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Cruciferous Sprouts as Healthy Foods: Elicitation of Phytochemicals and Functionality

Brotes de Crucíferas como Alimentos Saludables: Elicitación de Fitoquímicos y Funcionalidad

DOCTORAL THESIS

Nieves Baenas Navarro

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Autorizan,

La presentación de la Tesis Doctoral "**CRUCIFEROUS SPROUTS AS HEALTHY FOODS: ELICITATION OF PHYTOCHEMICALS AND FUNCTIONALITY**", realizada por Dª **Nieves Baenas Navarro**, bajo nuestra dirección y supervisión, en el Departamento de Ciencia y Tecnología de los Alimentos del CEBAS-CSIC, para la obtención del Grado de Doctor por la Universidad Miguel Hernández de Elche.

En Murcia, a 17 de Junio de 2016.

Dra. Dª. Cristina García Viguera Dr. D. Diego A. Moreno Fernández

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Dr. José Ramón Díaz Sánchez, Dr. Ingeniero Agrónomo, Catedrático de Escuela Universitaria y Director del Departamento de Tecnología Agroalimentaria de la Universidad Miguel Hernández,

CERTIFICA:

Que la Tesis Doctoral titulada "**CRUCIFEROUS SPROUTS AS HEALTHY FOODS: ELICITATION OF PHYTOCHEMICALS AND FUNCTIONALITY**" de la que es autora la Ingeniera Agrónoma **Nieves Baenas Navarro** ha sido realizada bajo la dirección de la **Dra. Cristina García Viguera**, Profesora de Investigación, y el **Dr. Diego A. Moreno Fernández**, Científico Titular, del Consejo Superior de Investigaciones Científicas (CSIC) en el Centro de Edafología y Biología Aplicada del Segura (CEBAS), la cual considero conforme en cuanto a forma y contenido para que sea presentada para su correspondiente exposición pública.

Y para que conste a los efectos oportunos firmo el presente certificado en Orihuela a diecisiete de junio de dos mil dieciséis.

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Regional Agency of Science and Technology, Séneca Fundation, Region of Murcia (11909/PI/09) Use of low quality water for producing crops with high nutritional value: characterization of the response to nutritional stress (boron and salinity). January 2010 - December 2014.

Research Group of scientific excellence in the Region of Murcia. Regional Agency of Science and Technology, Séneca Fundation, Region of Murcia (04486/GERM/06). CEBAS-CSIC. 2008 - 2013.

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RESULTS OBTAINED DURING THE RESEARCH PERIOD

Peer-reviewed indexed scientific publications derived from the present Doctoral Thesis:

Articles:

Baenas, N., Moreno, D.A., García-Viguera, C. 2012. Selecting sprouts of *Brassicaceae* for optimum phytochemical composition. *Journal of Agricultural and Food Chemistry*, 60, 11409-11420.

Baenas, N., García-Viguera, C., Moreno, D.A. 2014. Biotic elicitors effectively increase the glucosinolates content in *Brassicaceae* sprouts. *Journal of Agricultural and Food Chemistry*, 62, 1881-1889.

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Baenas, N., Villaño, D., García-Viguera, C., Moreno, D.A. 2016. Optimizing elicitation and seed priming to enrich broccoli and radish sprouts in glucosinolates. *Food Chemistry*, 204, 314-319.

Baenas, N., González-Trujano, M.E., Guadarrama, O., Pellicer, F., García-Viguera, C., Moreno, D.A. Antinociceptive effects of broccoli sprouts involves opioid neurotransmission in experimental models in rodents. *Phytomedicine*. Under review.

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Reviews:

Baenas, N., García-Viguera, C., Moreno, D. 2014. Elicitation: a tool for enriching the bioactive composition of food. *Molecules,* 19(9), 13541-13563.

Books chapters:

Baenas N.[*], Francisco M.[*], Velasco P., Cartea M.E., García-Viguera C., Moreno D.A. Bioactive Compounds from *Brassicaceae* as Health Promoters. Vol. 2, pp. 27-47. In: Natural Bioactive Compounds from Fruits and Vegetables as Health Promoters. ISBN (eBook): 978-1-68108-243-1. ISBN (Print): 978-1-68108-244-8. 2016. Eds. Silva, L.R. and Silva. B.M. Bentham Science Publishers – Sharjah, UAE. ([*]equal contribution).

Other peer-reviewed scientific publications:

Moita, E., Gil-Izquierdo, A., Sousa, C., Ferreres, F., Silva, L.R., Valentão, P., Domínguez-Perles, R., Baenas, N., Andrade, P.B. 2013. Integrated analysis of COX-2 and iNOS derived inflammatory mediators in LPS-stimulated RAW macrophages pre-exposed to *Echium plantagineum* L. bee pollen extract. *PLoS ONE*, 8 (3), e59131.

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Mena, P., Domínguez-Perles, R., Gironés-Vilaplana, A., Baenas, N., García-Viguera, C., Villaño, D. 2014. Critical Review: Flavan-3-ols, anthocyanins, and inflammation. *IUBMB Life*, 66(11), 745-758.

Teixeira[*], A., Baenas[*], N.Dominguez-Perles, R., Barros, A., Rosa, E., Moreno, D.A., Garcia-Viguera, C. 2014. Natural Bioactive Compounds from Winery By-Products as Health Promoters: A Review. *International Journal of Molecular Science*, 15(9), 15638-15678. ([*]equal contribution).

Barros, A., Gironés-Vilaplana, A., Teixeira, A., Baenas, N., Dominguez-Perles, R. 2015. Grape stems as a source of bioactive compounds: application towards added-value commodities and significance for human health. *Phytochemistry Reviews*, 14(6), 921-931.

Wagner, A.E., Piegholdt, S., Rabe. Baenas, N., Schloesser, A., Eggersdorfer, M., Stocker, A., Rimbach, G. 2015. Epigallocatechin gallate affects glucose metabolism and increases fitness and life span in *Drosophila melanogaster*. *Oncotarget*, 6(31), 30568-30578.

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Editorial board member of books or special issues:

Iberian-american fruits rich in bioactive phytochemicals for nutrition and health. 2014. ISBN: 978-84-15413-25-7. Eds. Gironés-Vilaplana, A., Baenas N., Villaño, D., Moreno, D.A.

Uchuva, *Physalis peruviana* L.: Fruta Andina para el mundo. 2014. ISBN: 84-15413-27-0 & 978-84-15413-27-1. Eds. Moreno, D.A. Ed. support: Sánchez G.N.A., Baenas N., Dominguez-Perles, R.

Future Trends in Phytochemistry in the Global Era of Agri-Food and Health. II. Phytochemistry Society of Europe (PSE). Abstract book. 2015. ISBN: 978-0-9565472-6-2. Eds. Baenas N., Marhuenda J., Moreno D.A., García-Viguera C.

Future Trends in Phytochemistry (PSE Young Scientists Meeting). Phytochemistry Reviews (Proceedings of the Phytochemical Society of Europe). 2015. Vol. 14, Issue 6, pp. 871-1072. Guest Eds. García-Viguera, C., Gil-Izquierdo, A., Moreno, D.A., Baenas, N. Ed. Springer Dordrecht. Biomedicine/Biology Editorial, Springer Netherlands.

Dissemination publications:

Domínguez-Perles, R., Baenas, N., García-Viguera, C., Carvajal, M., Moreno, D.A. December 2012. Alimentación y sostenibilidad: Aprovechamiento de los subproductos del bróculi para uso industrial. Grupo THM. URL: *http://publicaciones.poscosecha.com/es/poscosecha/8-alimentacion-y-sostenibilidad-aprovechamiento-de-los-subproductos-del-brocoli-para-uso-industrial.html*

Baenas, N., Moreno, D.A., García-Viguera, C. April 2013. Brotes de crucíferas: alimento natural y de gran riqueza fitoquímica. Grupo THM. URL: *http://publicaciones.poscosecha.com/es/poscosecha/12-brotes-de-cruciferas-alimento-natural-y-de-gran-riqueza-fitoquimica.html*

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Contributions to congresses and symposiums derived from the present Doctoral Thesis:

Baenas, N., Moreno, D.A., García-Viguera, C. Plant secondary metabolites of different *Brassicaceae* sprouts. Poster. September 2013. 1st European Conference on Natural Products. Dechema, Frankfurt am Main, Germany.

Baenas, N., da Silva, J. T., Moreno, D.A., García-Viguera, C. Increasing glucosinolates content in *brassicaceae* sprouts by biotic elicitors. Poster. November 2013. 10 Slaca. Latin American Symposium of Food Science, Brasil.

Baenas, N., Silván, J.M., Medina, S., García-Viguera, C., Moreno, D.A., de Pascual-Teresa, S. Antiproliferative effects and metabolism of sulforaphane and glucoraphanin from broccoli sprouts in human colon and liver cancer cells. Second poster award. October 2014. II International Congress "Food Technology, Quality and Safety" and XVI International Symposium "Feed Technology", Novi Sad, Serbia. Proceeding: ISBN 978-86-7994-043-8, pp.451-456. *Second Poster Award.*

Baenas, N., Ferreres, F., Cristina García-Viguera, C., Moreno, D.A. Anthocyanins profiling by HPLC-DAD-ESI/MSn after biotic elicitor treatments in brassicaceae sprouts. Poster. October 2014. II International Congress "Food Technology, Quality and Safety" and XVI International Symposium "Feed Technology", Novi Sad, Serbia. Proceeding: ISBN 978-86-7994-043-8, pp.446-450.

Baenas, N., Silván, J.M., Medina, S., de Pascual-Teresa, S., García-Viguera, C., Moreno, D.A. Metabolism and antiproliferative effects of broccoli sprouts as food matrix, glucoraphanin and sulforaphane in human carcinoma cells lines. Award for best oral communication. April 2015. Future trends in Phytochemistry in the global era of Agri-food and health II. A young Scientists meeting. San Pedro del Pinatar, Murcia, Spain. *First Oral Communication Award.*

Baenas, N., García-Viguera, C., Moreno, D.A. Efecto del elicitador metil jasmonato en la concentración de compuestos bioactivos en brotes de brócoli (*Brassica oleracea* var itálica) y rábano (*Raphanus sativus* cv. Rambo). Poster. June 2015. XIV Congreso Nacional de Ciencias Hortícolas. Retos de la Nueva Agricultura Mediterránea. Orihuela, Spain. Proceeding: ISBN 978-84-606-8547-0, pp.466-469, Actas de Horticultura 71. Actas del XIV Congreso Nacional de Ciencias Hortícolas- SECH.

Other contributions to congresses:

Gironés-Vilaplana, A., Baenas, N., Villaño, D., Speisky, H., García-Viguera, C., Moreno, D.A. Evaluation of Phytochemicals and Biochemical Activity of Native Iberian-American Fruits. Poster. September 2013. 1st European Conference on Natural Products. Dechema, Frankfurt am Main, Germany.

Gil-Izquierdo, A., Moita, E., Sousa, C., Ferreres, F., Silva, L.R., Valentão, P., Domínguez-Perles, R., Baenas, N., Andrade, P.B. Integrated analysis of COX-2 and iNOS derived inflammatory mediators in LPS-stimulated RAW macrophages pre-exposed to *Echium plantagineum* L. bee pollen extract. Poster. September 2014. 12th Euro Fed Lipid Congress, Oils, Fats and Lipids: From Lipidomics to Industrial Innovation. Montpellier, France.

Moreno, DA., Hassini, I., Baenas, N., Carvajal, M., Martínez-Ballesta, M.C. Effect of KCl-priming and methyl jasmonate on secondary metabolites production in two varieties (white and red) of *Brassica oleracea* L. under NaCL stress. Poster. October 2014. II International Congress "Food Technology, Quality and Safety" and XVI International Symposium "Feed Technology", Novi Sad, Serbia. Proceeding: ISBN 978-86-7994-043-8, pp.485-488.

