Biotechnological strategies to increase the production of natural compounds by using plant *in vitro* cultures

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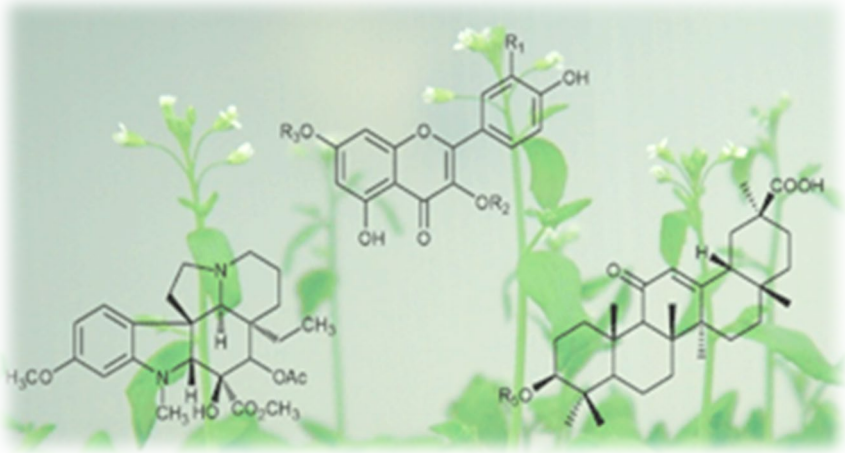
SECONDARY METABOLITES

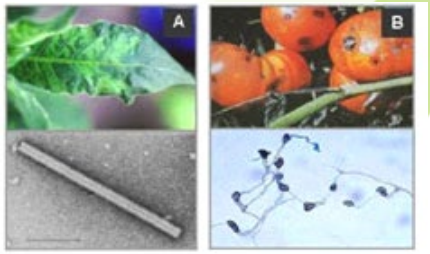


- Small amounts (<1% DW)
- Their biosynthesis depends on:
  - Plant genetic constitution
  - It is restricted to:

    Specific stages of plant development

    specialized cells and stress of different nature

FUNCTIONS OF SECONDARY METABOLITES IN PLANTS



DEFENSE AGAINST BIOTIC AND ABIOTIC STRESS

SECONDARY METABOLITES

BIOACTIVE COMPOUNDS

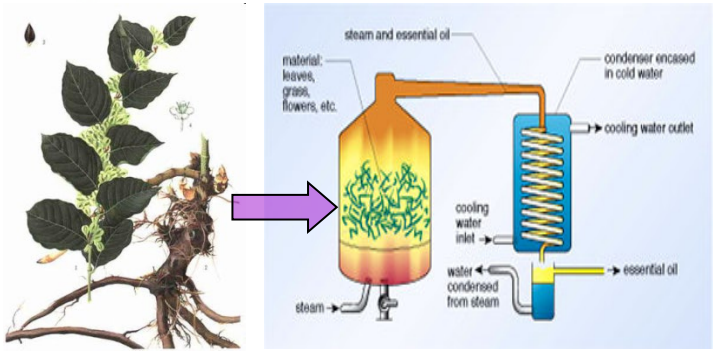
"Plant molecules that without being nutrients, have a beneficial effect on human health"

- Pharmaceutical industry
- Agrifood industry
- Cosmetic industry

These compounds synthesized under biotic and abiotic stress by plants are considered as high added value products

Strategies to increase secondary metabolite production

STRATEGIES TO PRODUCE SECONDARY METABOLITES: PLANT RAW MATERIAL AND CHEMICAL SYNTHESIS



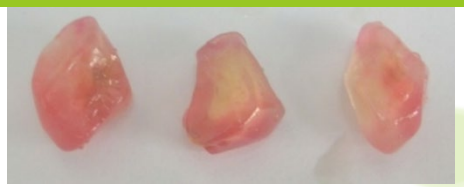
Polygonum cuspidatum

Plant seasonal nature
Compound heterogeneity
Plant extinction (wild plants)

Stereospecificity
Strict conditions
High costs

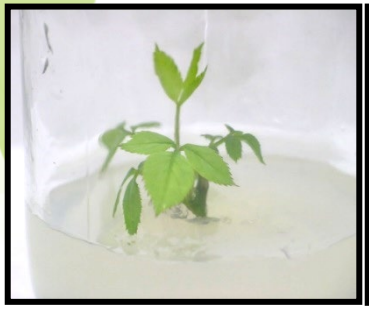
BIOTECHNOLOGICAL ALTERNATIVE: PLANT IN VITRO CULTURES

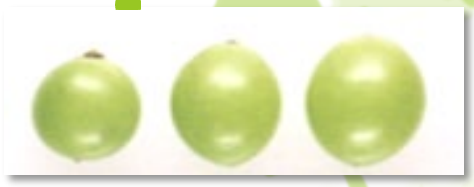
HOW IS POSSIBLE TO OBTAIN PLANT IN VITRO CULTURES AND KEEP THEM IN ENDLESS GROWTH?

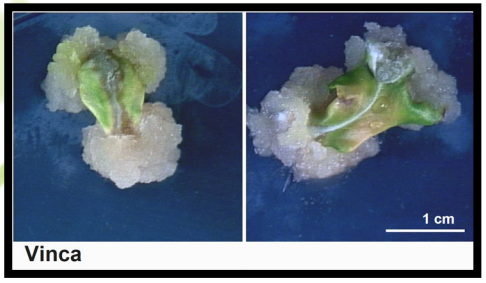


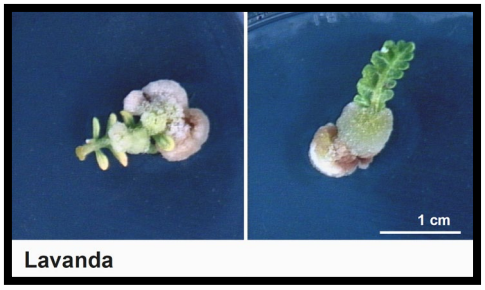
Cell culture

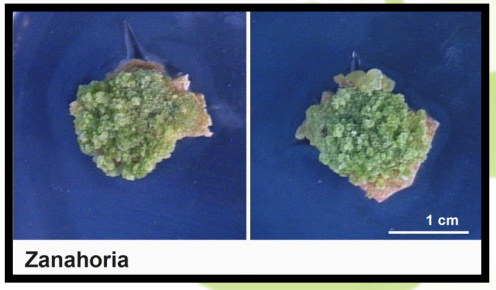
callus induction from explants









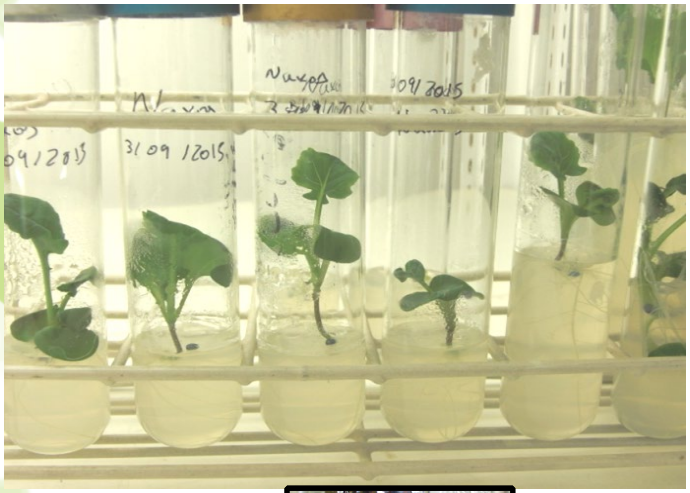


PLANT BIOMASS GENERATION AND ELICITATION TO INCREASE THE PRODUCTION OF BIOACTIVE COMPOUNDS

Plant biomass: vitroplants and cell cultures

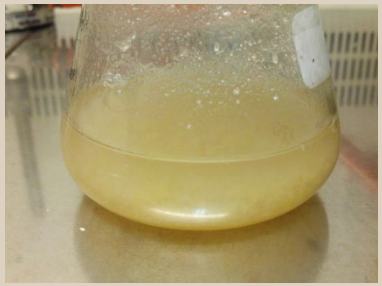
Vitroplants

in vitro germination



Apical bud or nodal segment

Cell cultures

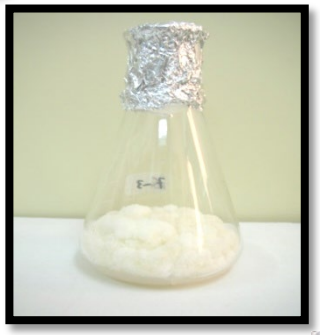


Biomass production

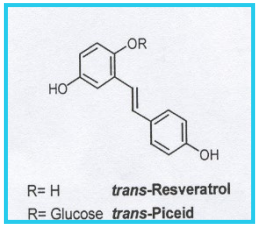
Elicitation

Bioactive compounds

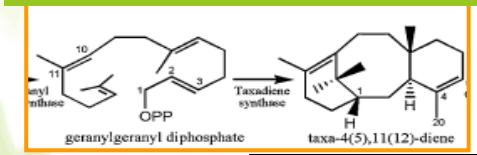
STRATEGIES TO INCREASE THE PRODUCTION OF BIOACTIVE COMPOUNDS



ELICITATION



METABOLIC ENGINEERING

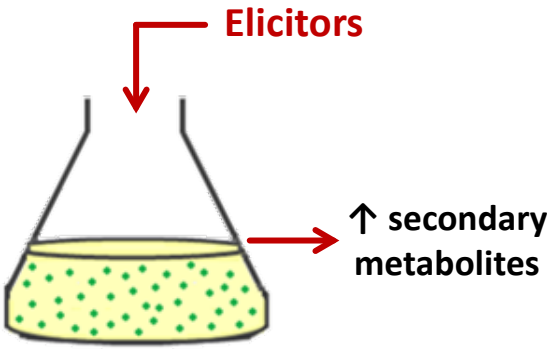


Phyton Biotech
A DFB PHARMACEUTICALS COMPANY

Bristol Myers Squibb

ELICITATION AS A STRATEGY TO INCREASE THE PRODUCTION OF BIOACTIVE COMPOUNDS

Elicitation:
is the induction of metabolite biosynthesis due to addition of elicitors



Elicitors:
molecules which introduced into a living cell system, at low concentrations, are capable of re-directing the metabolism, inducing the biosynthesis of a particular secondary metabolite

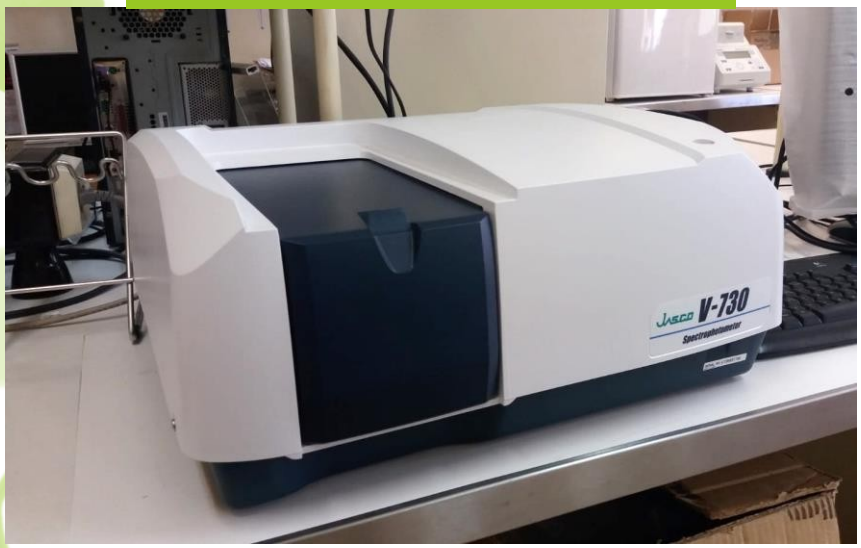
Radman et al., 2003; Biotechnol. Appl. Biochem.37:91

TECHNIQUES AND EQUIPMENTS FOR EXTRACTION, IDENTIFICATION AND QUANTIFICATION OF BIOACTIVE COMPOUNDS

LC-MS ESI/TOF

GC-MS

SPECTROPHOTOMETER

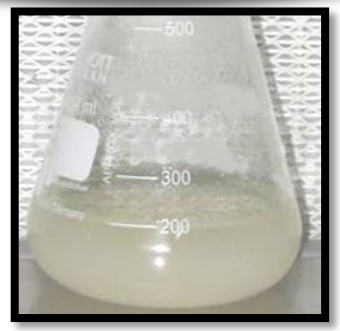


HPLC-DAD

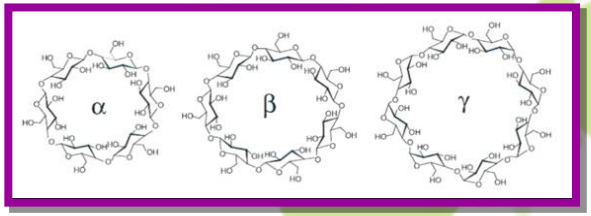
Case study: The production of *trans*-resveratrol in elicited grapevine cell cultures: patents WO2003062406A1 and WO2009106662A1

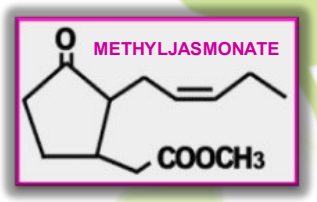
Elicitation with cyclodextrins (CDs) and/or methyljasmonate (MeJA)

grapevine cv Monastrell



β-CDs, MeJA





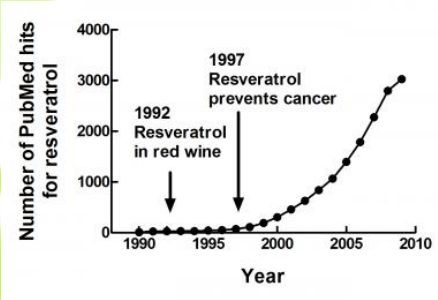
Viniferins: stilbene unit: *trans*-resveratrol

