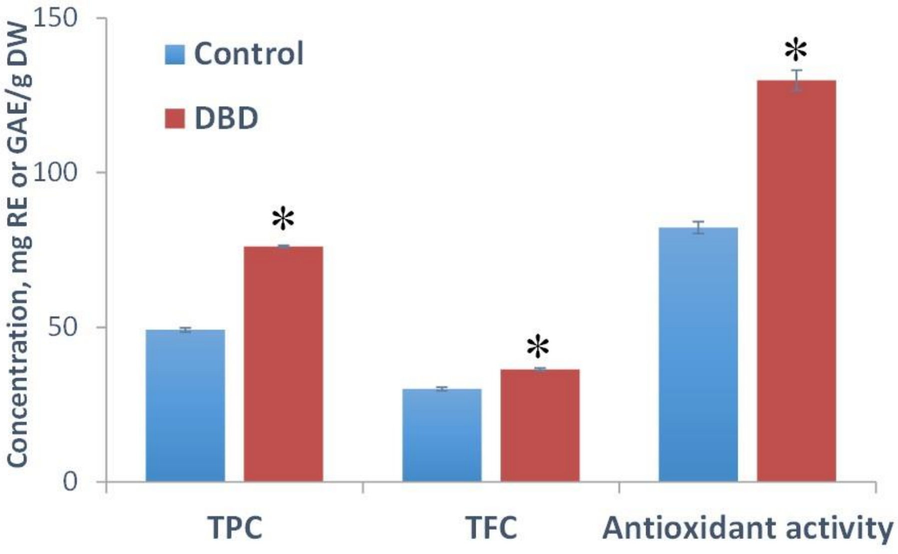


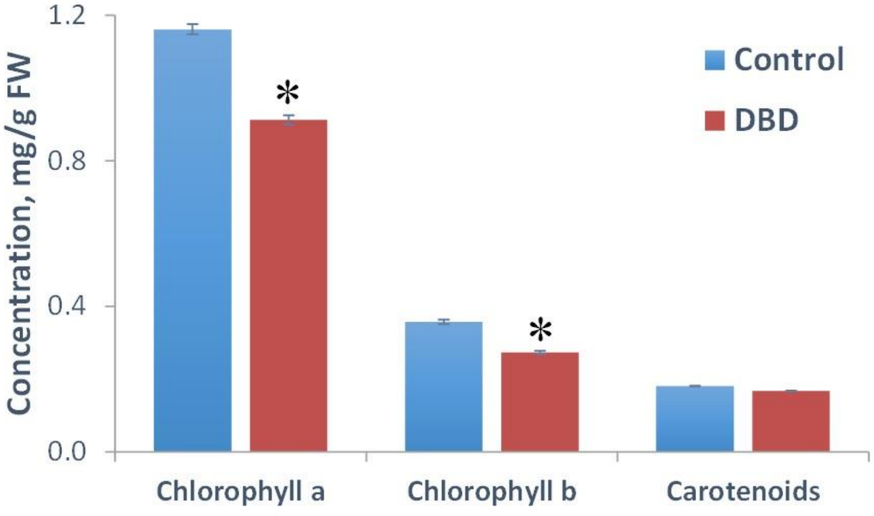


DBD5 + Aeroponics (pilot)

Group	Lenght, cm		Fresh weight, g			Dry matter, %		
	Stem	Roots	Leaves	Stem	Roots	Leaves	Stem	Roots
Control	9.25±0.75	33.50±3.50	13.99±1.35	2.70±0.67	9.34±0.27	16.56±0.37	13.72±0.58	10.53±1.09
DBD	8.00±2.50	27.25±0.25*	5.83±0.71*	1.03±0.16*	1.57±0.30*	23.95±0.56*	17.97±1.33*	12.79±0.59*

Although DBD treatment stimulated biosynthesis/accumulation of SGs per unit of dry or fresh leaf material, however, biomass growth was inhibited, plants were smaller, and SGs yield per plant or area unit was 35% lower.