Barros, A., Gironés-Vilaplana, A., Texeira, A., Baenas, N., Domínguez-Perles, R. Phytochemical and functional evaluation of grape (*Vitis vinifera* L.) stems towards innovative valorisation procedures: Pitfalls and opportunities. Poster. April 2015. Future trends in Phytochemistry in the global era of Agri-food and health II. A young Scientists meeting. San Pedro del Pinatar, Murcia, Spain.

García-Soto, Z. M., Baenas, N., Moreno, D.A., Zafrilla, P., Abellan, J. Cardiovascular effects of broccoli consumption in obese menopausal women. Poster. April 2015. Future trends in Phytochemistry in the global era of Agri-food and health II. A young Scientists meeting. San Pedro del Pinatar, Murcia, Spain.

Migues, I., Baenas, N., Gironés-Vilaplana, A., Villaño, D., Ruales, J., Heinzen, H., Speisky, H., Moreno, D.A. Characterization of phenolic compounds and antioxidant capacity in six Latin-American fruits. Poster. April 2015. Future trends in Phytochemistry in the global era of Agri-food and health II. A young Scientists meeting. San Pedro del Pinatar, Murcia, Spain. *First Poster Award.*

Supervisor in degree projects:

Title: Increase of bioactive compounds in broccoli sprouts (*Brassica oleracea* var. *italica*) and radish (*Raphanus sativus*) by elicitation. Student: Antonio Hernández Molina. Date: 09-08-2014. Grade: excellent. Degree: Technical Agricultural Engineer, Miguel Hernández University.

Title: Using elicitors to increase the content of bioactive compounds in broccoli (*Brassica oleracea* var *italica*) and red radish (*Raphanus sativus* cv. Rambo). Student: Juan Antonio Sotomayor Ballesta. Date: 07-09-2015. Grade: excellent. Degree: Food Science and Technology, University of Murcia.

Title: Bioavailability and metabolism of glucosinolates from radish sprouts in young free living women. Student: Alejandra Navarro Martínez. Date: Under execution. Degree: Biochemistry, University of Murcia.

Scientific seminars:

"Ensayos de capacidad antioxidante interlaboratorios". Jornada INSA-UB/CORNUCOPIA: Nutrición y seguridad I+D II Reunión de Coordinación de la Red CYTED CORNUCOPIA. Universidad de Barcelona (UB), Spain. Date: 04-12-2013.

"Investigación, otra salida profesional" XVII Jornadas de empleo, Miguel Hernández University (EPSO-UMH), Orihuela, Spain. Date: 02-19-2014.

"Studies of novel *Brassicaceae* sprouts rich in bioactive compounds" Seminar in the Institute for Human Nutrition, Faculty of Agricultural and Nutritional Sciences, Christian Albert University of Kiel, Germany. Date: 12-08-2014.

"Studies of novel cruciferous sprouts rich in bioactive compounds" Seminar in the Erasmus-Plus program. Catholic University of Murcia (UCAM), Spain. Date: 05-13-2015.

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DOCTORAL THESIS STRUCTURE

The present Doctoral Thesis corresponds to a compendium of publications, presented before in the section "Results obtained during the research period: Peer-reviewed indexed scientific publications derived from the present Doctoral Thesis", and has been prepared in agreement with the internal regulations of the Miguel Hernández University of Elche for the presentation of Doctoral Thesis with European Mention.

The Thesis structure is the following:

- Introduction (Chapter 1): Includes a literature review based on the bioactive compounds present in cruciferous food, including specific information about sprouts, as well as an extensive explanation of the elicitation techniques to enhance the amount of phytochemicals in the plants. Then, an overview of the quality of sprouts during shelf-life is included, and finally, the beneficial effects related to cruciferous consumption and attributed to the bioactive compounds are described along with their absorption and metabolism. Part of the information presented in this Chapter is included in the Annexes (Chapter 7).
- Objectives (Chapter 2): Based on the importance of the topics involved in the Chapter 1, the four specific objectives were established, which correspond to the four subsections showed in the publications on Chapter 4, achieving the general objective of the present Doctoral Thesis.
- General materials and methods (Chapter 3): Here, it is presented a summary of the plant materials and the different methods used in the works included in this Thesis, as well as the place where there have been carried out.

- Publications (Chapter 4): This Chapter is divided in four subsections according to the specific objectives established:

  Subsection 1. Selecting sprouts of *Brassicaceae* for optimum phytochemical composition (*Publication 1*): where a screening of 10 different sprouts species was carried out in order to highlight the most interesting sprouts in terms of health-promoting compounds.

  Subsection 2. Using elicitation to enhance the content of bioactive compounds in cruciferous sprouts: once the suitable sprouts varieties were selected, elicitation techniques were employed to enhance the content of phytochemicals in cruciferous sprouts. First, the study of the effect of the most effective elicitors found in literature over the bioactive compounds present in selected cruciferous sprouts was developed (*Publication 2*), then, an specific characterization of anthocyanins in red radish varieties was carried out (*Publication 3*), and finally a optimization of doses and seed priming of seeds with elicitors were performed (*Publication 4*).

  Subsection 3. Evaluation of *in vitro* and *in vivo* biological activities of broccoli and radish sprouts (*Publications 5, 6 and 7*). First, the metabolism and functionality of broccoli and radish sprouts are presented in *publications 5 in and 6 in* , respectively. Then, a study of the antinociceptive activity of broccoli sprouts was carried out (*Publication 7*).

  Subsection 4. Shelf-life quality and safety of eco-grown broccoli and radish sprouts (*Publication 8*): this work was performed in order to demonstrate that sprouts are safe and healthy foods for human consumption.

- General results and discussion (Chapter 5): First, a summary of the results and discussion related to the different publications presented in this Thesis is presented along with a descriptive figure of the work-flow performed. Then, a broader explanation of the results and their justification is included.
- Conclusions (Chapter 6): Here, the global conclusions related to all the results presented in this work are presented.
- Annexes (Chapter 7): In this chapter two publications are included: a book chapter about the bioactive compounds and nutrients present in cruciferous foods, following with a literature review about elicitation.
- References (Chapter 8): This final chapter includes the bibliography cites used in complementary sections to the publications.

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ABSTRACT

Cruciferous sprouts are fresh plant foods very interesting because of their higher levels of nutrients and bioactive compounds compared to adult plants. Germinating seeds for 8 days has been established as optimum for harvest and consumption, allowing manipulation while the content of phytochemicals remains higher than in other vegetables, even though, the bioactive compounds contents decrease during germination. Determining the bioactive compounds (phenolics, glucosinolates and isothiocyanates/indoles) in cruciferous sprouts, as well as selecting the suitable species and the germination time, have been found to be of great importance to maximize the health-promoting properties of sprouts for consumption.

Elicitation practices with phytohormones (MeJA, JA and SA), sugars (sucrose and glucose) and amino acids (methionine), by priming seeds and using exogenous spray applications enhanced the contents of glucosinolates, precursors of the bioactive isothiocyanates and indoles, which have been widely studied because of their anticarcinogenic, antioxidant and anti-inflammatory activities. Once broccoli and radish sprouts were selected due to their high content in glucoraphanin and glucoraphenin, respectively, among other health-promoting glucosinolates and phenolic compounds, certain biological activities were evaluated. The metabolism and antiproliferative effect of broccoli sprouts was studied *in vitro* using cell cultures. The effects of radish sprouts cv. Rambo modulating the energy metabolism was determined in the *Drosophila melanogaster* model, and the antinociceptive effect of broccoli sprouts was evaluated using rodent models.

Finally, shelf-life quality and safety of these sprouts was studied for 7 and 14 days under refrigerated storage. This multidisciplinary work open views to design studies of cruciferous foods for human nutrition, since their incorporation to diet and regular consumption will likely provide positive effects for health and disease prevention.

RESUMEN

Los brotes de crucíferas son alimentos de origen vegetal de gran interés debido a su mayor contenido en nutrientes y compuestos bioactivos en comparación con el vegetal adulto. No obstante, un objetivo en nuestra investigación es maximizar sus propiedades beneficiosas relacionadas con el contenido en compuestos bioactivos (glucosinolatos y compuestos fenólicos), para ello los estudios de la selección de la especie y el tiempo óptimo de germinación para su recolección y consumo, son factores fundamentales que nos permiten una adecuada manipulación y mantener un contenido en fitoquímicos más alto que el que encontramos en otros vegetales, a pesar de que el contenido en compuestos bioactivos disminuye con la germinación.

Para incrementar el contenido en glucosinolatos (precursores de los isotiocianatos e índoles), se empleó la elicitación con fitohormonas (MeJA, JA and SA), azúcares (sacarosa y glucosa), y amino ácidos (metionina) como inductores de semillas y aplicados en spray sobre los brotes. Con todo ello, se seleccionaron los brotes de brócoli y rábano por su alto contenido en glucorafanina y glucorafenina, respectivamente, así como en otros glucosinolatos y compuestos fenólicos, y se evaluaron algunas de sus actividades biológicas. Concretamente se estudió el efecto antiproliferativo de los brotes de brócoli así como la absorción y metabolismo de sus compuestos utilizando cultivos celulares. Por otro lado, se demostró el efecto de los brotes de rábano cv. Rambo sobre el metabolismo energético con el modelo *Drosophila melanogaster* y, por último, se evaluó el efecto antinociceptivo de los brotes de brócoli utilizando modelos de roedores.

Finalmente, y con el objetivo de estudiar la vida útil de los brotes, se analizó su contenido microbiológico y de compuestos bioactivos durante 7 y 14 días de

almacenamiento, estableciendo que los brotes bajo condiciones de refrigeración son alimentos seguros para los consumidores. Este trabajo multidisciplinar abre diferentes líneas de estudio sobre crucíferas para nutrición humana, ya que su incorporación en la dieta y consumo regular, proporcionará efectos beneficiosos en la salud y prevención de enfermedades.

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ABBREVIATIONS

˙NO	Nitric oxide radical
˙OH	Hydroxyl radical
˙OOH	Hydroperoxyl radical
4-HGB	4-Hydroxyglucobrassicin
ANOVA	Analysis of variance
Avr	Avirulence
C	Carbon
Ca	Calcium
CHS	Chalcone synthase
CO_2	Carbon dioxide
CoA	Coenzyme A
Cu	Cupper
CYP	Cytochrome P450
CYS	Cysteine
CYS-GLY	Cysteine-Glycine
DAD	Diode array detection
DNA	Deoxyribonucleic acid
DPPH˙	2,2-diphenyl-1-picrylhydracyl radical
DW	Dry Weight
EFSA	European Food Safety Authority
ESI	Electrospray ionization
ESP	Epithiospecifier proteins
ET	Ethylene
EU	European Union
FAOSTAT	Statistics Division of Food and Agriculture Organization of the United Nations
FDA	U.S. Food and Drug Administration
Fe	Iron
FRAP	Ferric reducing antioxidant power
FW	Fresh weight
GAP	Good Agricultural Practices
GER	Glucoerucin
GHP	Good Hygiene Practices
GLS	Glucosinolate/s
GMP	Good Manufacturing Practices
GRA	Glucoraphanin
GRE	Glucoraphenin

GSH	Glutathione
HACCP	Hazard Analysis Critical Control Point
HDAC	Histone deacetylases
HPLC	High performance liquid cromatography
I3C	Indole-3-carbinol
ISR	Induced systemic resistance
ITC	Isothiocyanate/s
JA	Jasmonic acid
K	Potassium
LDL	Low-density lipoprotein
MeJA	Methyl jasmonate
MeOH	Methanol
Mg	Magnesium
MGB	4-Methoxyglucobrassicin
Mn	Manganese
MRM	Multiple reaction monitoring
MS	Masa
NAC	N-acetylcysteine
NaCl	Sodium chloride
NCDs	Non-communicable diseases
NF-κB	Nuclear factor-kappa B
NGB	Neoglucobrassicin
Nrf2	NF-E2-related factor
NSP	Nitrile-specifier proteins
$O^{2\cdot-}$	Superoxide anion
P	Phosphorus
PAL	Phenylalanine ammonialyase
PAR	Photosynthetically active radiation
PR	Pathogenesis-related
RH	Relative humidity
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SA	Salicylic acid
SAR	Systemic adquired response
SFE	Sulforaphene
SFN	Sulforaphane
TFP	Thiocyanate-formein proteins
UGT	UDP-glycosyltransferase

UV	Ultraviolet
WHO	World Health Organization
Zn	Zinc

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Chapter 1. Introduction

Part of the information presented in this Chapter is included in the Annexes I and II

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1. PLANT-DERIVED FOODS AND HEALTH

1.1. Phytochemicals in a healthy diet context

One of the key facts that are presented by the WHO (World Health Organization) is that a healthy diet helps to protect against non-communicable diseases (NCDs), including diabetes, heart disease, stroke and cancer (WHO, 2015a). Based on this, several epidemiological studies have demonstrated that a diet rich in vegetables is decisively associated with health-promotion and disease prevention. The phytochemical content of plant foods, such as phenolic compounds, carotenoids, vitamins and glucosinolates (GLS) among others, has been widely investigated as responsible of these effects. Clinical studies based on interventions with nutraceuticals, including vitamins E and C, carotenoids, GLS and phenolic compounds, which have generally antioxidant capacity, preventing or diminishing the excessive oxidation in body cells (Miller *et al.*, 2014), have shown reduction in parameters related to Diabetes Mellitus and Metabolic Syndrome (Cicero and Colletti, 2016), cardiovascular risk factors (Kim *et al.*, 2008), reduction of pro-inflammatory cytokines (Surh and Na, 2008), and, basically, uncontrolled oxidation of lipids, DNA, and proteins, which is associated with the increase of chronic diseases in humans. Phytochemicals, either alone or in combination, showed promising results against various cancers through genetic and epigenetic modifications (Shukla *et al.*, 2014). The traditional Mediterranean-type diet, including plant foods and making an emphasis on plant protein sources, provides a well-accepted healthy dietary pattern to reduce NCDs.