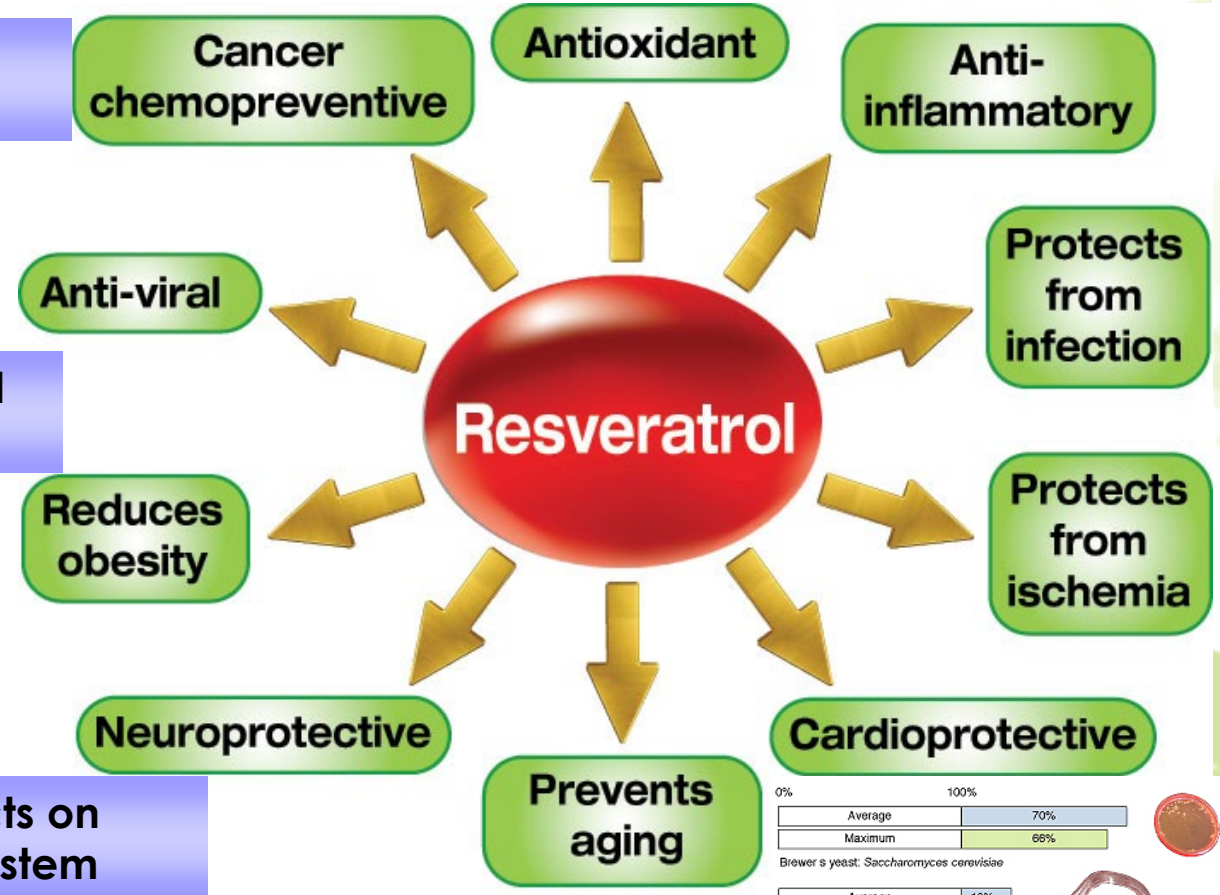
BENEFICIAL EFFECTS OF RESVERATROL ON HEALTH

Preventing carcinogenesis

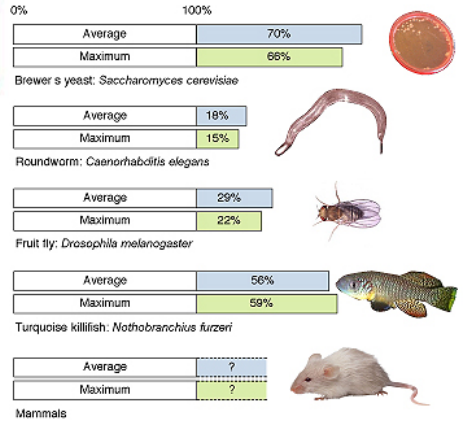
Obesity-related disorders

Beneficial effects on neurological system

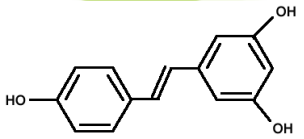




Age-related human diseases



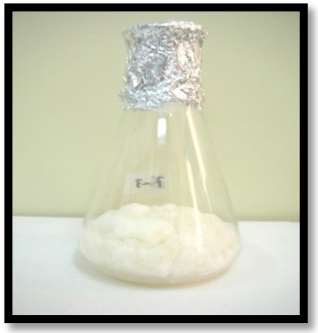
Beneficial effects on cardiovascular system



High value of RESVERATROL
High demand of its production
Great effort to produce it in high amounts

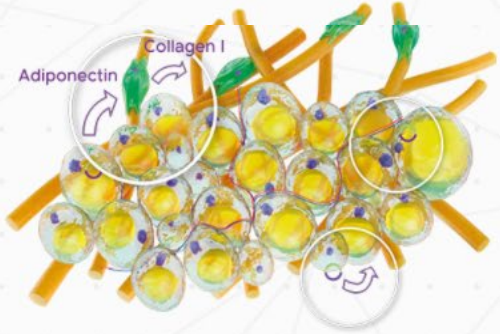
Development of new strategies to increase *trans*-resveratrol production using grapevine cell cultures

1990-now



Establishment of cell cultures from inmature fruits of *Vitis vinifera* cv Monastrell

https://lipotrue.com/es/productos/joybliss/



Rellenador de arrugas

Ilumina tu estado de ánimo

Exhala la inflamación

Crea tu propia felicidad

Joybliss™

The joy of plumping

La felicidad se define como un estado inconmensurable de dicha absoluta. Todos tenemos nuestra propia **lista** de cosas que sabemos que nos harán felices y nos ayudara a conseguir el objetivo de convertirnos en una versión más sana y más radiante de nosotros mismos. Nosotros, los **buscadores de la felicidad**, perseguimos la dicha absoluta de manera constante.

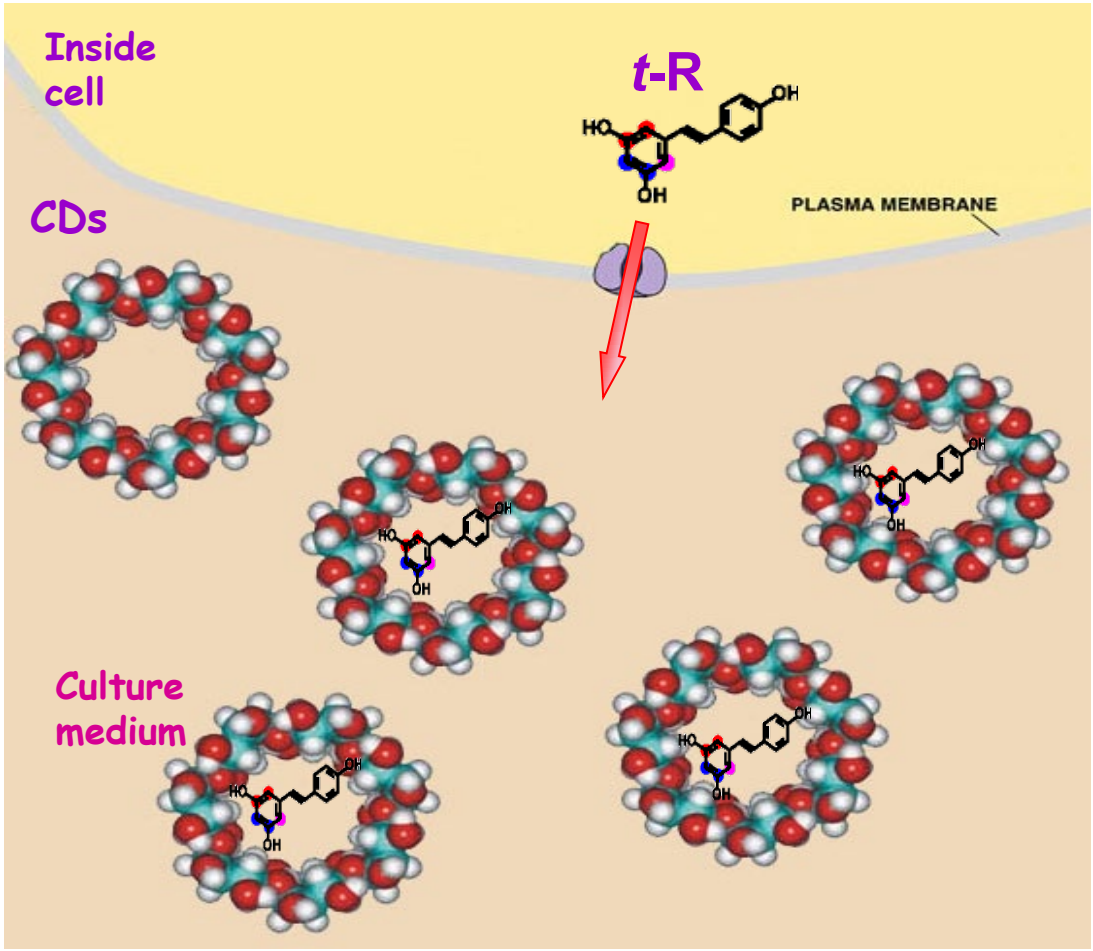
Según los psicólogos, la rueda de la vida debe estar lo más llena posible para aportar equilibrio a tu vida y generar felicidad y, de esta forma, hacer que el viaje a través de ella transcurra con facilidad. Que tu piel ofrezca un aspecto fresco, joven y descansado también depende de que la rueda del relleno esté lo más completa posible.

Joybliss™ es un ingrediente activo verde que ayuda a completar la rueda del relleno. **Joybliss™** induce la diferenciación en el proceso de adipogénesis y previene el envejecimiento del tejido adiposo modulando su inflamación y aumentando el contenido de colágeno I en la hipodermis gracias a la comunicación entre los adipocitos y fibroblastos.

Joybliss™ reduce las patas de gallo y las arrugas del pliegue nasolabial como ingrediente de relleno, alivia el eritema y aporta luminosidad a la piel. **Joybliss™** también aumenta el estado de ánimo positivo y disminuye los marcadores vocales de estrés para ayudarte a llegar a la dicha absoluta.

Una vez que tu rueda del relleno está completa, tu piel se ve más joven y la alegría que sientes fluye a tu alrededor.

GRAPEVINE CELL CULTURES AND ELICITATION WITH CYCLODEXTRINS

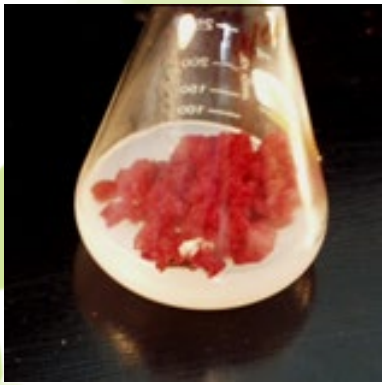


The role of CDs as elicitors that trigger defense responses as the biosynthesis of *t*-R

CDs chemically resemble the alkyl-derived pectic oligosaccharides released from cell walls during fungal attack (Bru et al., 2006; J. Agric. Food Chem. 54:65).

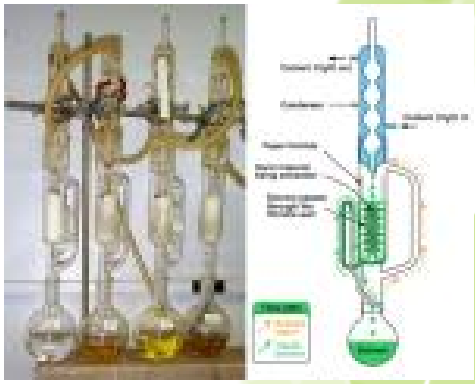
We discovered a way to produce and enhance the levels of *trans*-resveratrol using grapevine cells as true factories!!!!