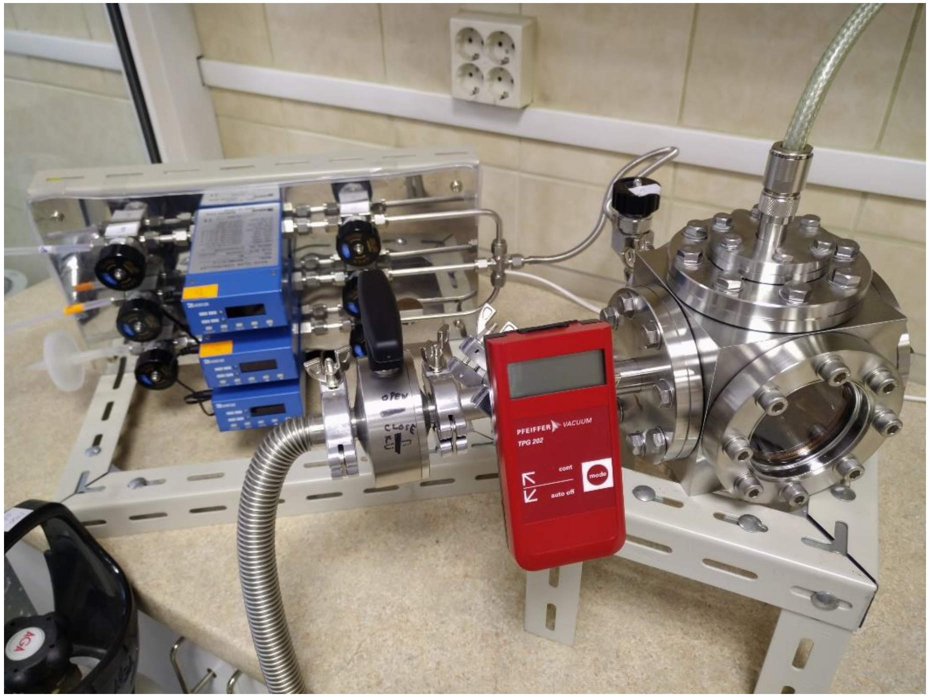




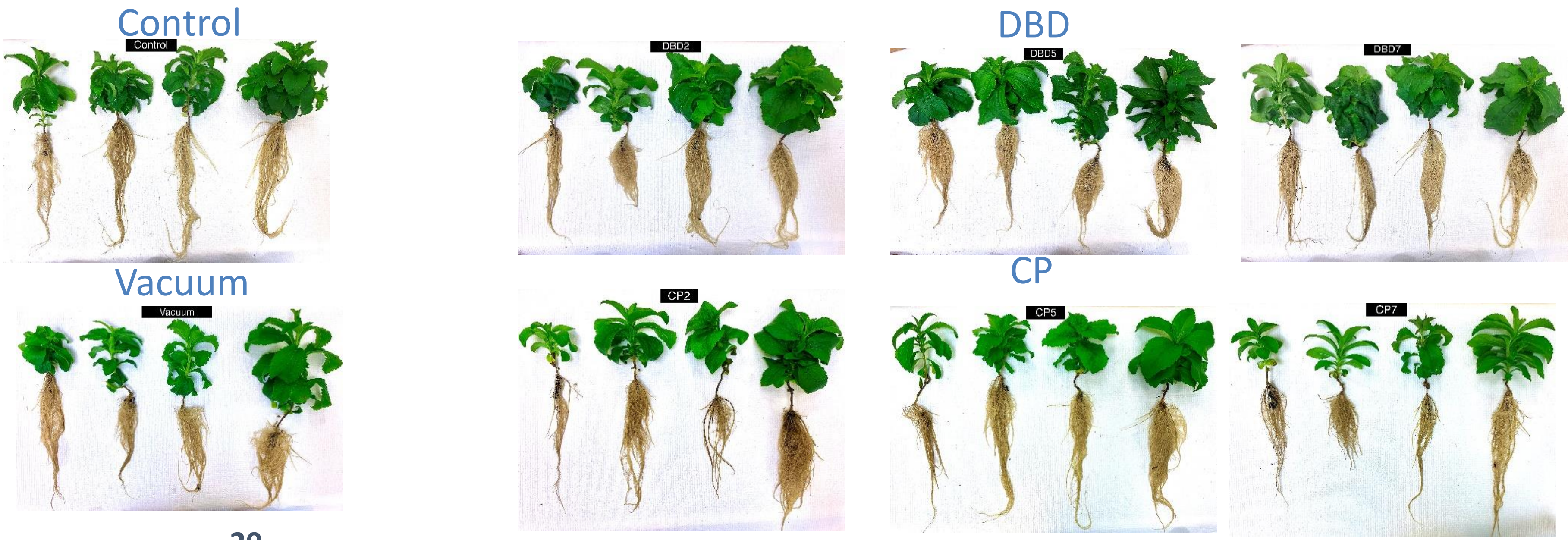
CP equipment

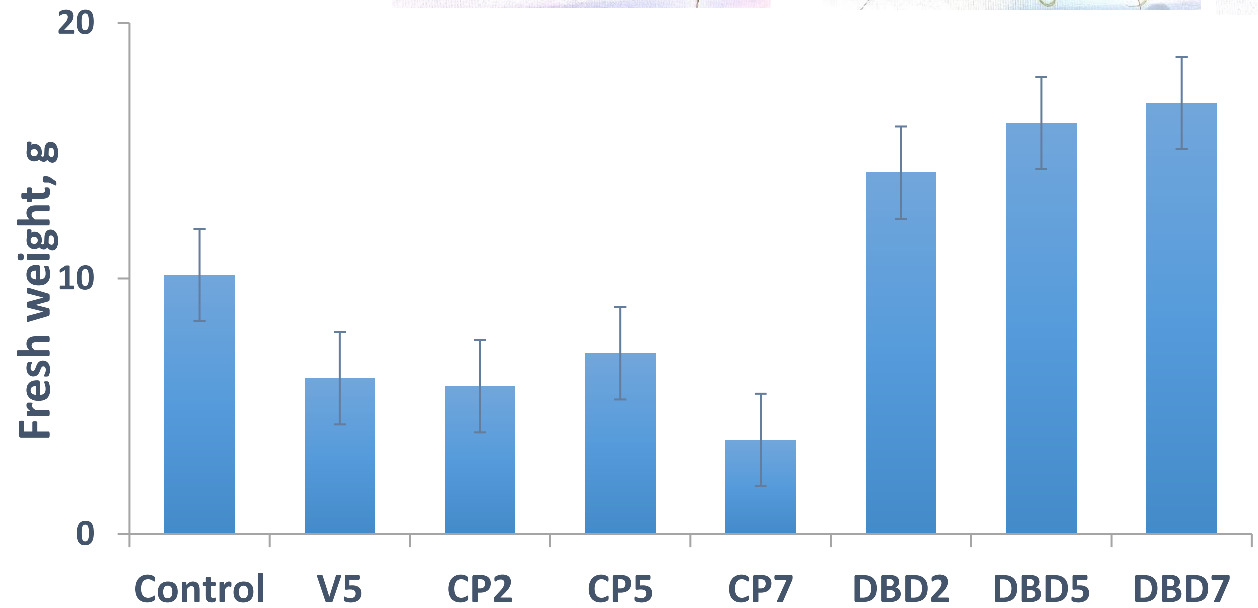
Experimental setup for seed treatment with CP (LP CC)

at Vytautas Magnus University, installed by prof. Shiratani M. and prof. Koga K. from Kyushu University, Japan.