Non-pharmacological treatments based on phytochemicals could involve the use of food ingredients to prevent diseases and promote wellbeing and healthy lifestyle.

1.2. Functional foods and consumers

Over the last decades, consumers have become more aware of how important is to increase the consumption of fruits and vegetables as well as doing any kind of regular physical activity in terms of health-promotion for preventing the development of diseases. According to the Regulation (EC) No. 1924/2006 of the European Union, there is a wide range of nutrients and substances including, but not limited to, vitamins, minerals (including trace elements), amino acids, essential fatty acids, fiber and various plants and herbal bioactive compounds with a nutritional or physiological effect, present in foods that can be the subject of a claim. Health claims describe a relationship between a food substance (a food, a food component or ingredient) and a reduced risk of a disease or health-related condition, according to the FDA (U.S. Food and Drug Administration) (FDA, 2013). The European Food Information Council (EUFIC) described functional foods as those foods which are intended to be consumed as part of the normal diet and that contain biologically active components which offer the potential of enhanced health or reduced risk of disease. Conducting well designed clinical studies to evaluate the bioactivity and efficacy of phytochemicals should be a strategy in order to reduce the social and economic costs of attempts to prevent or to treat different diseases.

Changing global food systems and dietary patterns, while empowering healthy food and consumer education policies, will be associated with improved food quality to a balanced diet (Khoo and Knorr, 2014; Willett *et al.*, 2006).

2. *BRASSICACEAE*

2.1. General aspects and nutritive value

Brassicaceae plants, also called crucifers, represent a monophyletic group including approximately 350 genera and 3,700 species, and are among the oldest cultivated crops, since have been cultivated from the Greeks and Romans (Janick, 2011) and continue to date for different uses. These vegetables has been the subject of much scientific interest due to their economic importance, most of them are produced as edible plants, including roots (kohlrabi, turnip), stems (radish), leaves (kale, collards), inflorescences (broccoli, cauliflower, cabbages, Brussels sprouts) and seeds (mustards, wasabi), and also are used for feed or oil extraction (rapeseed) (Figure 1.1).

This family includes very common known species such as *Brassica oleracea* (broccoli, cauliflower, kohlrabi, Brussels sprouts, cabbages, among others), *B. rapa* (turnip, Chinese cabbage, pak choi, among others), *B. napus* (rapeseed, leaf rape), *Sinapis alba* (white mustard), *Raphanus sativus* (radishes) and *Lepidium sativum* (garden cress).

Figure 1.1. *Common vegetables from the Brassicaceae family.*

The world production of crucifers (broccoli, cauliflower, cabbage and other crucifers for consumption) was 90 million tons in 2013, being almost 14 million tons the production of the European Union, and 700,000 tons the production in Spain (FAOSTAT, 2013). *Brassicaceae* crops are mainly distributed in temperate regions of the Northern Hemisphere: in areas of Southwestern and Central Asia, China and Japan, Europe, the Mediterranean basin and North America. *Brassicaceae* production has grown steadily and its vegetables represent a major item of the human diet worldwide. Despite the great diversity among the *Brassicaceae*, members of only a few genera are used in human diet (Fahey *et al.*, 2001).

There are numerous species with great potential for exploitation in the 21st century agricultural and food commodities, particularly as sources for nutrients and bioactive compounds.

The vegetables from the family *Brassicaceae* have been widely approved for its beneficial effects on human health through epidemiological studies (Jahangir *et al.*, 2009), as good sources of a variety of nutrients and phytochemicals that may work synergistically against certain types of cancer, cardiovascular diseases, neurodegeneration and diabetes (Figure 1.2) (Clarke, 2010; Dinkova-Kostova and Kostov, 2012). Although vegetable cruciferous plants are sources of fibre, folates, vitamins (A, E, C, and K) and minerals (Ca, Fe, K, Cu, Zn, P, Mn, and Mg, among others), the majority of the research literature is concentrated on the content of secondary metabolites, such as flavonoids and carotenoids, and, specially, GLS. These compounds are mainly present in the cruciferous family, within the *Brassicales* Order, and their hydrolysis products, isothiocyanates (ITC) and indoles, are the bioactive compounds which may be responsible of the anti-inflammatory and chemopreventive activity, reduction of metabolic disorders and reduction of the risk of a number of cancers, associated with the intake of crucifers. Besides, the beneficial effects of crucifers have been also partly attributed to nutrients and phytochemicals with antioxidant capacity, such as vitamins C and E, carotenoids and phenolic compounds (Avato and Argentieri, 2015; Bjorkman *et al.*, 2011).

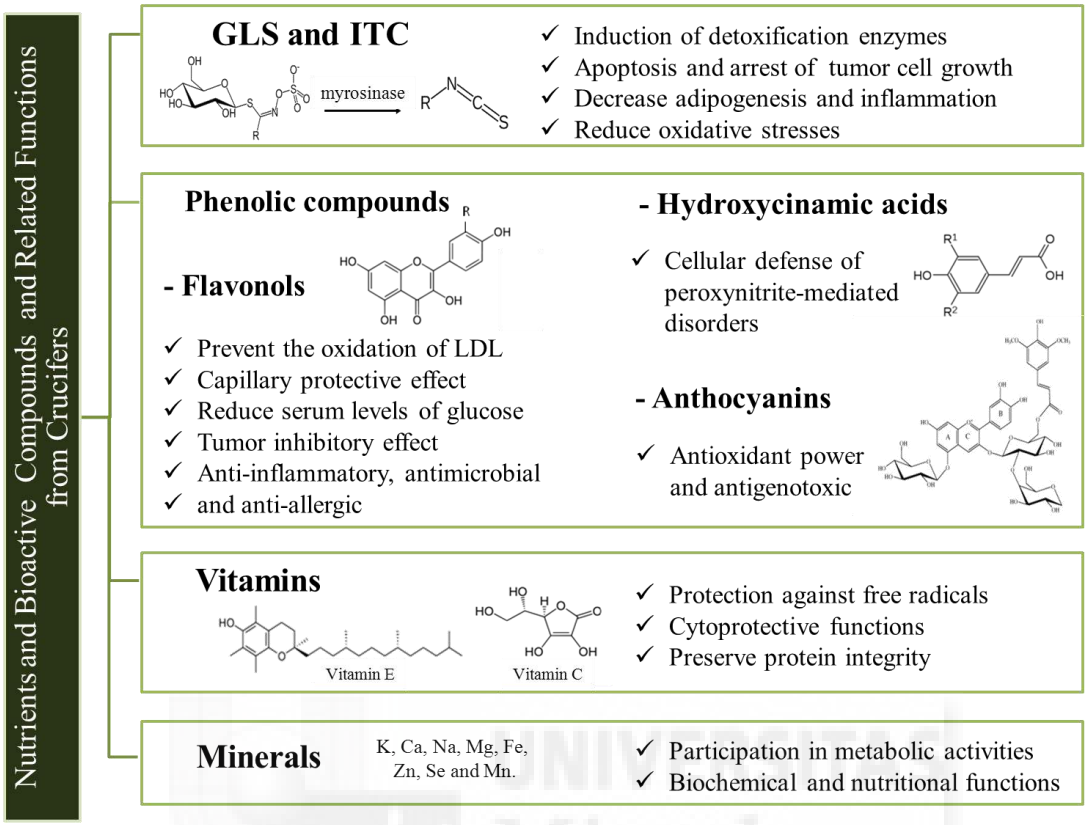

***Figure 1.2**. Nutrients and phytochemicals present in cruciferous foods and the associated beneficial physiological functions (for references, see Annex I).*

Cruciferous sprouts are usually collected and commercialized at 7 or 8 days of age after germination, considering this young physiological state the optimum for consumption in terms of biomass and size, which allow manipulation, as well as for their health-promoting compounds content, since sprouts have significantly greater concentrations of phytochemicals than mature plants (10-100 times) (Cevallos-Casals and Cisneros-Zevallos, 2010; Hanlon and Barnes, 2011; Pérez-Balibrea *et al.*, 2008). This is due to the high content in bioactives that is found in seeds, which are the storage organs of nutrients in the plant. On the other hand, over the growth period of sprouts a decrease of these compounds is found, as a result of a dilution effect of tissue expansion. The sprouts consist on the cotyledon, which may become the

embryonic primary leaves of a seedling, the hypocotyl and the radicle or root, being the cotyledons the organs with higher concentration of bioactive compounds (Pérez-Balibrea *et al.*, 2008), therefore, the harvest of the aerial portion of the sprouts (hypocotyls and cotyledon) for consumption is a reasonable practice from the perspective of capturing most of their bioactive compounds (Guo *et al.*, 2014; Pereira *et al.*, 2002). These edible sprouts could be consumed raw, and they are very low caloric foods, recommended for healthy diet.

Several intervention trials focuses on low-glycemic and low-fat food intake, generally based on minimally processed vegetables, have shown different benefits including weight loss, improvements in cardiometabolic biomarkers, reductions in cardiac events and mortality, improving insulin sensitivity, and diabetes control, as well as reductions of incidence of cardiovascular problems, inflammation and cancer, both in adults and children (Jéquier and Bray, 2002; Katz and Meller, 2014; Mirza *et al.*, 2013). Edible sprouts are a rich source of nutrients and phytochemicals (Dominguez-Perles *et al.*, 2011a), and in cruciferous sprouts, GLS and phenolics are cited as responsible of the health benefits derived from their consumption (Cartea *et al.*, 2011; Wagner *et al.*, 2013).

2.2. Glucosinolates and isothiocyanates

2.2.1. Chemical structure, classification and role in plants

The GLS are a relatively large group (> 120 described to date) of sulphur and nitrogen-containing compounds with a common structure which comprises a β-D-thioglucose group; a sulphonated oxime moiety; and a variable aglycone side-chain

derived from one of eight natural amino acids that determine the final chemical structure of the GLS, being methionine, tryptophan or phenylalanine the common known (Fahey *et al.*, 2001). Therefore, GLS can be classified by their precursor amino acids as aliphatic (derived from alanine, leucine, methionine or valine), aromatic (from phenylalanine or tyrosine) and indolic (from tryptophan) (Clarke, 2010).