TRADITIONAL METHODS: EXTRACTION FROM CELLS



CALLUS

CELL CULTURES



CELL EXTRACTION

BIOMASS PRODUCTION

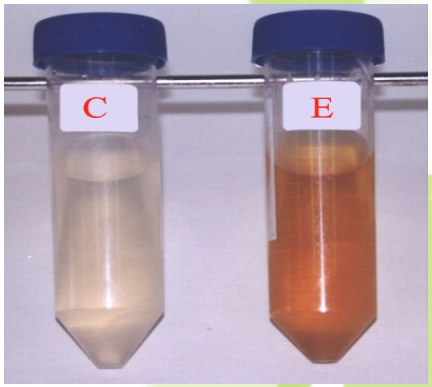
YIELDS
1-50 mg/L

BIOACTIVE EXTRACTS

NEW INNOVATIVE PROCEDURE: EXTRACTION FROM CULTURE MEDIUM

CALLUS

CELL CULTURES



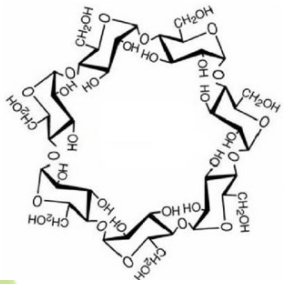
CONTROL/ELICITED CULTURE MEDIUM

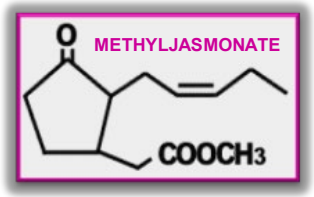
trans-resveratrol production

WO 2003062426A1 (300-1000 mg/L) (Cyclodextrins)

WO2009106662A1 (>3000 mg/L) (Cyclodextrins and methyljasmonate)

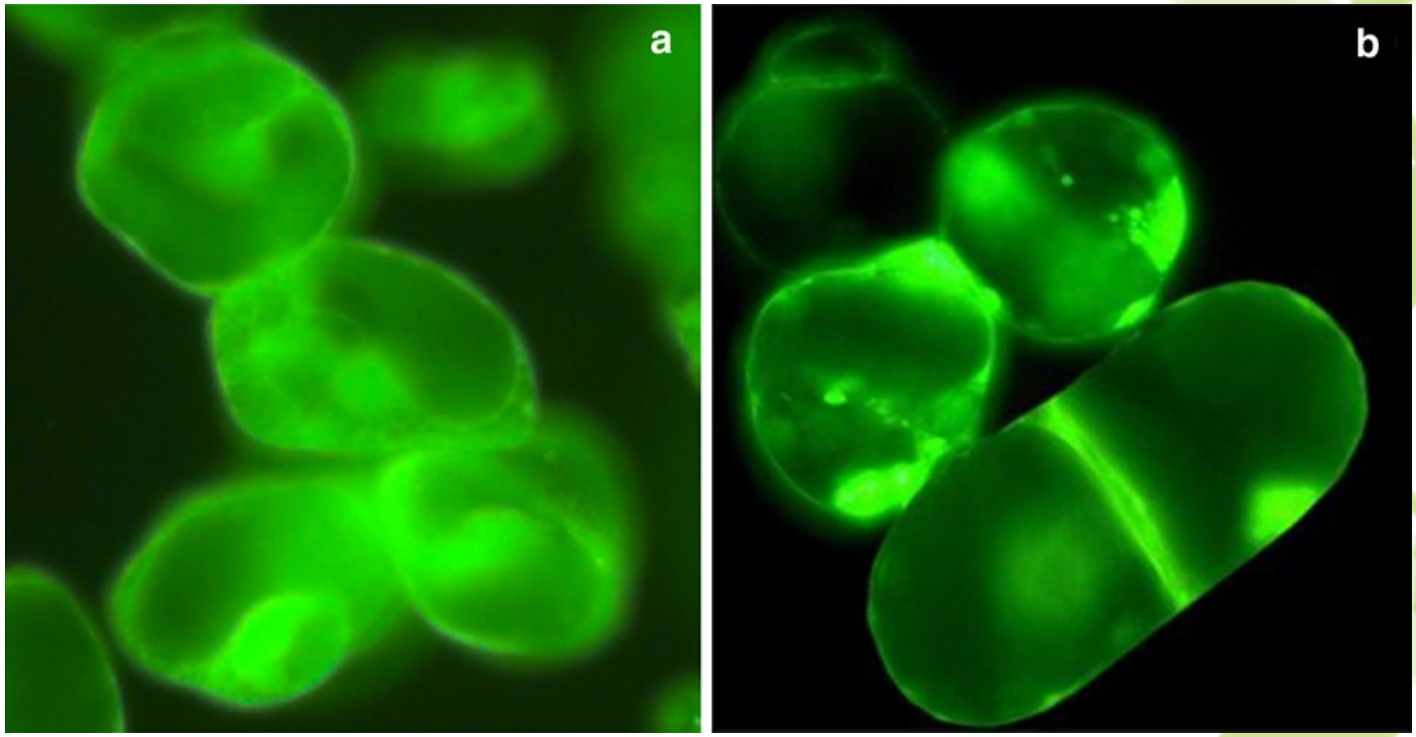
OUR CULTURE CONDITIONS





BIOACTIVE EXTRACTS

GRAPEVINE CELL VIABILITY AFTER 168 HOURS OF ELICITATION



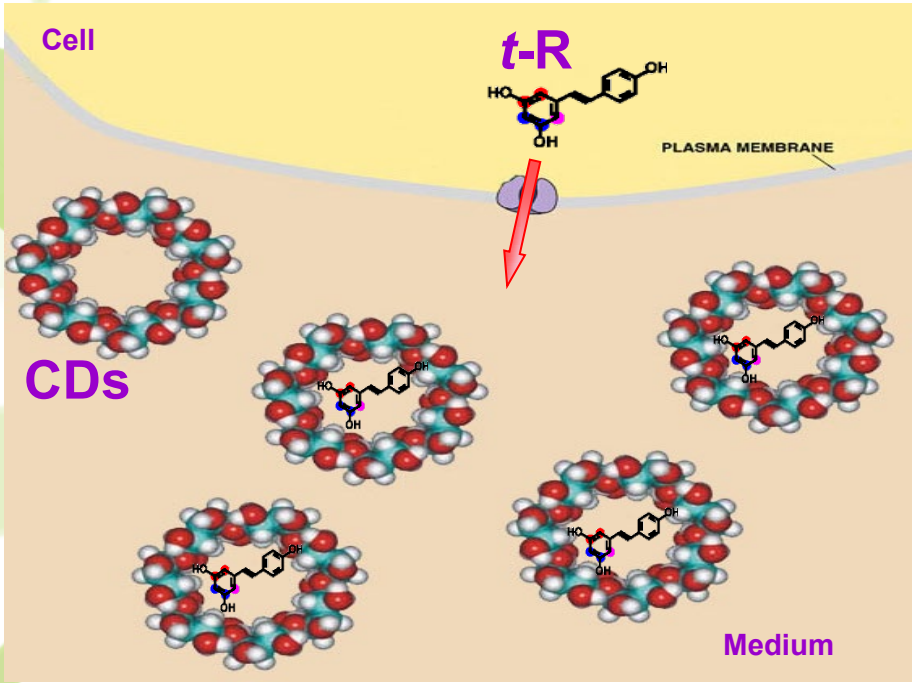
The high levels of *t*-R accumulated in the culture medium had no toxic effect on the cells, allowing cell growth

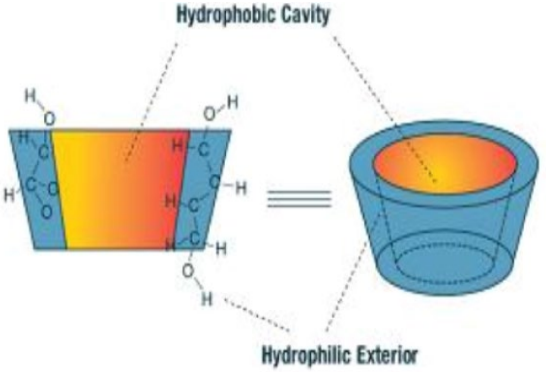
Belchí-Navarro et al. (2012) Plant Cell Rep 31:81–89

PHYSICO-CHEMICAL CYCLODEXTRIN PROPERTIES

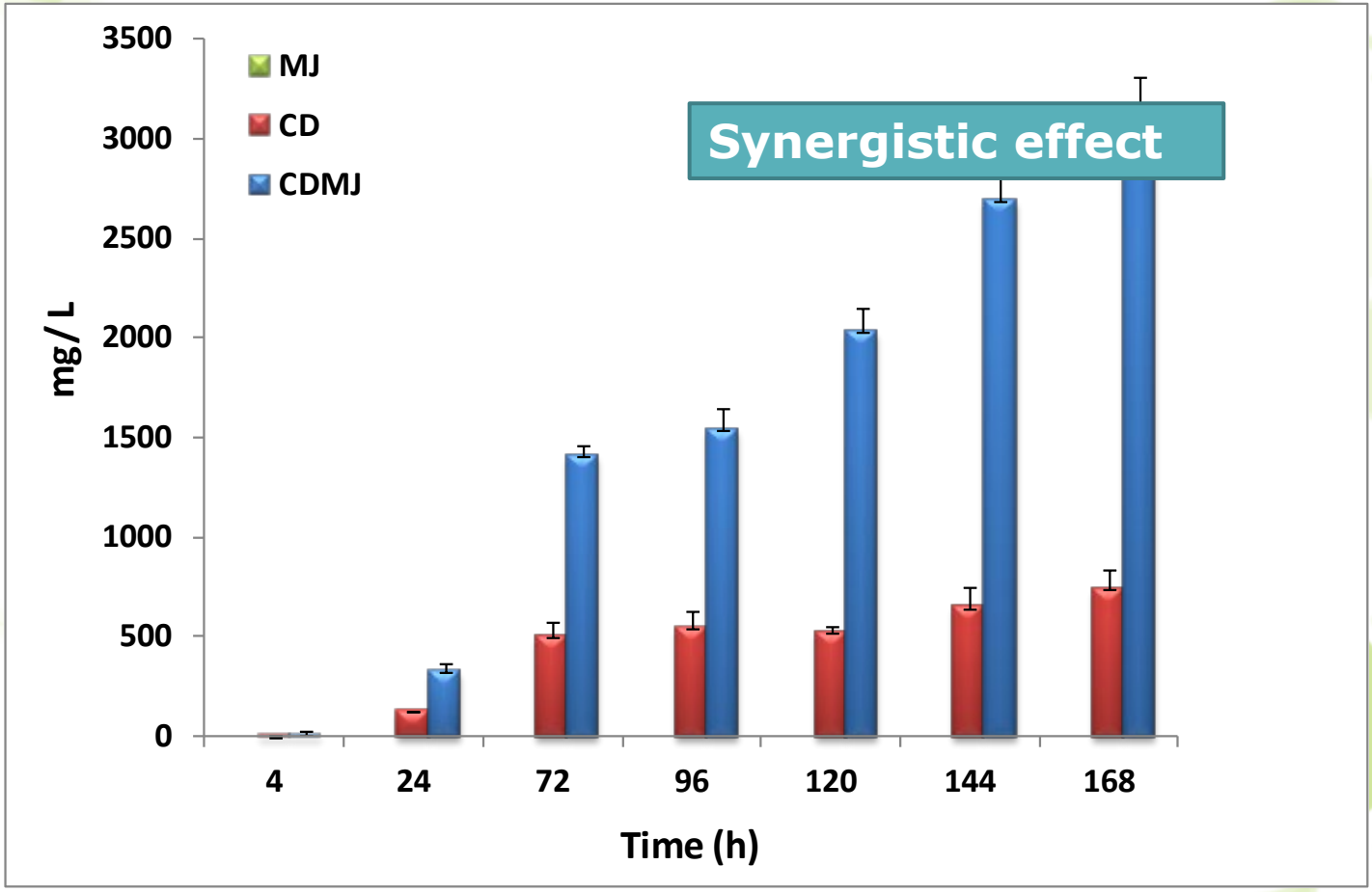
- **Hydrophilic surface and hydrophobic central cavity able to trap apolar compounds**
- **Inclusion complexes 1:1 type**

(Morales et al., 1998; Plant Cell Tissue Organ Cult. 53:179)





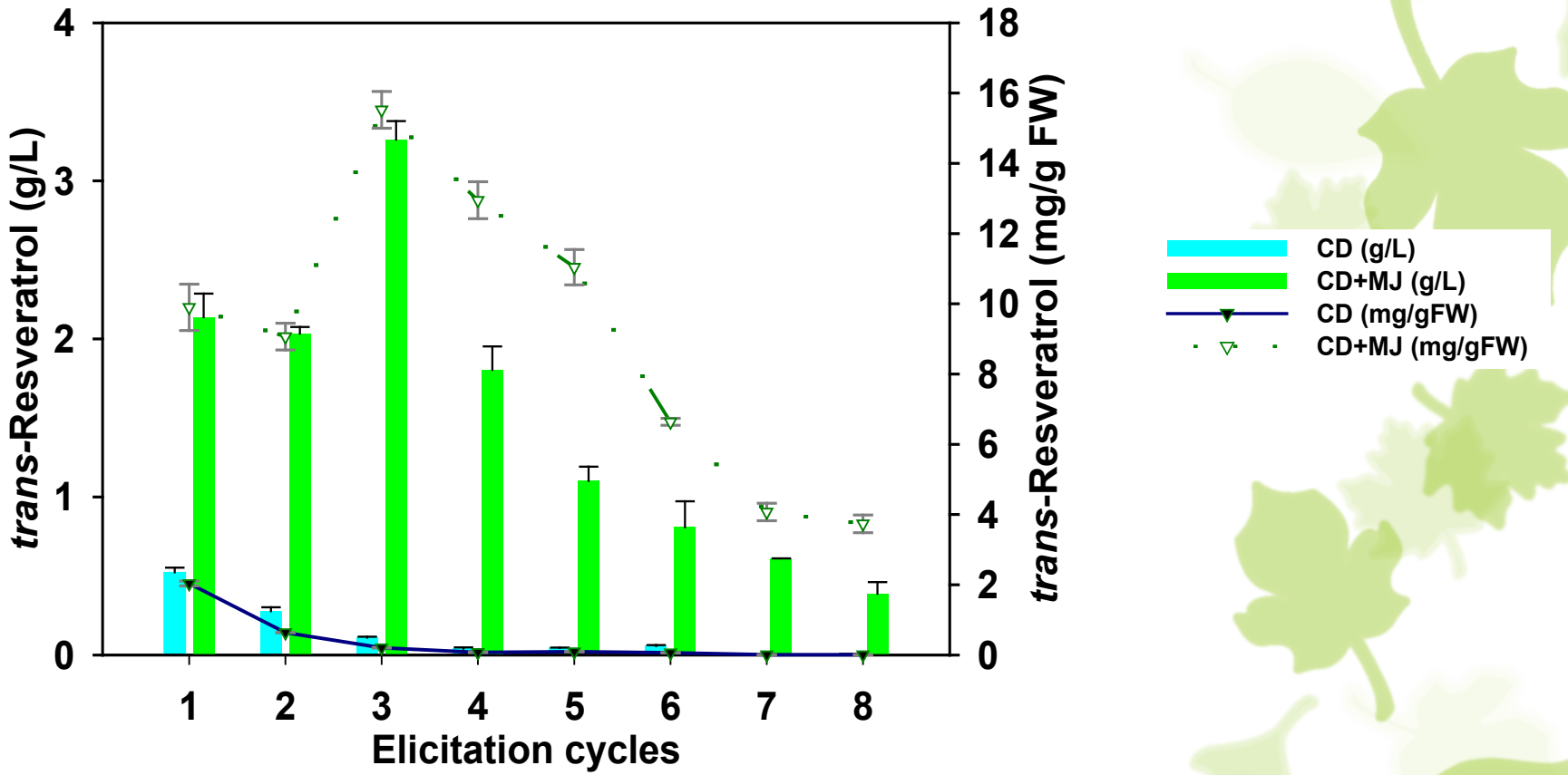
EXTRACELLULAR RESVERATROL PRODUCTION IN THE PRESENCE OF CDs AND/OR MJ



Resveratrol production is dependent on elicitation time. The joint action of CDs and MJ provokes a synergistic effect on resveratrol production (Lijavetzky, D., 2008. BMC Research Notes, 1: 132)

Almagro et al., 2013. In: Ramawat KG, Merillon JM (eds) Natural products: Handbook of natural products, Springer ISBN 978-3-642-22144-6

ELICITATION CYCLES



Variation of *trans*-resveratrol production through eight continuous elicitation cycles (96 hours each cycle).