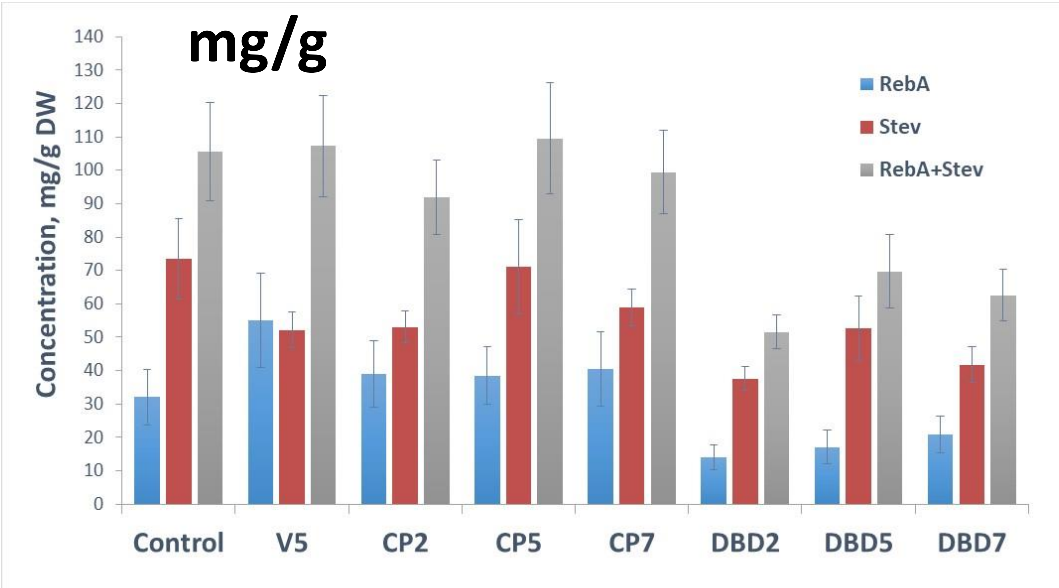


DBD or CC + Aeroponics

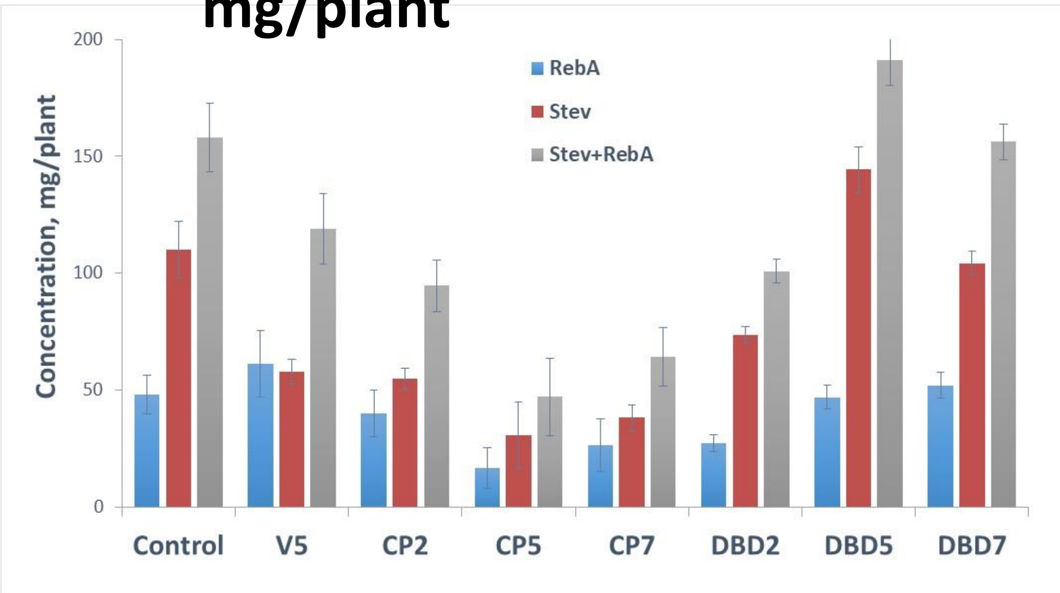




DBD or CC + Aeroponics



mg/plant



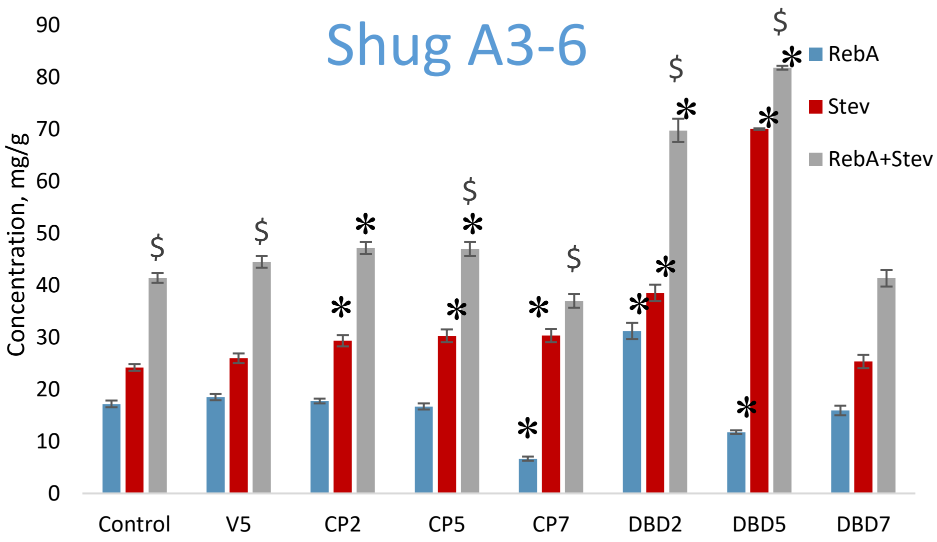
CC

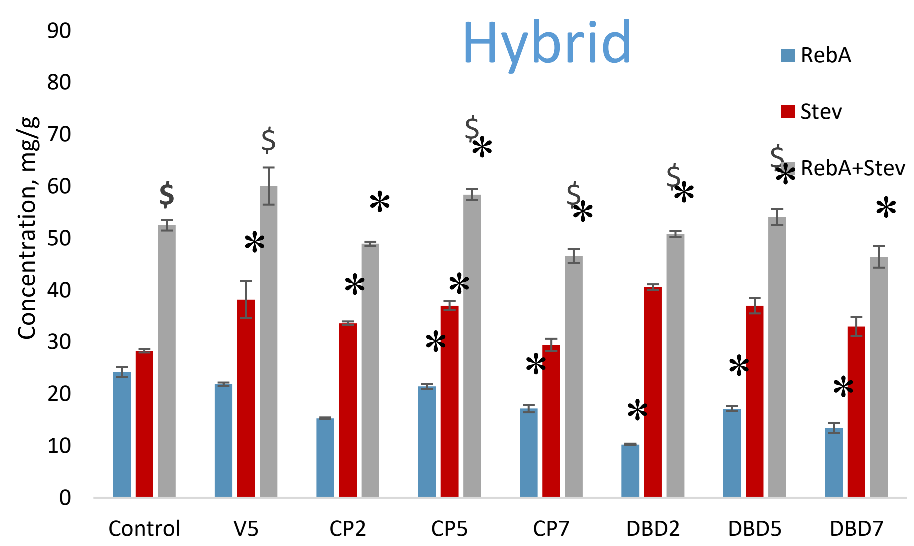
- decreased leaf DW by app. 30%
- didn’t change RebA and Stev, but increased RebA/Stev ratio 0.43 (control) up to 0.71
- increased TPC (1.6-2.4-fold), TFC (40-55%) and AA (17-52%).

DBD

- Increased leaf DW by 30-83%
- decreased SG concentration by 34-51%
- SGs amount per plant or area unit was 21% higher in DBD5
- decreased AA, didn’t change TPC and increased TFC (20%)

2 cultivars + soil





Conclusions from different experimental systems

- SGs were increased by seed treatment with low-pressure and DBD plasma.
- SGs were increased in different cultivars/cultigens.
- The effect was persistent at least for 14 weeks.
- More studies are required to evaluate the possibility of enhanced trait transfer by vegetative propagation by adjusting cutting time, growing conditions or cultivar selection.
- In older (3-year-old) seeds CP effects were weaker
- CP treatment combination with aeroponics does not stimulate SGs biosynthesis but increases TPC, TFC and antioxidant activity. However, CC inhibits the growth but increases the taste quality of SGs.

Aknowledgements

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