Biosynthesis of glucosinolates proceeds in three stages (Figure 1.3.): (i) side-chain elongation of amino acids through five reactions: initial transamination, condensation with acetyl-CoA to form a 2-alkylmalate derivative, isomerization, oxidative decarboxylation, and, finally a α-keto acid is elongated by one carbon, which can be transaminated to an homoamino acid; (ii) development of the core structure, where the amino acids are oxidated to aldoximes catalyzed by cytochrome P450 (CYP family), then, aldoximes are oxidized to reactive aci-nitro or nitrile oxide intermediates, next cleavage of the S-alkylthiohydroximate conjugate by a C-S lyase produces thiohydroximates, which are glucosylated by UGT74B1 to desulfo-glucosinolates, and the sulfation concludes the synthesis of primary glucosinolates (aglycones); and (iii) secondary side-chain modifications by oxidation, elimination, akylation or esterification, give rise to the formation of the diverse glucosinolates found in nature (Grubb and Abel, 2006).

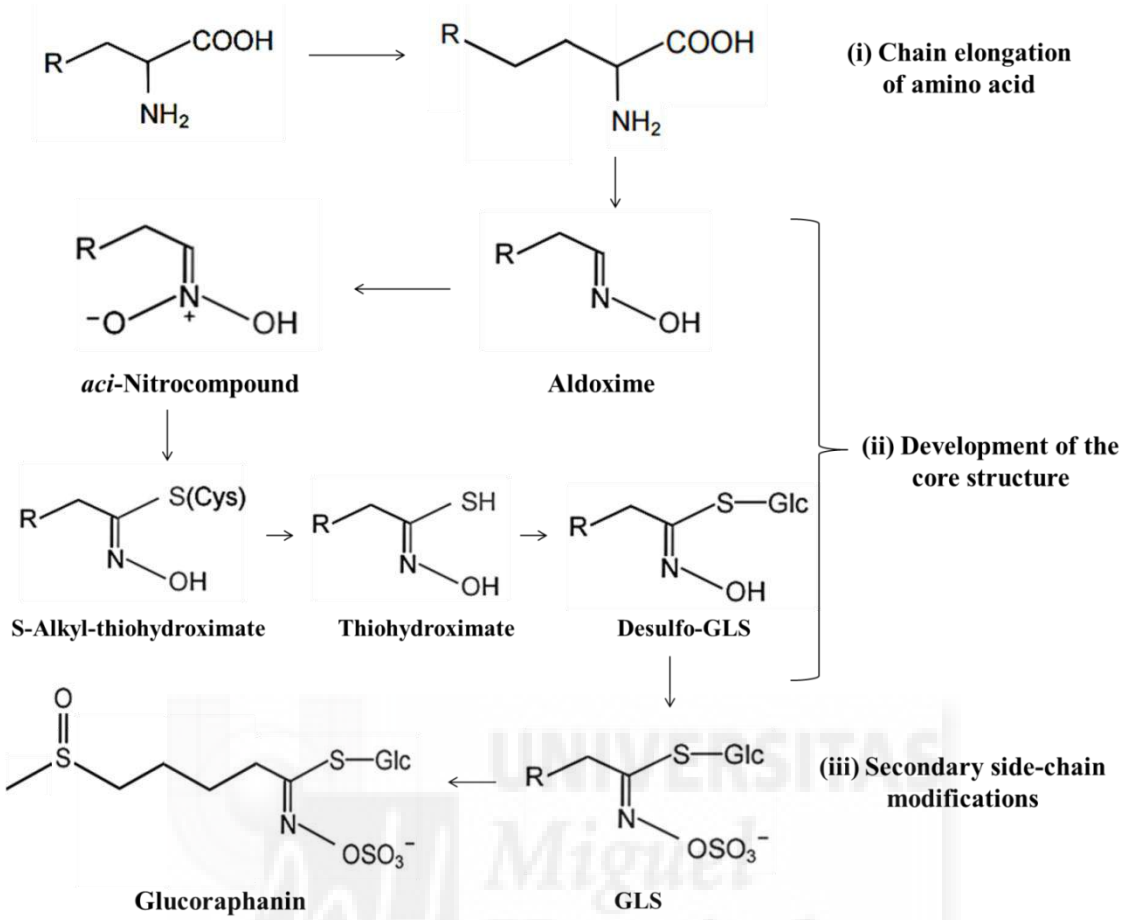

Figure 1.3. *Summary of stages of the biosynthesis of glucosinolates (Grubb and Abel, 2006).*

GLS are hydrolyzed to the biologically active ITC and indoles among other compounds, which can include oxozolidine-2-thiones, nitriles, epithionitriles, and thiocyanates. This hydrolysis takes place in presence of the enzyme myrosinase (thioglucoside glycohydrolase, EC:3.2.1.147), when there is a tissue disrupted by herbivory, insect or pathogen attack, crushing or chewing, since GLS are stored in vacuoles separated from myrosinase cells (Borgen *et al.*, 2010; Grubb and Abel, 2006). Also the action of the gut microflora upon human ingestion has myrosinase-like activity (Angelino and Jeffery, 2014).

The chemical nature of the hydrolysis products depends mainly on the structure of the GLS side chain, the plant species, the presence of epithiospecifier proteins (ESP), thiocyanate-formein proteins (TFP), and nitrile-specifier proteins (NSP), as well as the reaction conditions (pH, presence of Fe^{2+}, etc.) (Figure 1.4). The function of these compounds, other than ITC and indoles, is largely unknown, and consequently, the biological role of specifier proteins (ESP) has remained unclear (Burow and Wittstock, 2009). Intact GLS have not shown health promotion activity; thus, the bioavailability of ITC *in vivo* is dependent not only on ingestion of GLS, but also on their conversion rate prior to passage across the gut wall (Angelino and Jeffery, 2014).

GLS and their hydrolysis products play a role as mediators in plant-insect interactions. They can serve as poison or deterrent in the plant defense system against generalist insects, herbivores and certain microbial pathogens and also as attractants to specialist insects feeding on crucifers (Hopkins *et al.*, 2009). An increase of secondary metabolites (GLS) has been recorded in the damaged leaves (local) and in the adjacent leaves and stems (systemic) for several days after infection (Abdel-Farid *et al.*, 2010). Most of the degradation products of GLS are responsible for the characteristic taste and smell of cruciferous vegetables (Tang *et al.*, 2013). However, the presence of degradation products is not always beneficial, since the product hydrolysis of the GLS progoitrin, present in high concentrations in cabbages and turnips, the ITC goitrin, could produce goitrogenic effect in animals fed with high amounts of rapeseed meal (EFSA, 2008). The exact mechanisms underlying these results are not entirely elucidated.

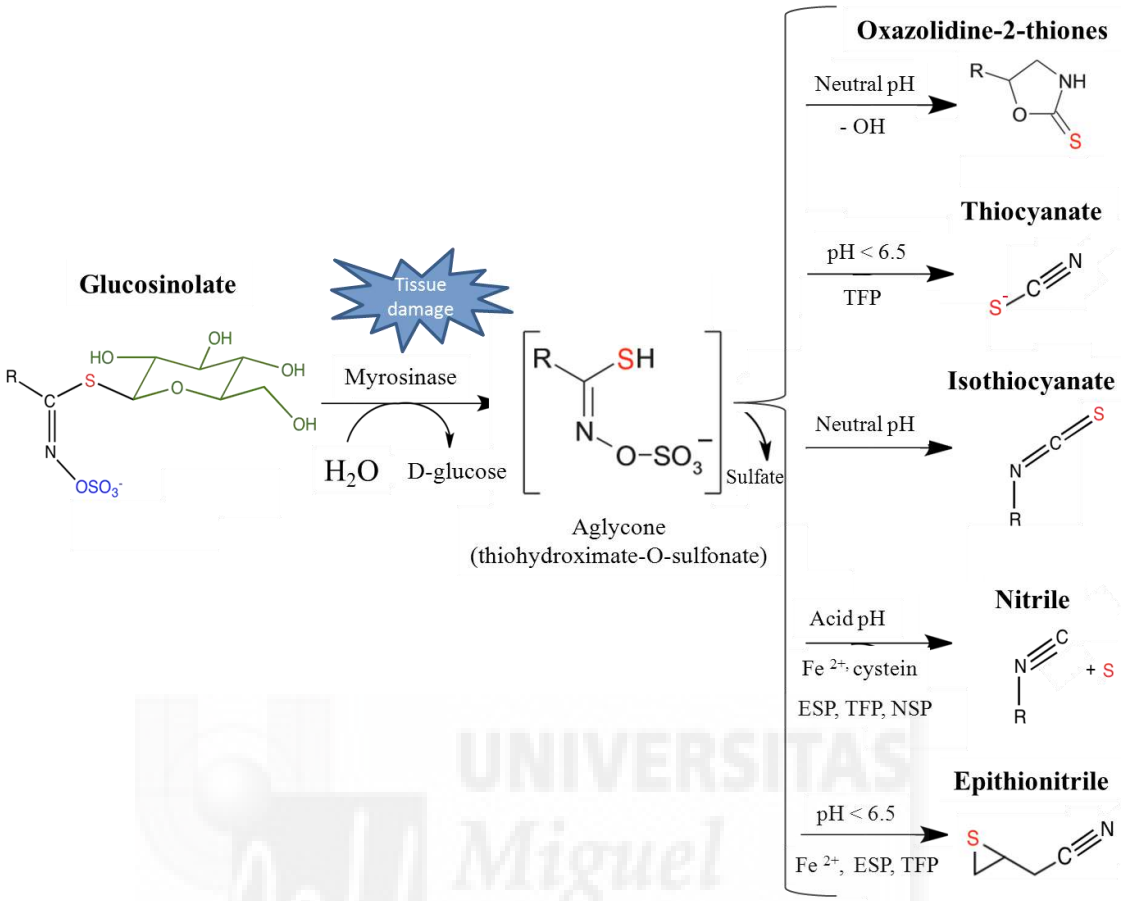

Figure 1.4. *Glucosinolate hydrolysis by myrosinase and released products (Borgen et al., 2010; Burow and Wittstock, 2009).*

On the other hand, increasing epidemiological evidence have shown that high intake, from 3 to 5 times per week, of cruciferous vegetables rich in GLS (i.e. broccoli), such as glucoraphanin, glucoiberin and glucobrassicin, is associated with a decreased cancer risk in humans (more details in Section 5) (Higdon *et al.*, 2007; Jeffery and Keck, 2008; Wagner *et al.*, 2013). Rapid development of molecular and genetic tools in combination with the availability of new data on the model plant *Arabidopsis thaliana* has greatly enhanced the gain of knowledge in recent years.

Further research work is needed to enrich plants in GLS to improve pest resistance and value of cruciferous plants for food and health.

2.2.2. Factors influencing the glucosinolates content

Comparative studies of GLS profiles indicate significant differences among species, varieties, developmental states, environmental (biotic or abiotic) factors, growth conditions, storage, and processing methods (Bjorkman *et al.*, 2011; Jahangir *et al.*, 2009; Vallejo *et al.*, 2002). The amount of GLS in plant tissues and organs has been shown to be highly variable, being seeds the part of the plant with the highest content of these compounds, followed by sprouts, and inflorescences, leaves and roots from the adult plants. This amount of GLS may range from 1 % of dry weight up to 10 % in the seeds of some species (Fahey *et al.*, 2001). Pre-harvest and/or post-harvest conditions are also known to affect bioactive compounds, since plants produce signalling molecules (e.g. salicylic acid, jasmonic acid etc.) after wounding and/or pathogen attack and following stress, that cause a direct or indirect activation of metabolomics pathways (Ren and Dai, 2012). Besides, the exposure of *Brassicaceae* plants to exogenous treatments with phytohormones, such as SA, JA and MeJA, resulted in an increase in these secondary phytochemicals (Ku *et al.*, 2014; Ramakrishna and Ravishankar, 2011). Fertilization with sulphur and nitrogen supply, as well as other exogenous factors such as drought, UV light, temperature, CO_2 and NaCl, also influences GLS concentrations (Martínez-Ballesta *et al.*, 2013; Ramakrishna and Ravishankar, 2011). Different studies indicate that indole GLS are mainly altered by environmental changes, whereas the aliphatic GLS appear to be primarily genetically and not environmentally controlled (Mikkelsen *et al.*, 2003). In

order to prevent the gradually decrease of bioactive compounds during postharvest handling procedures, storage and transport, crucifers should be maintained at refrigeration temperatures (4 – 8 °C) and should be consumed as soon as possible upon harvest (Jones *et al.*, 2006; Song and Thornalley, 2007; Vallejo *et al.*, 2003a). Moreover, the effect of different cooking methods on the GLS content in *Brassicaceae* foods have been studied, being hard boiling the treatment which showed significant losses of GLS by leaching into cooking water, while cooking by steaming or stir-frying did not produce this significant loss. In general, the steaming led to the lowest loss of total GLS (Kapusta-Duch *et al.*, 2016; Vallejo *et al.*, 2002).