SCALING-UP

***Vitis vinifera* cv Gamay cell cultures**

Bioreactors 2 L

Bioreactors 20 L

We are able to perform the scaling-up from flasks 1-2 L to commercial bioreactors 2-20 L

COMPARISON BETWEEN COMMERCIAL AND CUSTOMIZED BIOREACTORS

BAL

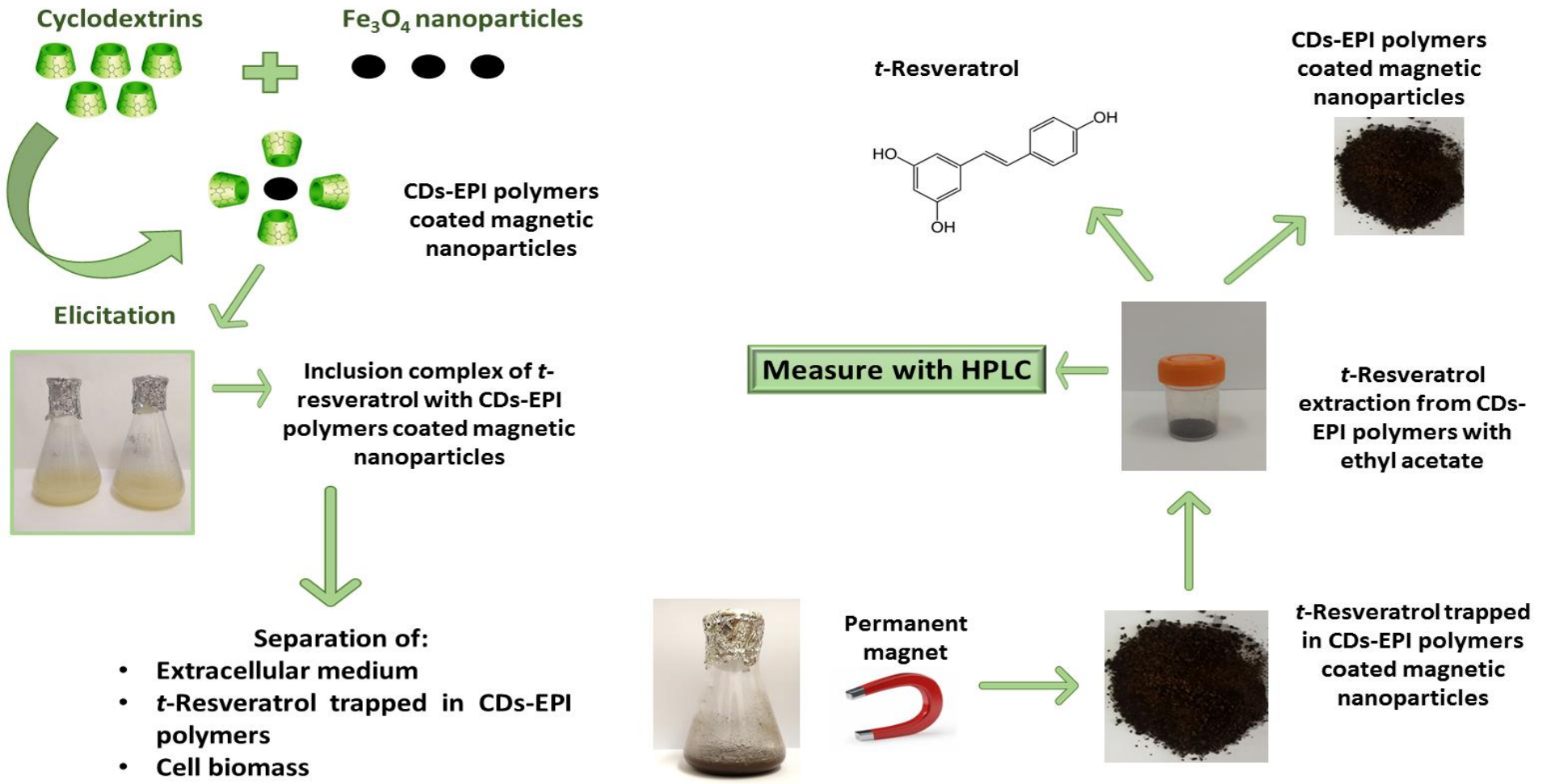
BTAA

trans-R production (mg/gFW)	Flasks 2 L	BTAA (Biostat B)	BAL (customized)
CD	1.5 ± 0.3 (100%)	1.3 (86.6%)	0.9 (60%)
CD+MeJa	2.8 ± 0.4 (100%)	11.7 (417%)	14.3 (510%)

Vera-Urbina et al. (2013) Production of Grapevine Cell Biomass (*Vitis vinifera* L. Gamay) and Resveratrol in Custom and Commercial Bioreactors using Cyclodextrins and with Methyl-Jasmonate Elicitors in Resveratrol: Sources, Production and Health Benefits. Nova Publishers, NY

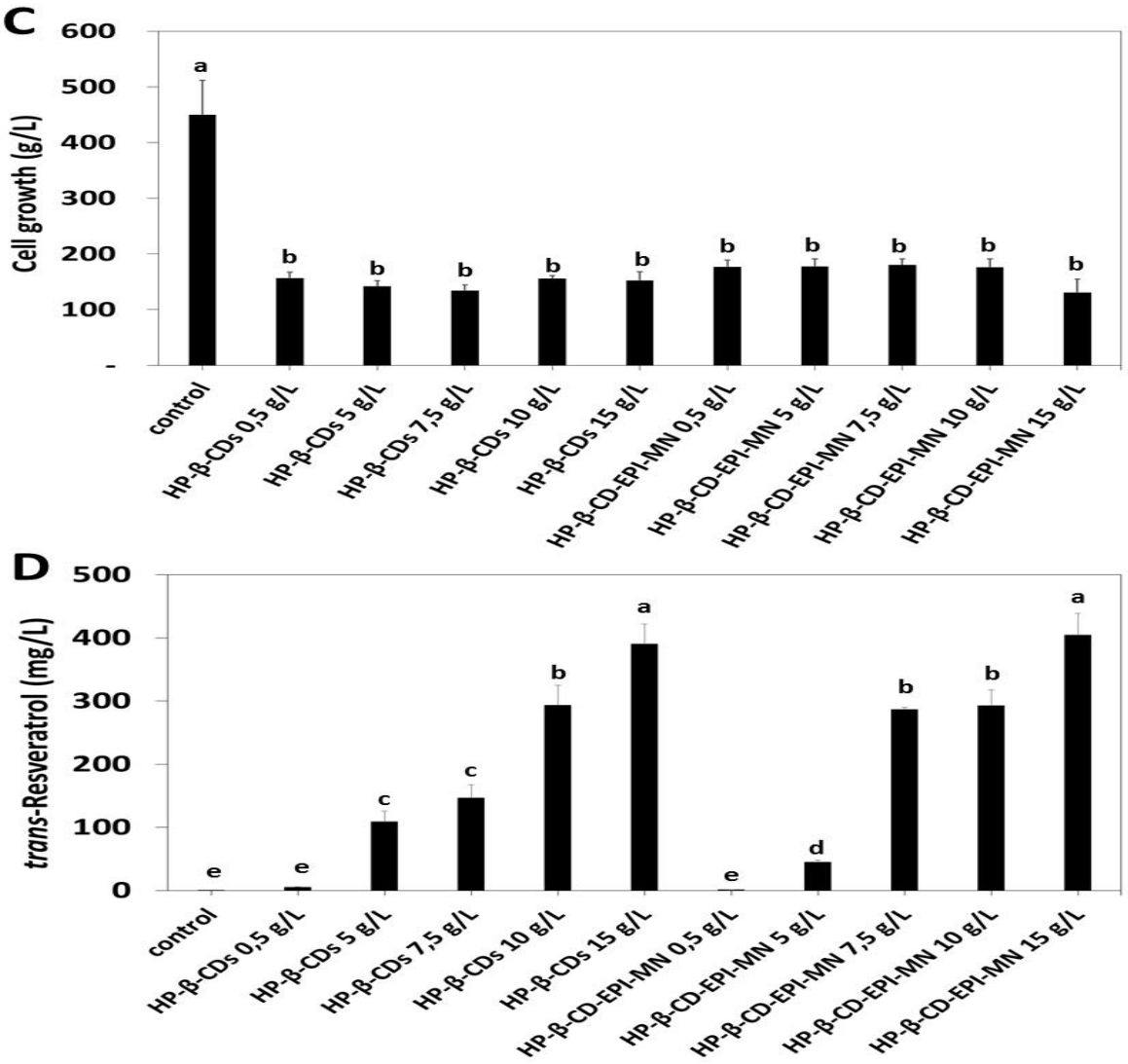
Method to recover cyclodextrins by designing nanosorbents:

Use of magnetic particles functionalized with cyclodextrins to form cyclodextrin/Fe_3O_4 nanocomposites

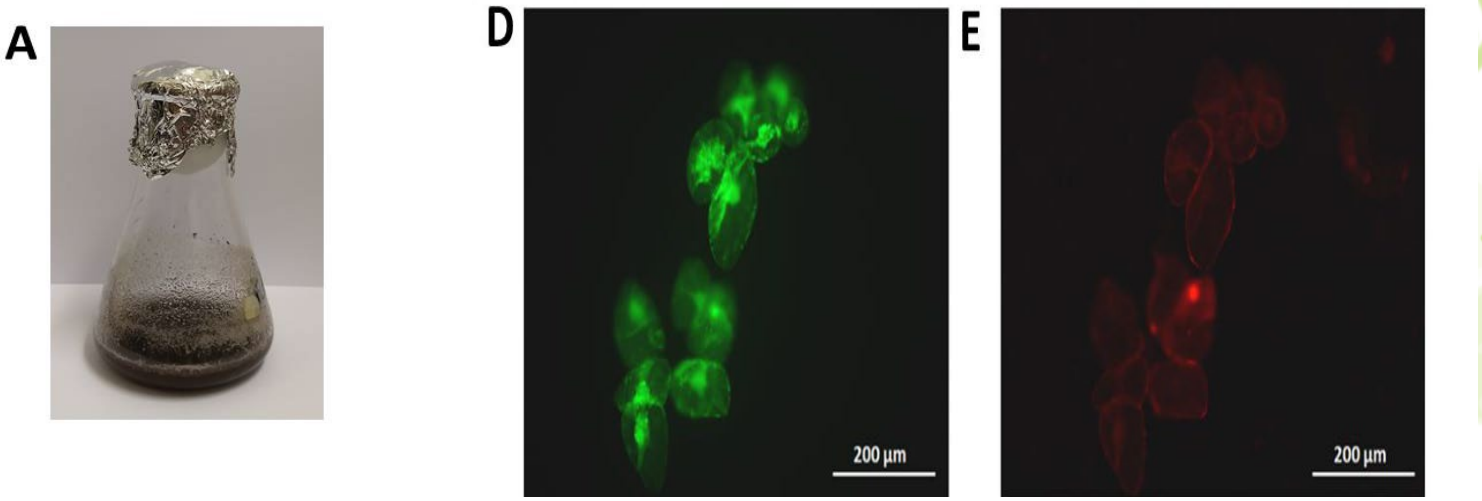


Almagro et al., 2020. A Smart Strategy to Improve t-Resveratrol Production in Grapevine Cells Treated with Cyclodextrin Polymers Coated with Magnetic Nanoparticles. (2020). *Polymers*, 12, 991 DOI:10.3390/polym12040991

Effect of different concentrations of hydroxypropyl-β-cyclodextrins-epichlorohydrin (HP-β-CD-EPI-MN) polymer in combination with 100 µM methyl jasmonate (MJ) on both **cell growth (C)** and ***t*-resveratrol production (D)** of *Vitis vinifera* suspension-cultured cells treated for 144 h.



Cell viability assessed with fluorescein diacetate and propidium iodide

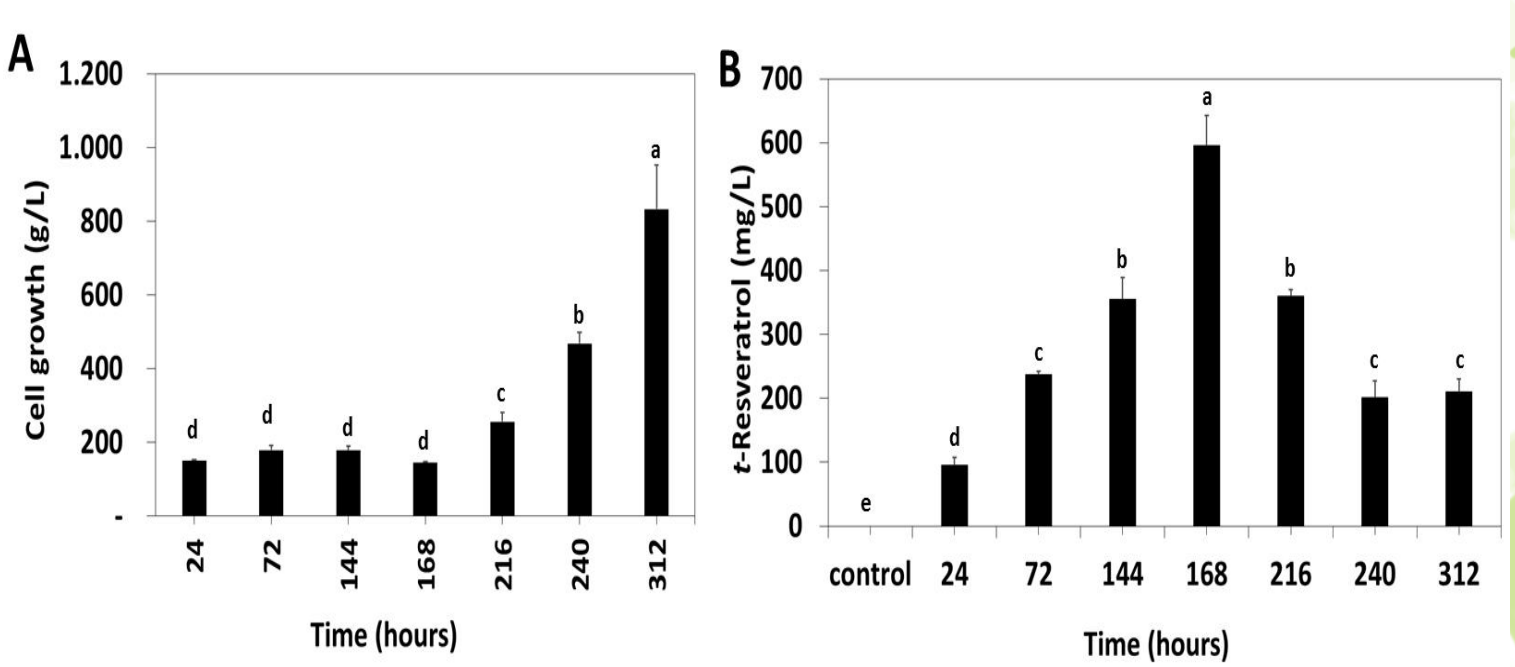


A. Cell growth observed in a flask containing *V. vinifera* suspension-cultured cells treated with 15 g/L hydroxypropyl-β-cyclodextrins-epichlorohydrin + methyl jasmonate.