2.3. Phenolic compounds: contents and functions

More than 8000 compounds divided into 12 subclasses belong to this group of natural compounds, characterized by having at least one aromatic ring with one or more hydroxyl groups attached and produced via shikimic acid pathway. The aromatic amino acid phenylalanine act as a precursor for their biosynthesis and the enzyme involved is known as phenylalanine ammonialyase (PAL) (Winkel-Shirley, 2001). Phenolics range from simple, low molecular-weight, single aromatic-ringed compounds to large and complex tannins and derived polyphenols. The number and arrangement of their carbon atoms are classified in flavonoids (flavonols, flavones, flavan-3-ols, anthocyanidins, flavanones, isoflavones and others) and non-flavonoids (phenolic acids, hydroxycinnamates, stilbenes and others), and are commonly found conjugated to sugars and organic acids (Cartea *et al.*, 2011; Del Rio *et al.*, 2013). These compounds perform a variety of functions in the plant, generally centered on responses to pathogen attack and UV protection, attracting insects for pollination and

seed dispersion, as well as contributing towards the color and sensory characteristics of vegetables (Crozier *et al.*, 2007).

Brassicaceae foods are generally rich in polyphenols, although the profile and content of those compounds in the plant may vary depending on climatic conditions and harvest season (Gorinstein *et al.*, 2009; Vallejo *et al.*, 2003b), as well as genetics, since total phenolic contents ranged from 15.3 $mg \cdot 100g^{-1}$ fresh weight in white cabbage, 27,8 $mg \cdot 100g^{-1}$ in cauliflower, 119 $mg \cdot 100g^{-1}$ in Chinese cabbage, to 337 $mg \cdot 100g^{-1}$ in broccoli heads; and within plant organs and plant stage, since total phenolics in broccoli could vary from 34.5 to 337.0 $g \cdot 100g^{-1}$ F.W. (Dominguez-Perles *et al.*, 2011b; Podsędek, 2007). Generally, these vegetables contain higher amounts of hydroxycinnamic acids, mainly sinapic acid and chlorogenic acid derivatives, than flavonols, specifically quercetin, kaempferol and isorhamnetin glycosides (Francisco *et al.*, 2011). Phenolic acids are predominant in seeds and sprouts, mainly sinapic acid derivatives, having these organs higher amounts of total phenolics than the adult plant (Pajak *et al.*, 2014; Sousa *et al.*, 2007). On the other hand, two-month leaves, three-month leaves, consumed organs and by-products samples of several *Brassica* vegetables were composed mostly by kaempferol and sinapic acid derivatives (Soengas *et al.*, 2012).

Diets rich in foods containing phenolic compounds, such as cruciferous foods, have been reported to possess many useful properties for prevention of non-transmissible chronic diseases and promotion of health, including anti-inflammatory, enzyme inhibition, antimicrobial, antiallergic, vascular and cytotoxic antitumor activity, but the most important action of phenolics is their contribution to the antioxidant protection in the human body (Crozier *et al.*, 2009; Finley *et al.*, 2011).

Phenolic compounds can play an important role to scavenge free radicals and up-regulate certain metal chelation reactions. Various reactive oxygen species (ROS), such as singlet oxygen, peroxynitrite and hydrogen peroxide, must be continually removed from cells to maintain healthy metabolic function. Diminishing the concentrations of ROS could have several benefits possibly associated with ion transport systems and so may affect redox signaling (Landete, 2012). Despite the beneficial effects of phenolic compounds it must be taken into account that only a small percentage of dietary phenolic compounds reaches the tissues, and very little of this absorbed bioactives retains the structure found in the plant. Plasma concentrations reached after polyphenol consumption varies highly according to the nature of the polyphenol and the food source, in the range of 0.3–0.75 μmol/L after consumption of 80–100 mg quercetin equivalents (Manach *et al.*, 2004). Moreover, phenols are modified during the first part of their metabolism and the most important modifications involve conjugation to produce glucuronide or sulphate conjugates by intestinal and/or hepatic detoxification enzymes. However, the major part of these molecules is metabolized by the colonic microflora rendering the so called microbial metabolites. Those microbial metabolites can be detected in the blood and urine after ingestion, but only a very small fraction of non-conjugated phenolics in their original form can be found. This implies that these microbial metabolites rather than the native phenolics are responsible for the beneficial biological effects in the body (Crozier *et al.*, 2009).

2.3.1. Phenolic acids

Phenolic acids in plants consist of two subgroups, the hydroxybenzoic and hydroxycinnamic acids. Hydroxybenzoic acids include gallic, p-hydroxybenzoic, protocatechuic, vanillic and syringic acids, which in common have the C6–C1 structure, but they are not cited in *Brassicaceae* foods. Hydroxycinnamic acids are the most common in cruciferous foods, and they are aromatic compounds with a three-carbon side chain (C6–C3), with caffeic, ferulic, *p*-coumaric and sinapic acids (Figure 1.5.), practically always found in conjugation with sugars or other hydroxycinnamic acids (Ferreres *et al.*, 2009). The higher antioxidant activity of the hydroxycinnamic acid could be due to the CH=CH–COOH group, which ensures greater H-donating ability and radical stabilisation than the –COOH group in the hydroxybenzoic acids (Terry, 2013).

Significant levels of hydroxycinnamic acids (hydroxycinnamoyl gentiobiosides and hydroxycinnamoylquinic acids) have been reported in *B. oleracea* crops, like kale, cabbage, broccoli, and cauliflower (Soengas *et al.*, 2012). However, the predominant phenolic acids in seeds and sprouts in these vegetables were sinapic acid derivatives, such as sinapic acid esters (1-sinapoylglucose, sinapoylmalate and 6,3'-disinapoylsucrose) (Lim, 2014; Takaya *et al.*, 2003). The most common glycoside of sinapic acid in *Brassicaceae* species is sinapoyl glucose (1-*O*-*β*-*D*-glucopyranosyl sinapate).

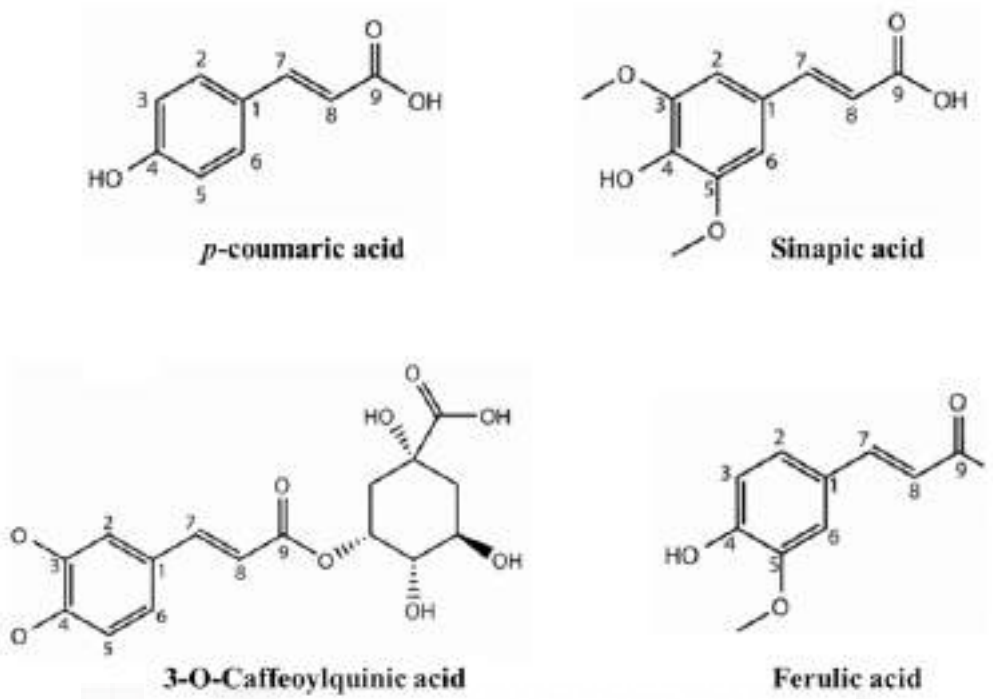

Figure 1.5. *Hydroxycinnamic acids commonly found in Brassicaceae foods.*

The profile of hydroxycinnamic acids varies with genetics and the developmental stage of the plant, showing seeds the higher amount of sinapic acid derivatives compared to leaves and inflorescences from *Brassica oleraceae*, since these compounds are precursors of lignin biosynthesis, important in the first plant stages to rigidifying cell walls and rendering them impermeable to water (Francisco *et al.*, 2015). The antioxidant scavenger properties of *Brassicaceae* extracts rich in phenolic acids have also been proved *in vivo*. Sinapic acid has shown to contribute to the cellular defense avoiding oxidation, through scavenge of $^{\cdot}OH$, $O^{2\cdot-}$, $^{\cdot}OOH$ and $^{\cdot}NO$ radicals and inflammation, through inhibition of the NF-κB, and consequently, the expression of proinflammatory mediators such as inducible nitric oxide synthase, cyclooxygenase-2, tumor necrosis factor-α, and interleukin-1β (Chen, 2016; Yun *et al.*, 2008).

2.3.2. Flavonoids

Flavonoids are low molecular weight compounds, consisting of fifteen carbon atoms with two aromatic rings A and B, connected by a three-carbon bridge (C6–C3–C6) configuration usually in the form of a heterocyclic ring, C. Over 5000 different flavonoids have been described to date and they are classified into at least 10 chemical groups, depending on the substitution pattern to rings A and B give rise to the different compounds, among them, flavones, flavonols, flavanols, flavanones, anthocyanins and isoflavones are particularly common in the human diet (Kumar and Pandey, 2013). These compounds have interesting biological activities connected to cancer-prevention, and cardiovascular system protection, including inhibition of oxidative damage (Del Rio *et al.*, 2013). In *Brassicaceae* foods, flavonoids reached higher contents at flowering (19.02 $\mu mol \cdot g^{-1}$ D.W.) rather than in leaves (9.28 $\mu mol \cdot g^{-1}$ D.W.). Seeds usually show very low quantities of flavonoids (Francisco *et al.*, 2015).

2.3.2.1. Flavonols

Flavonols in *Brassicaceae* vegetables are mainly represented by *O*-glycosides of quercetin, kaempferol and isorhamnetin (Figure 1.6.), conjugated with different organic acids (Ferreres *et al.*, 2004). Conjugation occurs most frequently at the 3 position of the C-ring, but substitutions can also occur at the 5, 7, 4´, 3´ and 5´ positions (Francisco *et al.*, 2009). The occurrence, position, structure, and total number of sugar moieties in flavonols (glycosides) play an important role in antioxidant activity, being aglycones more potent antioxidants than their corresponding glycosides. Besides, bioavailability is sometimes enhanced by a

glucose moiety (Kumar and Pandey, 2013). To date, more than 20 flavonols have been described in crucifers such as kale, white cabbage, cauliflower, and broccoli as well as in *B. napus* and *B. rapa* leaves. Among them, the main flavonols were identified as kaempferol and quercetin 3-*O*-sophoroside-7-*O*-glucoside and its combinations with different hydroxycinnamic acids, mainly kaempferol and quercetin 3-O-(caffeoyl/sinapoyl)-sophoroside-7-*O*-glucoside.

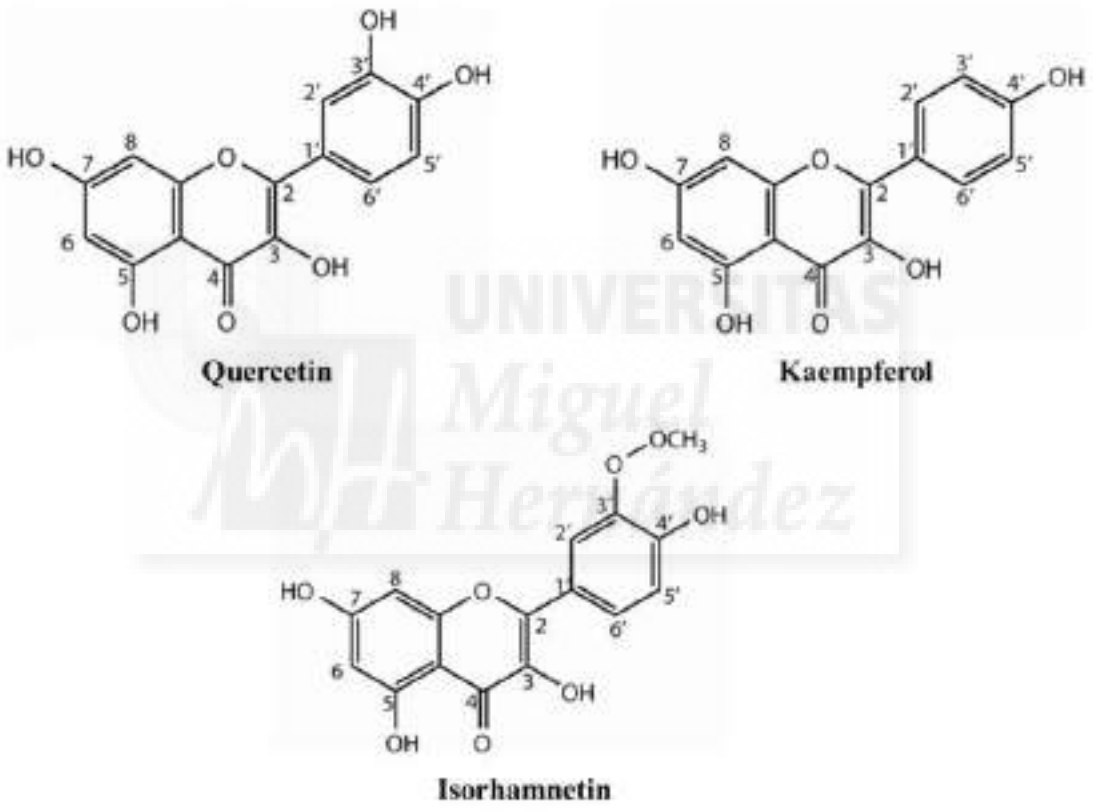

Figure 1.6. *Flavonoid aglycones commonly found in Brassicaceae foods.*

In the *B. rapa* group, in addition to quercetin and kaempferol derivates, it can be found derivatives of the flavonol isorhamnetin (Romani *et al.*, 2006). The glycosylated flavonols, such as 3-sophoroside-7-glucosides of kaempferol, are increasingly attributed beneficial health effects such as a reduced risk of age-related

chronic diseases, like cancers and cardiovascular diseases (Park *et al.*, 2009). In addition, quercetin has received considerable attention as a major representative of the flavonol subclass, found at high concentration in broccoli. This flavonol has displayed the ability to prevent the oxidation of LDL by scavenging free radicals and chelating transition metal ions. As a result, quercetin may aid in the prevention of certain diseases, such as cancer, atherosclerosis and chronic inflammation by retarding oxidative degradation and inducing enzymes that detoxify carcinogens (Ackland *et al.*, 2005; Batra and Sharma, 2013). Furthermore, isorhamnetin isolated from mustard leaf showed a strong activity in reducing serum levels of glucose in Diabetes Mellitus through an antioxidant activity tests (Yokozawa *et al.*, 2002).

2.3.2.2. Anthocyanins

The presence of these compounds causes the red pigmentation of some vegetables, such as red cabbage, red radish, purple cauliflower and purple broccoli. The major anthocyanins identified in these crops are cyanidin derivatives (Figure 1.7.), consisting of a cyanidin as aglycon, glycosylated mainly with glucose and/or sophorose (diglucoside), which are acylated with various aromatic and aliphatic acids. The chromatographic profile of anthocyanins of red crucifers, such as red cabbage and red radish, are one of the most complicated, because of the high number of different anthocyanins, which are highly conjugated cyanidin glycosides with several aromatic and aliphatic acids.

In red cabbage and broccoli sprouts the major anthocyanins were identified as cyanidin 3-*O*-(sinapoyl)(feruloyl) diglucoside-5-*O*-glucoside and cyanidin 3-*O*-(sinapoyl)(sinapoyl) diglucoside-5-*O*-glucoside (Moreno *et al.*, 2010). Anthocyanins

have been found to have a high antioxidant power and antigenotoxic properties (Posmyk *et al.*, 2009). These authors suggest that a mixture of anthocyanins not only prevents and limits but also repairs the cytological injury caused by Cu^{2+} stress on lymphocytes.

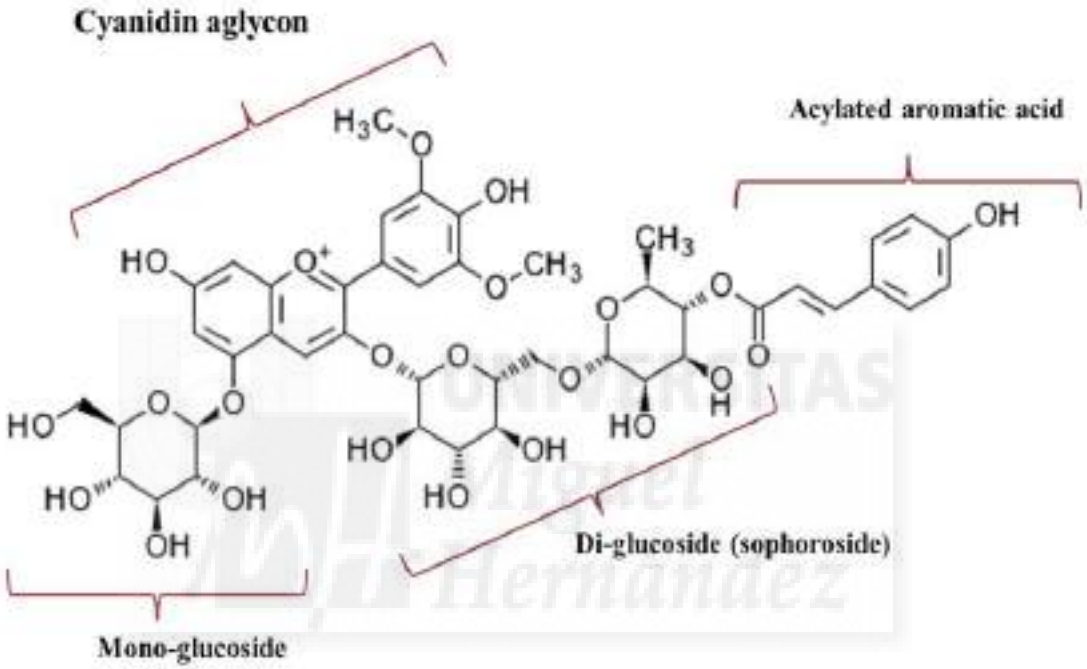

Figure 1.7. *Chemical structure type of acylated anthocyanins in Brassicaceae foods.*

The absorption, gastrointestinal transit and plasma elimination are dependent on anthocyanin structure. Absorption efficiencies of acylated cyanidins were lower than those for non-acylated anthocyanins. Also the acylated anthocyanins exhibited a shorter half-life for gastrointestinal absorption than the non-acylated ones. Fractional elimination of non-acylated was slower than for acylated anthocyanins (Novotny *et al.*, 2012).

3. ENRICHING FOODS IN HEALTHY BIOACTIVES: ELICITATION

Elicitors are physical or chemical stimuli which induce physiological changes in the plant. Plants respond to these stressors by activating an array of mechanisms, similar to the defense responses to pathogen infections or environmental stimuli, affecting the plant metabolism and enhancing the synthesis of phytochemicals. The first biotic elicitors were described in the early 1970s (Keen, 1975). Since then, numerous publications have accumulated evidence for pathogen-derived compounds that induce defense responses in intact plants (Doughty *et al.*, 1995; Pérez-Balibrea *et al.*, 2011) or plant cell cultures (Smetanska, 2008). The use of elicitors as a tool to enhance the phytochemical content in plants, applied alone or in combinations at selected time points of the vegetable growth, should not be confused with those administered during the plant production cycle or pre-harvest, such as conventional fertilization.

3.1. Classes of elicitors

Elicitors could be classified as biotic or abiotic compounds, also plant hormones may be considered as elicitors (Table 1.1.) (Angelova *et al.*, 2006; Poulev *et al.*, 2003; Radman *et al.*, 2003). Biotic elicitors, such as chitosan and alginate, have biological origin, often originated as a result of fungi, bacteria, virus or herbivore infections (exogenous elicitors), and in some cases are released from the attacked plant by the action of enzymes of the pathogen (endogenous elicitors) (Ebel and Cosio, 1994).

Biotic Elicitors
Lipopolysaccharides Polysaccharides (e.g. pectin, chitosan and alginate) Oligosaccharides (galacturonides, guluronate and mannan) Proteins (e.g. cellulose and glycoproteins) Complex composition (e.g. fungal spores, mycelia cell walls and pathogen toxins).
Abiotic Elicitors
Chemical: acetic acid, benzothiadiazole ethanol, ethene, inorganic salts (e.g. NaCl, $CuSO_4$ and $CaCl_2$) and metal ions (e.g. Co^{2+}, Fe^{2+}, Al^{3+} and Mn^{2+}). **Physical**: chilling, CO_2, O_2, drought, temperature shock, high pressure, high or low osmolarity, UV radiation, wounding.
Plant Hormones
Jasmonic acid (JA), methyl jasmonate (MeJA), methyl salicylate (MeSA), salicylic acid (SA), ethylene, cytokinin, gibberellin GA_3.

Table 1.1. *Elicitor classification based on their origin (Angelova et al., 2006; Poulev et al., 2003; Radman et al., 2003).*

Salicylic acid (SA) and jasmonic acid (JA) and its methyl ester, methyl jasmonate (MeJA), are widely known to elicit a wide range of compounds by inducing the expression of plant genes for various biosynthetic pathways. These small signaling molecules also defined as “hormones”, are induced in the cells in response to wounding or pathogen attack in plants, and they can induce cellular responses at low concentrations distant from their site of synthesis.

Abiotic elicitors are produced by factors responsible for environmental stress. These factors can be of chemical (inorganic salts, metal ions and others which disturb the membrane integrity) and physical origin (UV radiation, wounding, saline stress, ozone etc.) (Radman *et al.*, 2003).

Apart from the classification of elicitors according to their nature, they can also be classified upon their interaction with the host plant, as "general elicitors", such as carbohydrates, cell wall proteins, oligosaccharides etc., which induce non-specific mechanisms for the induction of defense response in different plant cultures, and "specific elicitors" from fungal, bacterial, viral or plant origin, which affect only a specific host cultivar since the presence of its corresponding resistance gene in the host plant is directly associated with specific gene pathogen (Thakur and Sohal, 2013). The defense mechanisms triggered by general elicitors have been studied as remarkable similar than the innate immunity of animals, and it is tempting to speculate that the recognition of general elicitors subsequently leads to plant innate immunity (Nurnberger and Brunner, 2002).

3.2. Mechanisms of action

In plant defense systems cells have acquired the capability to respond to pathogens and environmental stresses by building up a defense response. Plant response is determined by several factors, mainly depending on their genetic characteristics and physiological state. In the majority of cases, plant resistance to diseases is known to be genetically controlled by plant resistance (R) genes and the complementary pathogen avirulence (Avr) genes (gene-for-gene interaction concept), that codifies a product recognisable by the plant (Surico, 2013). However, triggering

resistance is not always due to specific Avr products but, instead, proceeds from the action of general elicitors, able to activate defenses in different cultivars of one or many species (Thakur and Sohal, 2013).