D. *Vitis vinifera* cells elicited with 15 g/L hydroxypropyl-β-cyclodextrins-epichlorohydrin + 100 µM methyl jasmonate treated with fluorescein diacetate.

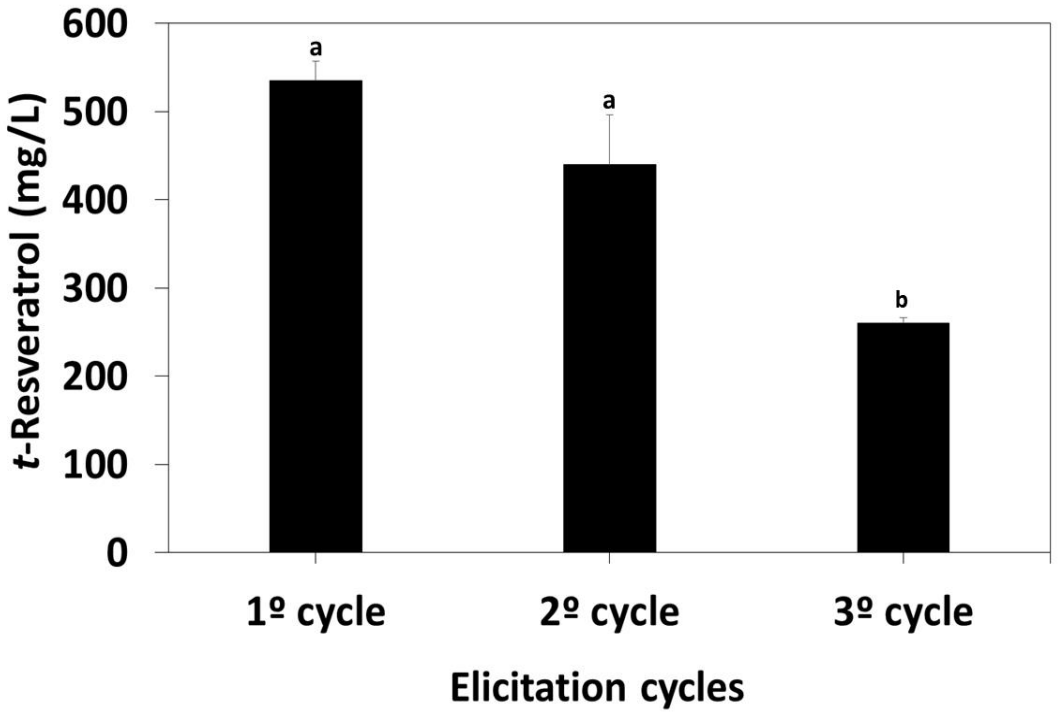
E. *Vitis vinifera* cells elicited with 15 g/L hydroxypropyl-β-cyclodextrins-epichlorohydrin + 100 µM methyl jasmonate treated with propidium iodide.
All images were taken at 144 h of treatment.

Effect of **elicitation time** on both **cell growth** (A) and ***t*-resveratrol production** (B) in *V. vinifera* suspension-cultured cells treated with 15 g/L hydroxypropyl-β-cyclodextrins-epichlorohydrin (HP-β-CD-EPI-MN) polymer in combination with 100 µM methyl jasmonate (MJ)



Almagro et al., 2020. A Smart Strategy to Improve t-Resveratrol Production in Grapevine Cells Treated with Cyclodextrin Polymers Coated with Magnetic Nanoparticles. (2020). *Polymers*, 12, 991 DOI:10.3390/polym12040991

Reuse of hydroxypropyl-β-cyclodextrins-epichlorohydrin polymer during three elicitation cycles (168 h each) with methyl jasmonate

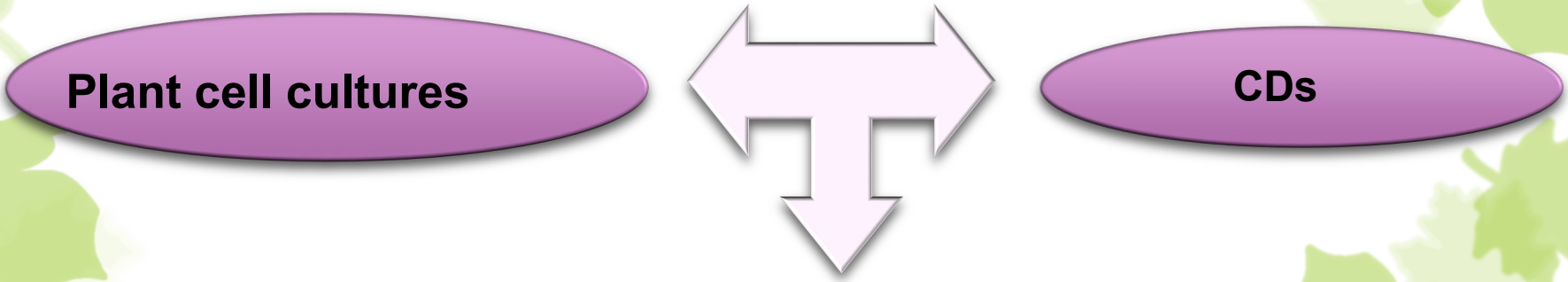


t-Resveratrol production through three continuous elicitation cycles (**168 h** each) using the same hydroxypropyl-β-cyclodextrins-epichlorohydrin polymer but changing grapevine cells which, in turn, were elicited with the recovered hydroxypropyl-β-cyclodextrins-epichlorohydrin polymer in combination with 100 µM methyl jasmonate.

Almagro et al., 2020. A Smart Strategy to Improve t-Resveratrol Production in Grapevine Cells Treated with Cyclodextrin Polymers Coated with Magnetic Nanoparticles. (2020). *Polymers*, 12, 991 DOI:10.3390/polym12040991

Case study: The production of phytosterols in elicited cell cultures: patent WO 2010049563 A1

Elicitation with cyclodextrins (CDs)



Phytosterols: β-sitosterol, campesterol, fucosterol, stigmasterol

BENEFICIAL EFFECTS OF COMPOUNDS PRODUCED BY CARROTS ON HUMAN HEALTH

Daucus carota

CAROTENOIDS → **β-carotene (provitamin A)**

α-TOCOPHEROL → **Vitamin E**

Antioxidants

PHYTOSTEROLS →
- Cholesterol-lowering
- Antitumoral effect
- Anti-inflammatory
- Anti-oxidative

Phytosterols mixed with dairy fat

EXTRACTION FROM BANANA FRUIT (5 Kg)

↓

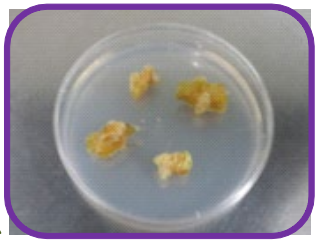
0.5 mg phytosterols/g DW

A novel fruit drink

AMC GROUP

USE OF CYCLODEXTRINS TO ENHANCE PHYTOESTEROL PRODUCTION USING CARROT CELL CULTURES

Roots



Explants

Callus

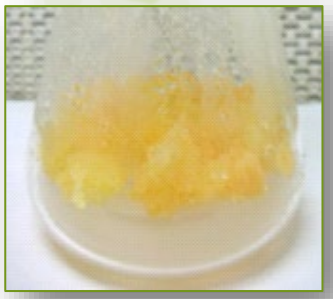
Cell cultures

Phytosterols

8 mg total phytosterols/ g DW
Productivity 13 mg/L day

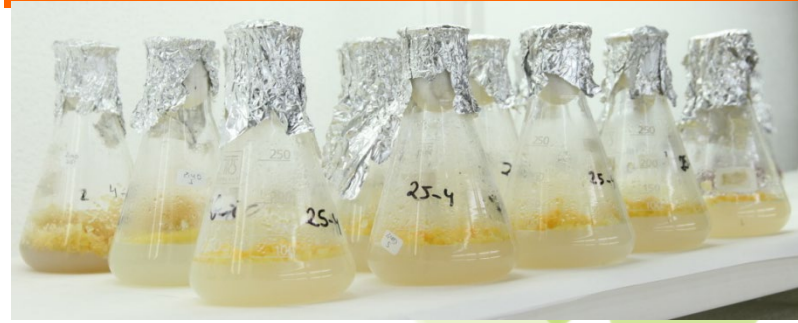
Patent nº
WO 2010049563 A1

CARROT CELL BIOMASS GENERATION



Subcultures each 20 days (in solid medium)

Exponential biomass production from calli



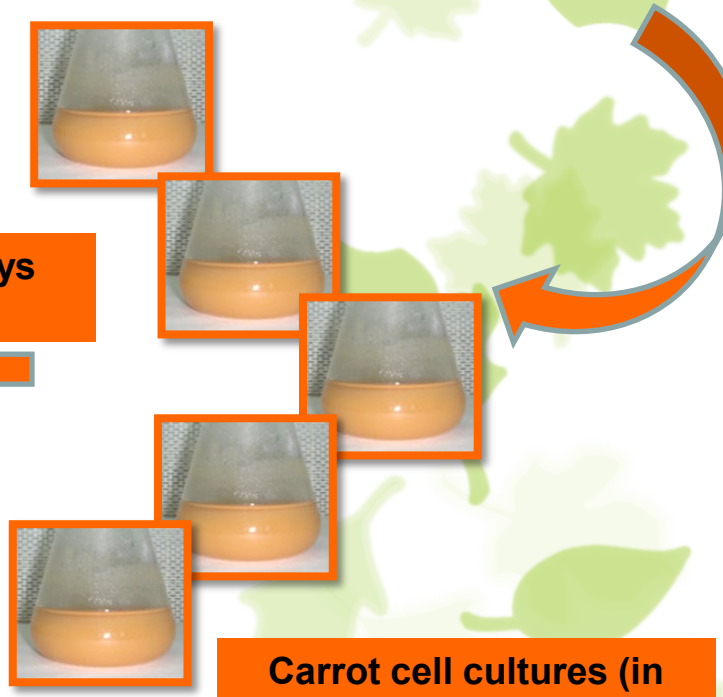
Production of non-elicited cells:

729 gFW/m²day

Production of elicited cells:

444 gFW/m²day

Subcultures each 9 days (in liquid medium)



Exponential biomass production from cell suspensions

Carrot cell cultures (in liquid medium)