First step in the response of plant against elicitors is the stimulus perception by receptors localized in plasma membranes of the plant cell, like protein kinases, which represent one of the most important players in pathogen perception for a number of fungal elicitors, or could be localized within the cell to initiate signaling processes that activate plant defenses, as for certain bacterial elicitors (Ebel and Mithöfer, 1998). The elicitor signal transduction is an important area of investigation. Plants respond to elicitors by activating an array of defense mechanisms on the surface of the plasma membrane (Figure 1.8.): induction of pathogenesis-related proteins (PR) and enzymes of oxidative stress protection; hypertensive responses characterized by rapid cell death in the immediate vicinity of the point of exposure to the pathogen; production of reactive oxygen species (ROS) and reactive nitrogen species (RNS); activation of defense-related genes; changes in the potential of plasma membrane cell and enhanced ion fluxes (Cl^- and K^+ efflux and Ca^{2+} influx), responsible for a transient cytoplasm acidification that can act as a signal for the production of secondary metabolites; rapid changes in protein phosphorylation and dephosphorylation (changing enzyme activity, cellular location or association with other proteins), and lipid oxidation; structural defensive barriers, such as reinforcement and lignification deposition in cell wall; and, the activation and the *de novo* biosynthesis of transcription factors, which directly regulate the expression of genes involved in the production of secondary metabolites (Ferrari, 2010; Zhao *et al.*, 2005).

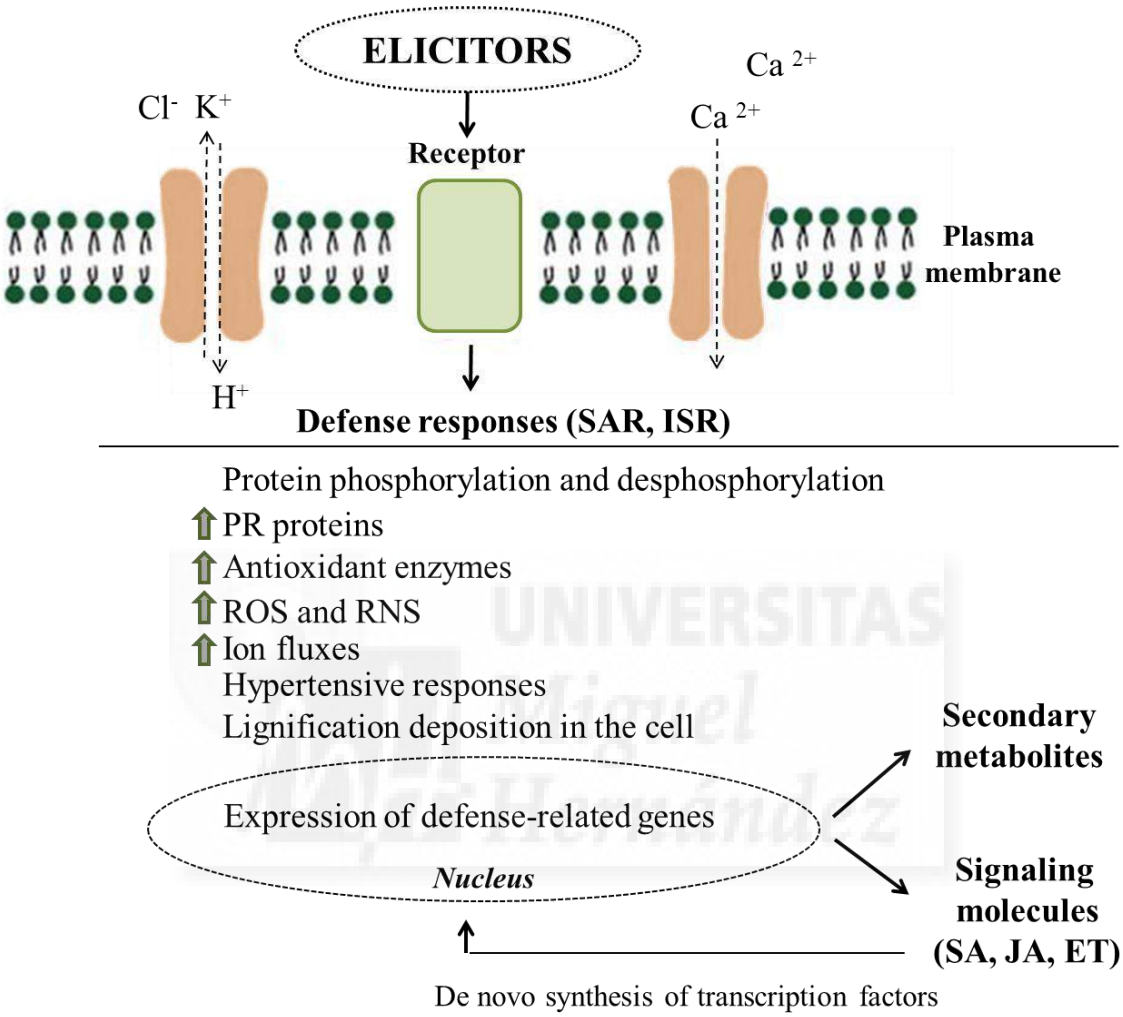

Figure 1.8. *General mechanism after elicitor perception (Ferrari, 2010; Zhao et al., 2005).*

3.3. Elicitors application

Elicitation could be used as preharvest or postharvest treatment. Among preharvest treatments, seed priming consists on soaking seeds in a water solution with the elicitor, inducing the cellular plant defense responses (SAR and ISR). There is evidence that seeds of parsley treated with jasmonates showed cells with induced early oxidative burst and various phenylpropanoid defence responses (Conrath *et al.*, 2015; Conrath *et al.*, 2002). Elicitors can be also applied as a gas in an enclosed environment (such as MeJA), on a liquid form to a hydroponic solution, or by exogenous sprays (Wasternack, 2014). The combination of seeds priming and exogenous application of elicitors, could be highly effective to enhance bioactive compounds production in the plant, as primed cells react more quickly and efficiently to subsequent elicitor treatment or pathogen attack (Conrath, 2011).

In postharvest practices, specific elicitor treatments have been used to enhance the phytochemical content and food quality, such as application of temperature shocks, UV radiation or gas combinations before commercialization (Terry and Joyce, 2004).

Elicitor nature, dosage and time of treatment, and combined application of compounds strongly affects the intensity of the plant response, stimulating different classes of secondary metabolites in different concentration levels, being more dependent on plant genetics (species and cultivars) and organ treated than on the elicitor nature (Ku *et al.*, 2014).

3.4. Effects on the content of bioactive compounds

Much effort has been put into identifying transcription factors and revealing the signal transduction steps underlying elicitor activation of plant secondary metabolism and also into the manipulation of regulatory and biosynthetic genes to enhance the production of target secondary metabolites. The molecular mechanisms by which elicitors regulate the expression of these transcription factors have not been yet determined, since the metabolic pathway that is affected is not linear, but through an extensive network of cellular responses. Cross-talk of multiple signaling pathways is very common and important for accumulation of plant secondary metabolites and other defense responses, but how different signaling pathways are integrated into the single cellular process (such as phytoalexin biosynthesis) is still not very clear. The regulation of cellular processes commonly occurs at several different levels including transcription, RNA processing, and translation, as well as by post-translational modification such as protein phosphorylation. However, the majority of studies show that transcriptional modulation of related genes is a common response to pathogens or elicitor signals and, studies on DNA sequences demonstrated that several elicitor-response elements in these genes are involved in the biosynthesis of secondary metabolites (Zhao *et al.*, 2005).

Elicitor activation of defense genes requires action of transcription factors, which can recruit the general transcription machinery to stimulate gene expression. As a consequence, in plant tissues is observed the production of antioxidant molecules, compounds of technological interest in healthy foods, such as phenolic compounds, which are accumulated in plant cells after elicitor treatment due to the activation of the phenylpropanoid pathway through specific transcription factors,

such as PAL and CHS activities (Zhao *et al.*, 2005). GLS also can be increased by treatments with elicitors, however, the underlying regulatory mechanisms responsible for this alteration are largely unknown, different resistance responses of the plant have become a target of investigation as responsible of the *de novo* biosynthesis of these compounds, such as the overexpression of the CYP79 gene family, implicated in the biosynthesis of the GLS structure (Mikkelsen *et al.*, 2003) and the study of gene expression quantitative trait locus (QTLs), which are sections of DNA related to a phenotype, where MYB28 and MYB29 expression activated GLS biosynthetic genes (Pino Del Carpio *et al.*, 2014).

Examples of induction of both phenolic compounds and GLS around 40 - 50 % is reported in radish sprouts treated with NaCl (100 mM) (Yuan *et al.*, 2010) and broccoli sprouts treated with sucrose and mannitol (176 mM) (Guo *et al.*, 2011a) after sowing seeds. Other bioactive compounds such as carotenoids, betalains, vitamins and folates, as well as nutrients involved in the primary metabolism of the plant (proteins, carbohydrates or lipids) are also influenced by elicitor applications, affecting plant growth and productivity (Gómez *et al.*, 2010). It is expected that a better understanding of the signal transduction pathways, linking plant cell stimulation and biosynthesis of natural compounds will help to develop new strategies to induce the production of target compounds, by either activation or suppression of certain metabolic pathways (Ferrari, 2010).

4. SHELF LIFE OF EDIBLE SPROUTS

In recent years, the production of sprouted seeds has increased as a result of increased consumer demand for this type of fresh products. Postharvest practices include the management and control of variables such as temperature, relative humidity, light and time of storage, to maximize their organoleptic, functional and microbial quality, avoiding physiological and physical disorders, such as freeze injury, softening or discoloration and the loss of health-promoting phytochemicals. Light conditions have shown to produce quality deterioration and weight loss in sprouts, being the dark storage a better method of preservation for extending quality and shelf life (Xiao *et al.*, 2014). Rapid cooling is essential to achieve the full storage potential of sprouts. According to the "Recommendations for Maintaining Postharvest Quality" of University of California (UC Davis, USA) cruciferous adult plants should be stored at 0 °C to optimize their shelf-life (21-28 days), since broccoli heads stored at 5 °C or 10 °C can have a storage life of 14 days or 5 days, respectively (Cantwell and Suslow, 2015). Regarding sprouts, due to the high respiration rates and perishable nature, distribution and storage should be carried out also at 0 °C (5 to 9 days) (Pérez-Balibrea *et al.*, 2015). However, the common temperatures of refrigeration found during commercialization and storage of fresh products at home are definitely higher (4 – 10 °C).

4.1. Microbial content

It is particularly important to prevent microbial contamination during the production of seeds because of the potential for pathogens to grow during the sprouting process. High quality seed, proper germination process and postharvest refrigeration are the primary controls, but washing sprouts before consumption, as fresh produce, in chlorinated or ozonized water (or other effective and approved disinfectant) will help control the possible decay and spoilage. Total plate counts as high as 10^8 – 10^9 CFU/g are frequently reported in sprouts due to the intrinsic microflora of the seeds (Gabriel *et al.*, 2007; Martínez-Villaluenga *et al.*, 2008; Soylemez *et al.*, 2001).