EXTRACTION AND PURIFICATION OF PHYTOSTEROLS FROM CARROT ELICITED CULTURE MEDIUM

Filtration

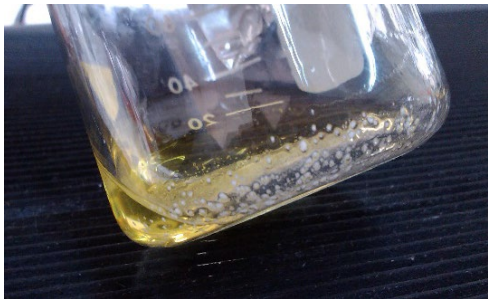
Elicited culture medium

Extraction from the medium and vacuum evaporation

Dried cells enriched in carotenoids

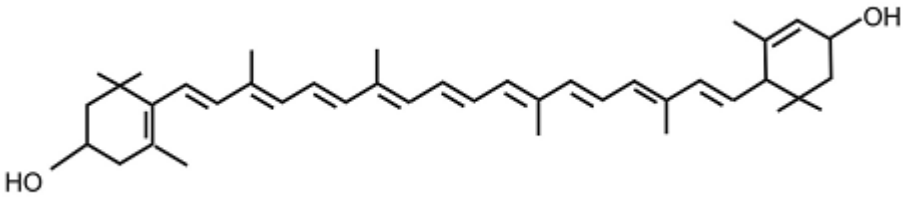
Dry extract enriched in phytosterols

Purification



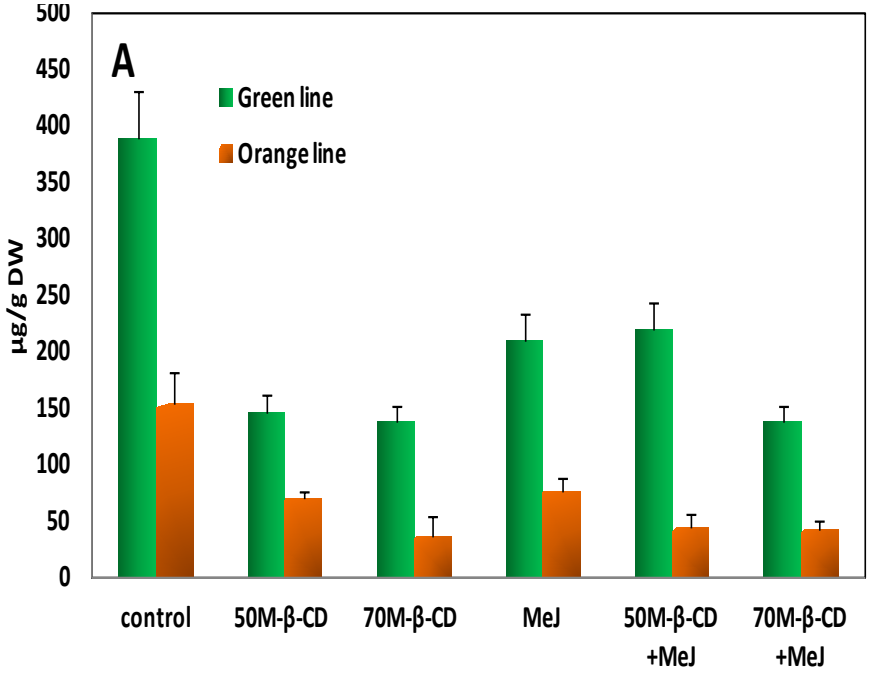
Phytosterol purification

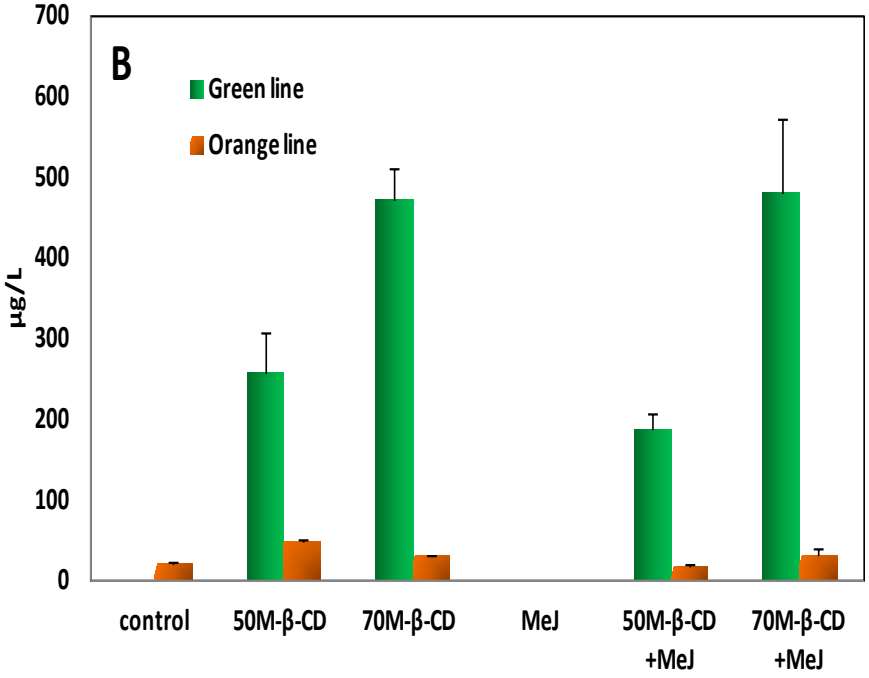
Phytosterols >92% purity



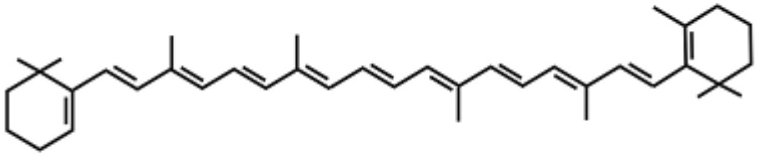
Luteína

LUTEIN



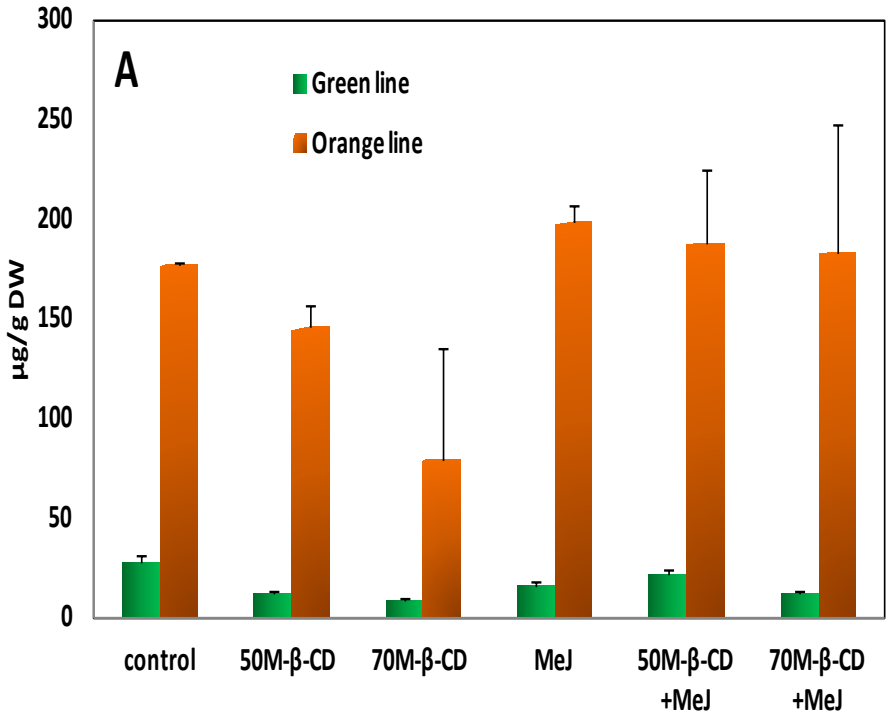


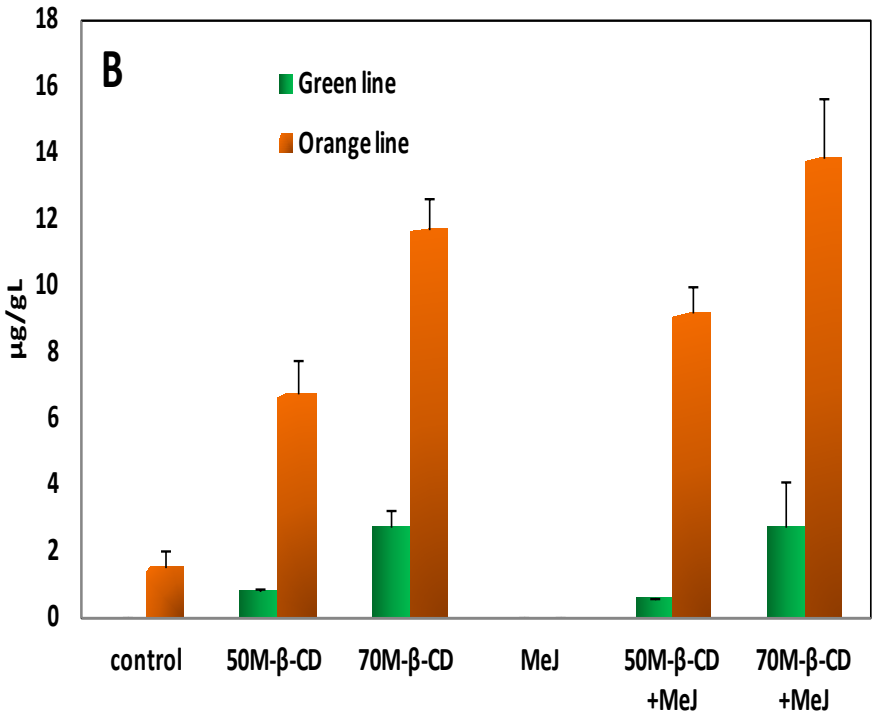
Accumulation intra- (A) and extracellular (B) of lutein in orange and green carrot cell lines elicited with CD alone or in combination with MeJ



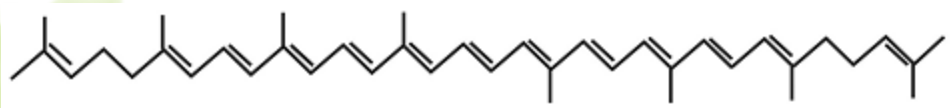
ß-caroteno

β-CAROTENE



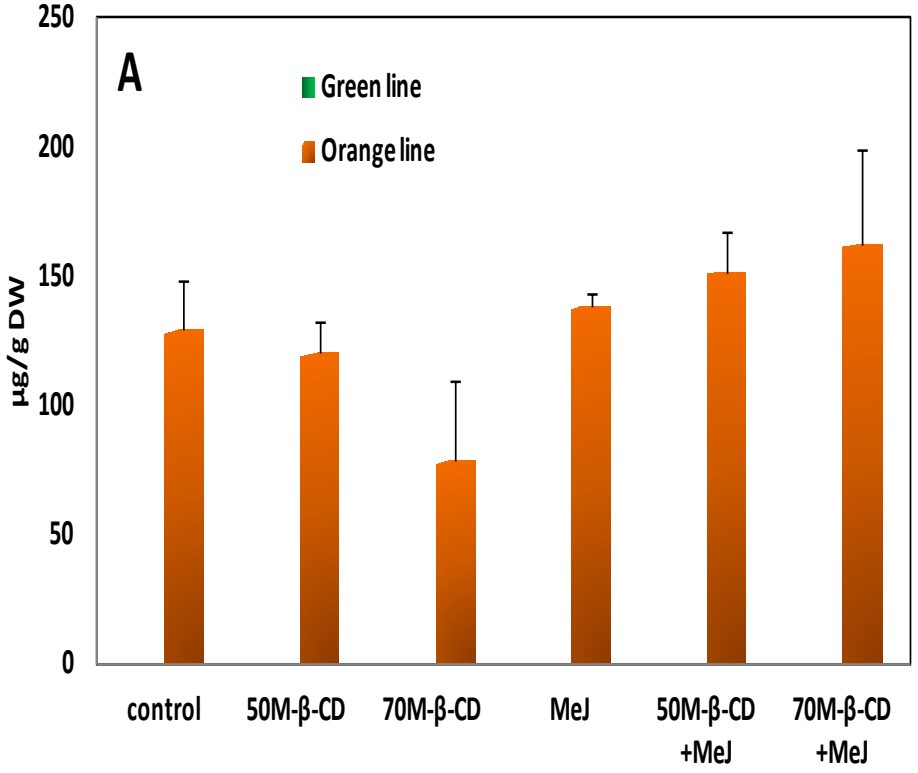


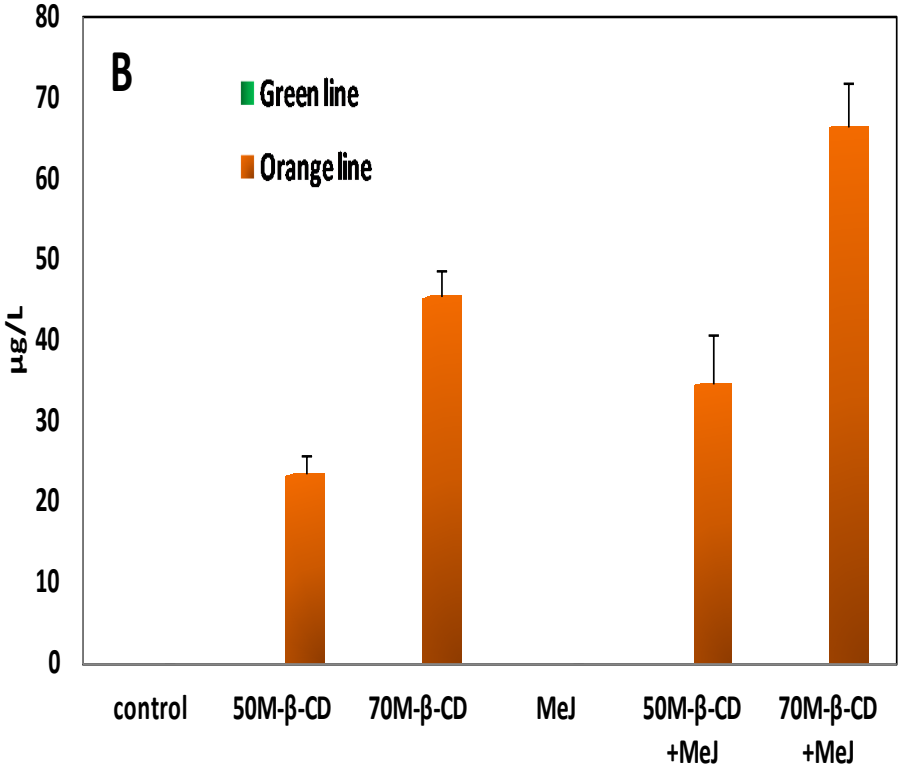
Accumulation intra- (A) and extracellular (B) of β-carotene in orange and green carrot cell lines elicited with CD alone or in combination with MeJ.



Licopeno

LYCOPENE





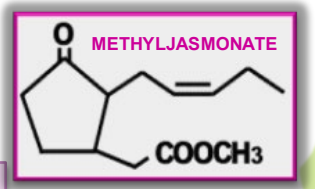
Accumulation intra- (A) and extracellular (B) of lycopene in orange and green carrot cell lines elicited with CD alone or in combination with MeJ.

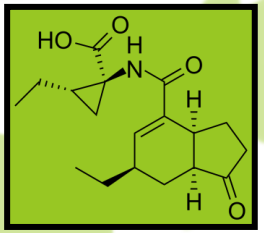
Case study: The production of glucosinolates in elicited broccoli cell cultures: patent ES 2694706 A1

Elicitation with methyl jasmonate or coronatine

broccoli cell cultures

MJ or coronatine





Glucosinolates:

glucobrassicin, 4-methoxy-glucobrassicin, 4-hydroxy-glucobrassicin, neoglucobrassicin

Intracellular signaling pathways similar to methyl jasmonate

STILBENES

TAXANES

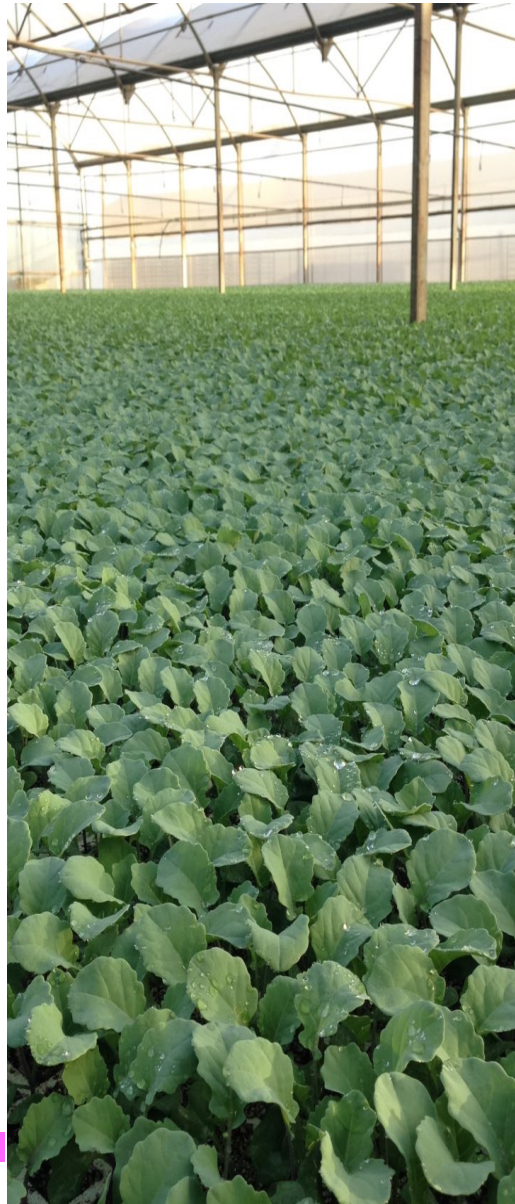
Almagro et al. 2015. Plant Physiology and Biochemistry DOI:10.1016/j.plaphy.2015.10.025;

Ramírez-Estrada et al. 2016. Molecules DOI:10.3390/molecules21020182

Brassicaceae family (Cruciferous)

Broccoli (*Brassica oleracea* L. var. *italica*)

- Vitamin C
- Calcium
- Magnesium
- Carotenoids
- Proteins
- Fiber
- **Phenolic Compounds**
- **Glucosinolates**



Sánchez-Pujante, P. J., Borja-Martínez, M., Pedreño, M. Á., & Almagro, L. (2017). Biosynthesis and bioactivity of glucosinolates and their production in plant in vitro cultures. *Planta*, *246*(1), 19-32.

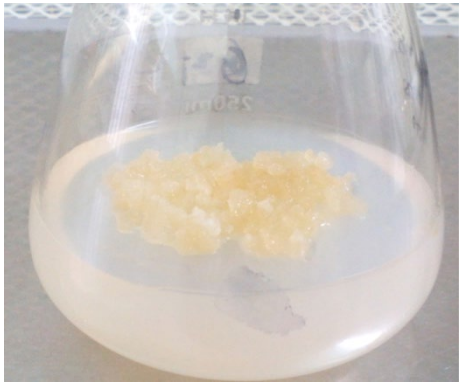
BROCCOLI CELL CULTURES AS BIOFACTORIES TO ENHANCE THE PRODUCTION OF GLUCOSINOLATES

in vitro germination

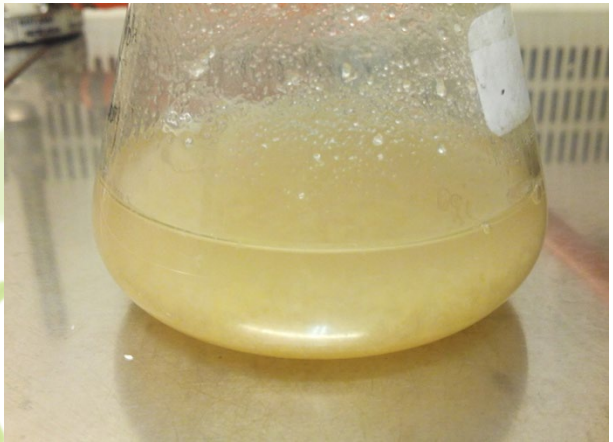
callus induction

optimization of callus growth

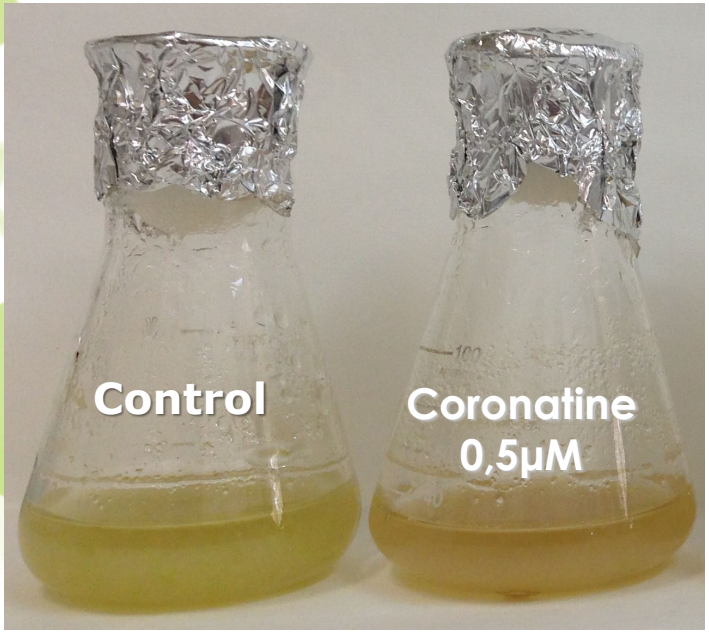
friable callus



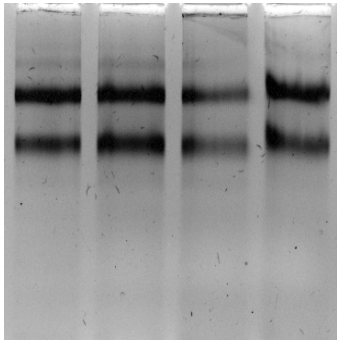
Cell cultures



Elicitation

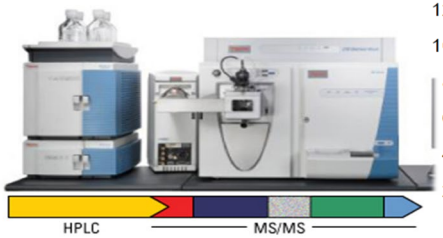


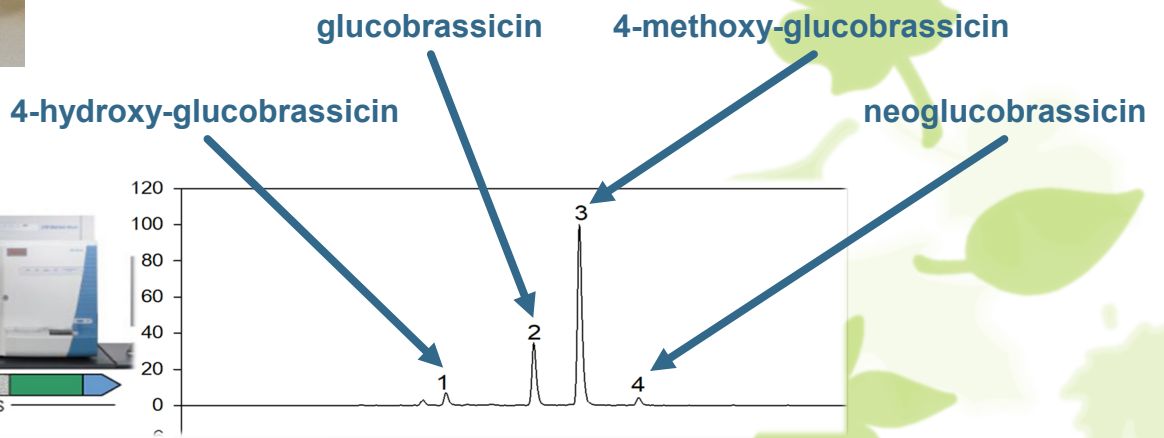
ARN



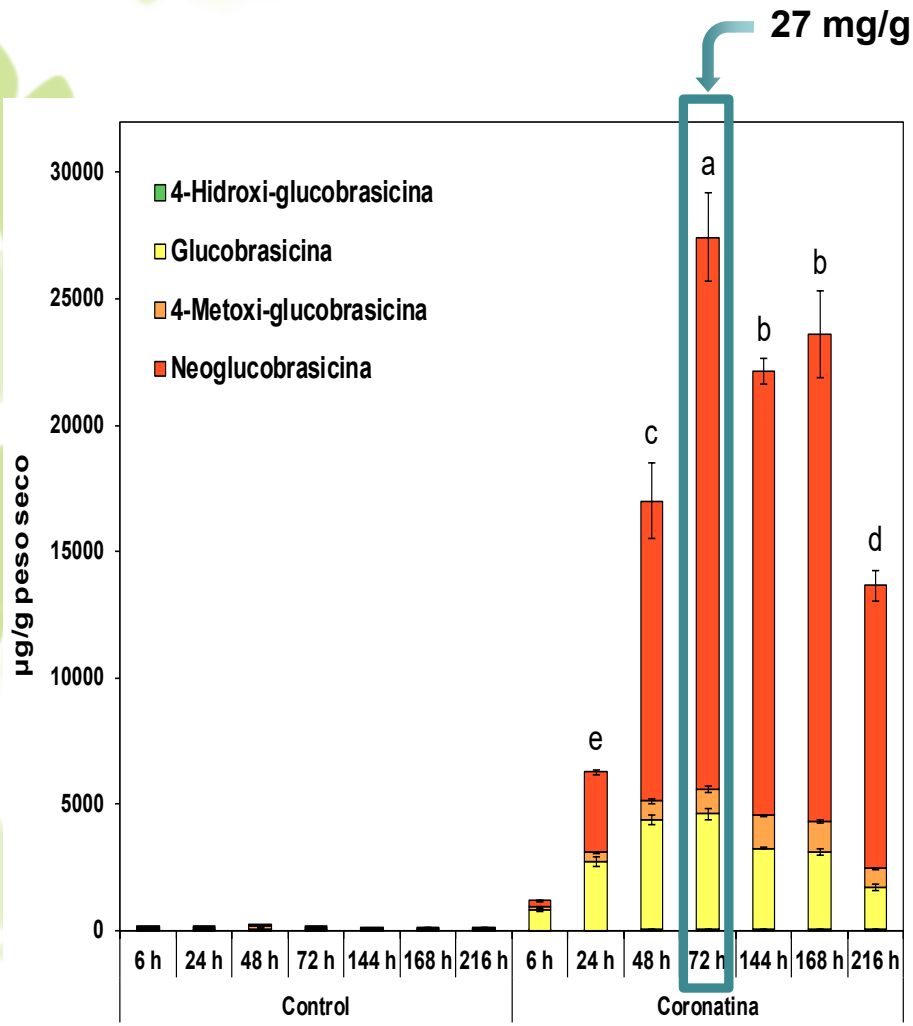
ARN isolation, ADNc synthesis and qPCR real time

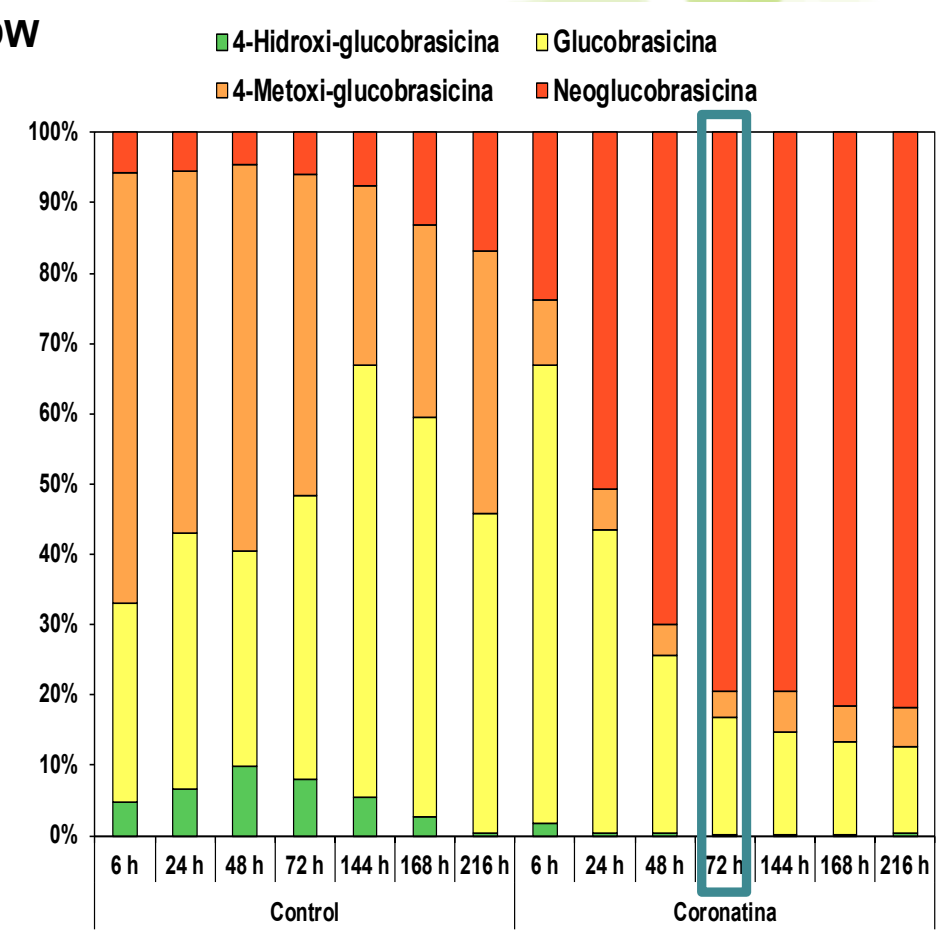
Extraction, identification and quantification of glucosinolates





Production of glucosinolates in exponential phase: 7 day-old broccoli cell cultures



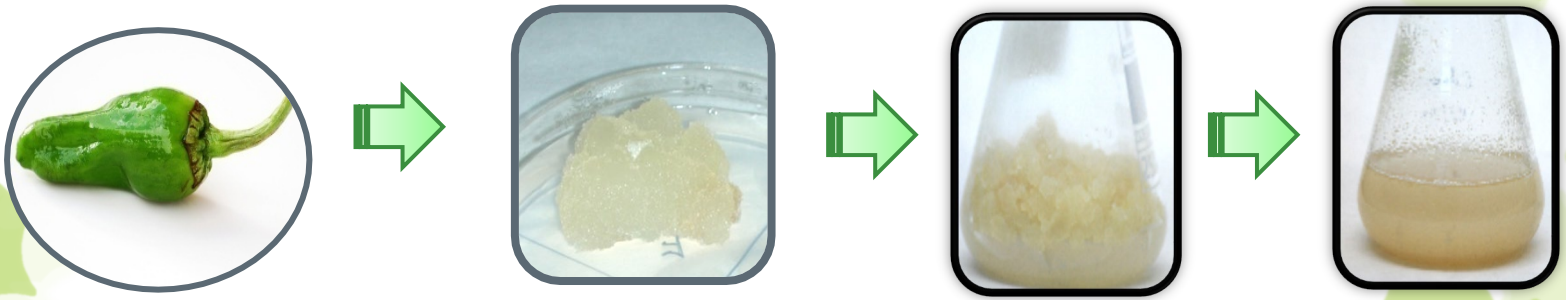


Neoglucobrassicin: 79,52%
Glucobrassicin: 16,73%
4-methoxy-glucobrassicin: 3,62%
4-hydroxy-glucobrassicin: 0,13%

Sánchez-Pujante, P. J., Sabater-Jara, A. B., Belchí-Navarro, S., Pedreño, M. A., & Almagro, L. (2018). ***Journal of Agricultural and Food Chemistry***, *67*(1), 102-111.

USE OF CYCLODEXTRINS AND/OR METHYL JASMONATE TO ENHANCE METABOLITE CONTENT USING PEPPER CELL CULTURES OBTAINED FROM FRUITS

Family: Solanaceae
Genus and specie: *Capsicum annuum*
Name: pepper



Sesquiterpenes:
aromadendrane 2353 µg/gDW and solavetivone 1094 µg/gDW
Phytosterols:
β-sitosterol 47 mg/L (1942 µg/gDW), campesterol 8 mg/L (700 µg/gDW)

Sabater-Jara et al., 2010. Induction of sesquiterpenes, phytosterols and extracellular pathogenesis-related proteins in elicited cell cultures of *Capsicum annuum*. Journal of Plant Physiology 167: 1273–1281.

USE OF CYCLODEXTRINS AND/OR METHYL JASMONATE TO ENHANCE METABOLITES CONTENT USING TOMATO CELL CULTURES FROM FRUITS

Family: Solanaceae
Genus and specie: *Solanum licopersicum*
Name: tomato

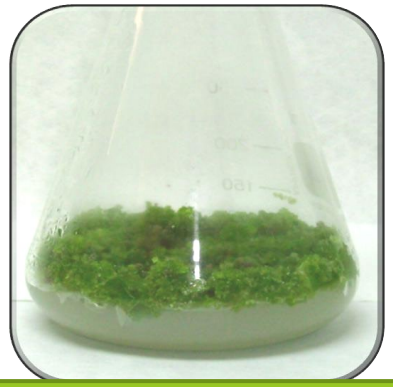
Triterpene:
Taraxasterol: **220 µg/gDW (6.2 mg/L)**
Phytosterols:
β-sitosterol: **25 µg/gDW (0.6 mg/L) Isofucosterol 30 µg/gDW (0.7 mg/L)**

Briceño et al., 2012. Enhancement of phytosterols, taraxasterol and induction of extracellular pathogenesis-related proteins in cell cultures of *Solanum lycopersicum* cv Micro-Tom elicited with cyclodextrins and methyl jasmonate. Journal of Plant Physiology. 169: 1050–1058

Taxonomy:

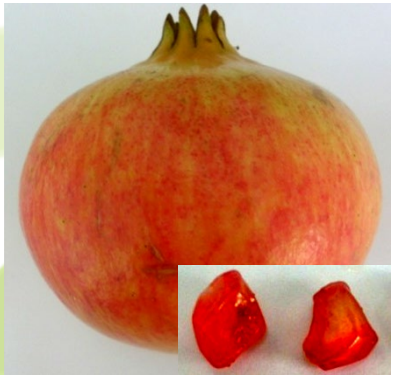
- Genus: *Daucus*
- Specie: *D. carota*

Metabolitos secundarios:
eugenol, isoeugenol, vanillina, 6-metoximeleina y derivados del ácido benzoico.
Fitoesteroles (110 mg/L): β-sitosterol, campesterol, estigmasterol y fucosterol

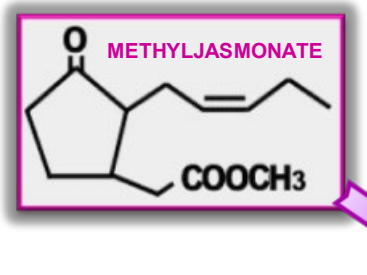


GREEN CARROT CELLS

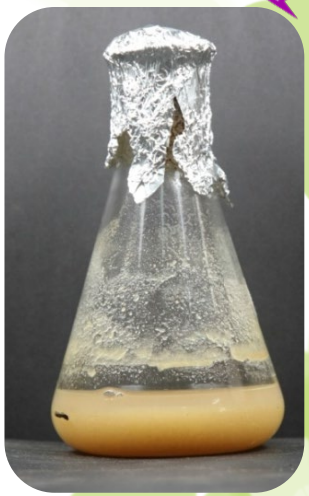
USE OF CYCLODEXTRINS AND METHYL JASMONATE TO ENHANCE THE PRODUCTION OF METABOLITES USING *Punica granatum* CELL CULTURES



AMC GROUP



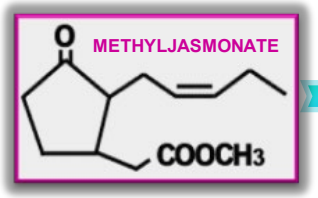
Explants

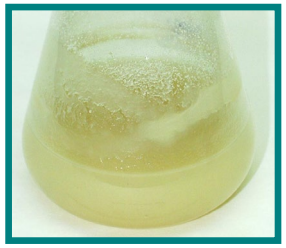


vytrus biotech

Punicalagin
Ellagic acid
Pelargonidin
Quercetin
Kaempherol
Luteolin

USE OF CYCLODEXTRINS AND METHYL JASMONATE TO INCREASE INDOLE ALKALOIDS USING *Catharanthus roseus* CELL CULTURES OBTAINED FROM VITROPLANTS





Indole alkaloids

Ajmalicine and catharanthine (900 mg/L y 150 mg/L)

Other alkaloids: tabersonine, lochnericine and serpentine

Spanish Patent P200802437

Viveros Bermejo

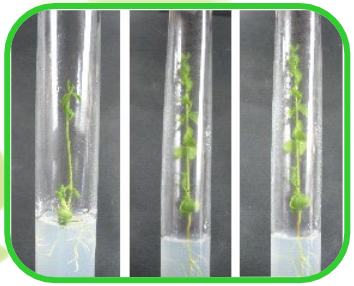
USE OF CYCLODEXTRINS AND/OR METHYL JASMONATE TO ENHANCE METABOLITE CONTENT USING *Ginkgo biloba* CELL CULTURES

Family: Ginkgoaceae
Genus and specie: *Ginkgo biloba*
Name: Ginkgo

Metabolites :
ginkgolides and bilobalides
kaempherol and catechin (flavonoids)

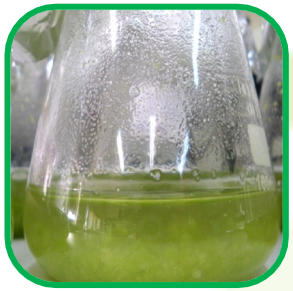
Sabater-Jara et al., 2013. Biotechnological approaches to enhance the biosynthesis of ginkgolides and bilobalide in *Ginkgo biloba*. Phytochemistry reviews 12(1):191-205

USE OF CYCLODEXTRINS ENHANCE METABOLITE CONTENT USING LINEN CELL CULTURES OBTAINED FROM SEEDS



Vitroplants

Callus



Elicited linen cell cultures

13.2 mg total phytosterols/ L:
β-sitosterol: 5 mg/L
Fucosterol: 0.2 mg/L
Campesterol: 7 mg/L
Stigmasterol: 1 mg/L

58 µg total phytosterols/ g FW

Tocopherols identified and quantified

Family: Linaceae
Genus and specie: *Linum usitatissimum*
Name: linen

FITOACTIVA

Almagro et al., 2016. New strategies for using *Linum usitatissimum* cell factories for the production of bioactive compounds. Plant Physiology and Biochemistry 99:73-78.

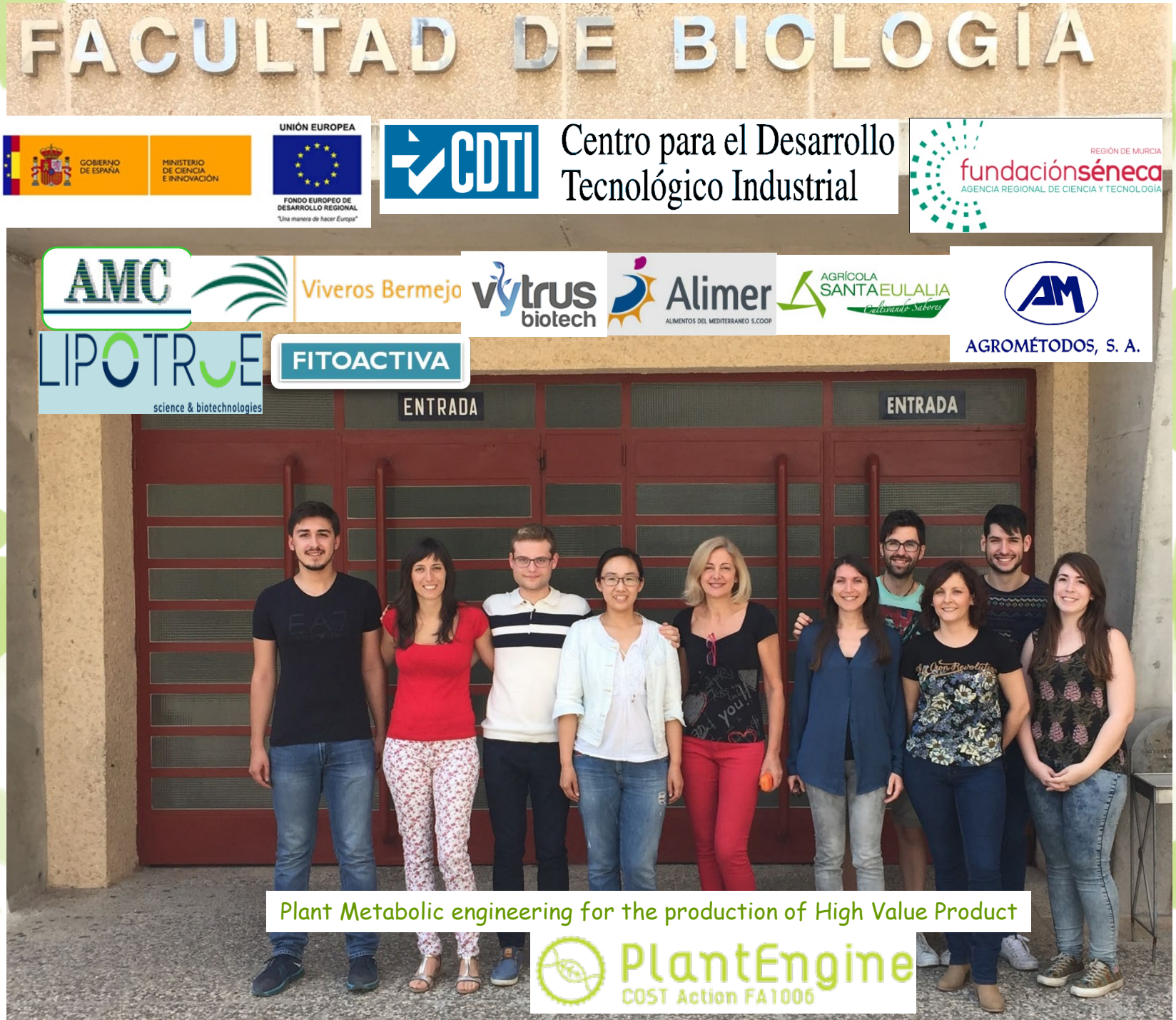
Plant in vitro cultures as biofactories

UNIVERSIDAD DE MURCIA

Dr. Maria A. Pedreño: mpedreno@um.es

We offer our technology (elicitation and metabolic engineering) for the production of bioactive compounds with pharmaceutical, nutraceutical and cosmetic purposes, and the production of plant biomass (plant micropropagation: vitroplants, and establishment and maintenance of cell cultures).

We offer our knowledge for the extraction , identification and quantification of plant extracts by different methodologies.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GOBIERNO DE ESPAÑA
MINISTERIO DE CIENCIA E INNOVACIÓN
UNIÓN EUROPEA
FONDO EUROPEO DE DESARROLLO REGIONAL
"Una manera de hacer Europa"
CDTI
Centro para el Desarrollo Tecnológico Industrial
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AGENCIA REGIONAL DE CIENCIA Y TECNOLOGÍA
AMC
Viveros Bermejo
vytrus biotech
Alimer
ALIMENTOS DEL MEDITERRANEO S.COOP
AGRÍCOLA SANTA EULALIA
AM
AGROMÉTODOS, S. A.
LIPOTRUE
science & biotechnologies
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ENTRADA
ENTRADA
Plant Metabolic engineering for the production of High Value Product
PlantEngine
COST Action FA1006