Seeds have been linked to outbreaks of food poisoning in humans. In fact, after the outbreak caused by *E. coli* in May 2011 in the EU, consumption of sprouts was identified as the most likely source of this food crisis. The European Food Safety Agency (EFSA) issued a scientific opinion on the risk caused by toxin-producing *E. coli* Shiga and other pathogenic bacteria in seeds and sprouted seeds in October 2011. In that opinion, EFSA said that the most likely initial source of the crisis aforementioned food is contamination of seeds with pathogenic bacteria and that these bacteria in the seeds can multiply during germination and be a risk to public health due to the high humidity and the favorable temperatures reached during the same process. In order to avoid or reduce potential risks of contamination of germinated seeds, thus ensuring protection of public health in the EU, it is necessary to observe the opinion of EFSA adopted in March 2013 Implementing Regulation (EU) No 208/2013 of the Commission, the 11th March 2013 for requirements on the traceability of sprouts and seeds intended for sprouts production. EFSA concluded

that the contamination of dry seeds with bacterial pathogens was the most likely initial source of the sprout-associated outbreak (EFSA, 2011). The World Health Organization (WHO, 2015b) stated that when food supplies are insecure, people tend to shift to less healthy diets and consume more "unsafe foods". Therefore, contamination with pathogenic bacteria must be minimized by the application of Good Agricultural Practices (GAP), Good Manufacturing Practices (GMP), Good Hygiene Practices (GHP), and Hazard Analysis Critical Control Point (HACCP) principles at all steps of the food production chain (EFSA, 2011). These challenges put greater responsibility on food producers and research to ensure food safety and quality.

4.2. Influence on bioactive compounds

The specific role of bioactive compounds in human metabolism has encouraged food technologists and researchers to develop new processes and technologies for preserving these health-promoting compounds during the shelf life of fruits and vegetables. *Brassicaceae* sprouts continue their metabolic activities upon harvest and during shelf-life, and their composition would change according to storage factors such as temperature, water availability and time. Storage temperature may be considered as the most important factor, which directly affects the physiology of vegetables and consequently the cellular constituents (Jones *et al.*, 2006). Even though the contents of bioactive compounds of cruciferous sprouts may be high at the time of harvest, decreases of their concentrations during storage are found, particularly if produce is not cooled effectively. Besides, there is not accurate

data that document the stability of these phytochemicals during storage. Variations in phytochemical contents responsible of the antioxidant capacity of the plant, such as phenolics, GLS and vitamins, may be due to the constant changes in the plant physiology and metabolism during storage, as a result of oxidative stress, which may include structural changes in synthesis or antioxidant compounds (Xiao *et al.*, 2014). Also bioactive compounds could serve as nutrients for specific microbial population present in the seeds and sprouts, being subjected to biotransformation and, therefore, giving rise to a decrease of phytochemical contents in the plant (Vale *et al.*, 2015).

5. CONNECTING CRUCIFERS AND HEALTH

Vegetables belonging to cruciferous family have long been studied as rich source of nutrients and bioactive compounds with attributable beneficial effects on human health, like antibacterial, antifungal, antitumor, anti-mutagenic, anti-inflammatory, neuroprotective and antioxidative activities, which have been demonstrated through many *in vivo* and *in vitro* studies, as well as in epidemiological evidences. The promotion of consumption of these plant foods is highly desirable in recent times in order to manage chronic diseases and enhance wellbeing and health through life.

5.1. Absorption and metabolism of glucosinolates/isothiocyanates (GLS/ITC)

The bioavailability of GLS/ITC is measured by the mercapturic acid pathway which acts as an indicator to measure the bioavailability of the breakdown products ITCs, which gives rise to N-acetylcysteine conjugates (Angelino and Jeffery, 2014). GLS are hydrolyzed to ITC when come into contact with the enzyme myrosinase from chewing and continue also with the action of the gut microbiota. An initial reaction between the electrophilic central carbon of the –N=C=S group of the ITC and the cysteine sulfhydryl group of glutathione (GSH) can take place spontaneously and is enhanced by glutathione S-transferase (GST), forming a dithiocarbamate GSH conjugate (Figure 1.9.). Then, cleavage of glutamine and glycine by the enzymes γ-glutamyl transpeptidase (GTP) and cysteinylglycinase (CGase) respectively yields L-cysteine conjugates, forming cysteinylglycine- (-CYS-GLY) and cysteine- (-CYS)

metabolites and, finally, the N-acetyl-cysteine (-NAC) conjugate is formed in the kidney.

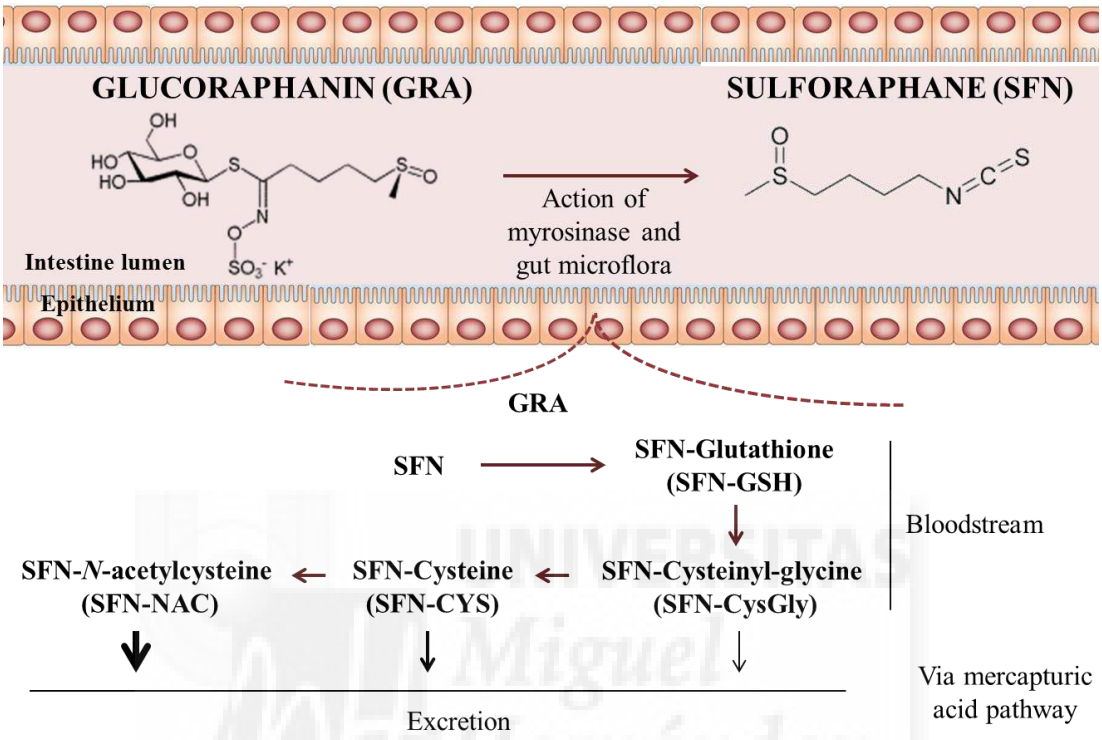

Figure 1.9. *Metabolism of sulforaphane (Angelino and Jeffery, 2014; Dominguez-Perles et al., 2014).*

The metabolites derived from sulforaphane (SFN), the predominant ITC found in broccoli and hydrolyzed from the GLS glucoraphanin (GRA), have been the most studied in terms of bioavailability and health benefits. SFN metabolites have been found in plasma and urine (Atwell *et al.*, 2015; Dominguez-Perles *et al.*, 2014). SFN-NAC has been studied as the major metabolite found in urine, used as a marker of bioavailability, following by SFN-CYS and SFN-GSH. These compounds could be analyzed by distinct HPLC-MS/MS methods up to nanomolar concentrations (Angelino and Jeffery, 2014; Egner *et al.*, 2008).

Clarke *et al*., (2011) observed limited SFN absorption in healthy adults after consuming GRA supplements with inactivated myrosinase, which was 7-fold lower than when subjects consumed equivalents levels of GRA from fresh broccoli sprouts containing the active enzyme. Urinary and plasma SFN metabolites appearance was faster after consuming broccoli sprouts than using broccoli powder lacking myrosinase (Cramer and Jeffery, 2011). ITC are absorbed rapidly, reaching peak concentrations of 0.94 – 2.27 μM in plasma, serum and erythrocytes 1 hour after consumption. The higher levels of ITC detected in plasma and urine have been reported at 2-3 h and 4-5 h, respectively, after consumption of broccoli sprouts, and ITC were mostly excreted within 24 hours in concentrations around 20 – 100 μmol/24 h depending on the amount consumed (Angelino and Jeffery, 2014). Vermeulen *et al*. (2006), observed 11 % higher excretion of SFN metabolites following consumption of raw *versus* cooked broccoli. Therefore, inactivation of the plant myrosinase by high temperature, as occurs during cooking, decreases bioavailability of ITC. Thus, boiling or steaming for more than 3-5 min and blanching prior to freezing will lead to lost activity (Tiwari *et al.*, 2015).

Other interesting work showed in plasma and urine higher levels of total SFN metabolites (3-5 times) in fresh broccoli sprouts consumers, compared to a myrosinase-treated broccoli sprouts extract containing SFN but not GRA; therefore, GRA conversion to SFN is not the only factor influencing SFN absorption, but also other compounds present in broccoli sprouts, such as minerals, phytochemicals and fibre may enhance SFN transport across cell membranes (Atwell *et al.*, 2015). In SFN bioavailability, also the total amount of SFN estimated could derive from the interconversion of erucin, from glucoerucin, to SFN *in vivo* (Clarke *et al.*, 2011).

5.2. Current evidences of biological activities: *in vitro* and *in vivo* studies

In vitro and *in vivo* studies have reported that ITC must be potentially triggers of the Keap1/Nrf2/ARE pathway, which regulates the transcription of many antioxidant genes that preserve cellular homeostasis and detoxification genes that process and eliminate carcinogens and toxins before they can cause damage (Stefanson and Bakovic, 2014). Thereby, stimulating expression of genes that regulate an extensive network of inducible cytoprotective phase II detoxification enzymes, such as glutathione S-transferases (GST), UDP-glucuronosyl transferases, and quinone reductase, that protect cells against reactive oxygen species (ROS), inflammation, and DNA-damaging electrophiles (Baird and Dinkova-Kostova, 2011). Also the chemoprotective activity of ITC may involve inhibition of phase I enzymes, being the cytochromes P450 the most important in mammals, blocking chemically-induced carcinogenesis (Clarke *et al.*, 2008). GST enzymes can metabolize the products of phase I activity through formation of water-soluble conjugates which are excreted in urine. Even though urinary levels of total ITC metabolites may be an excellent biomarker of exposure to ITC in general, cancer preventative potency varies widely for individual ITC actions.

The ITC SFN present in broccoli sprouts is a multi-faceted chemopreventive agent, with the ability to act not only in modulation of enzymes, but also blocking or suppressing carcinogenic stages, by eradication of infection, inhibition of growth promotion, alteration of signaling pathways, cell cycle arrest and apoptosis, and inhibition of recurrence (Myzak and Dashwood, 2006). More recently, SFN

metabolites were reported to inhibit histone deacetylases (HDAC), which remove acetyl groups from proteins, altering gene expression and protein function (Myzak *et al.*, 2007). Therefore, ITC can act to reduce the incidence or progression of various cancers, including colon (Byun *et al.*, 2016), kidney (Hsu *et al.*, 2007), breast (Pawlik *et al.*, 2013) and prostate (Wong *et al.*, 2014) through various molecular targets. Therefore, a diet of 3-5 servings per week of *Brassicaceae* vegetables (40-50 grams) is sufficient to cause a 30 % or 40 % decrease in risk for a number of cancers (Jeffery and Keck, 2008).

Induction of Nrf2-dependent antioxidant defense is reported to inhibit NF-κB activation, a transcription factor that activates expression of multiple genes related to inflammation (Oeckinghaus and Ghosh, 2009). The presence of chronic inflammation contributes to the development of type 2 diabetes, cardiovascular disease, neurodegeneration and cancer. Several bioactive compounds present in crucifers have been found to suppress proliferation of many cancer cell lines and to suppress activation of NF-κB and the associated inflammation, protecting against oxidative stress and inflammatory damage, as in the case of ITC, which are among the most potent phytochemicals activating Nrf2 and antioxidant enzymes (Stefanson and Bakovic, 2014).

Not only SFN, but also erucin, from the precursor GLS glucoerucin, iberin from GLS glucoiberin, sulforaphene from GLS glucoraphenin (which differs from GRA by a double bond) and phenethyl ITC from GLS gluconasturtiin, have been studied because their bioactivity, triggering the transcription factor Nrf2 into de nucleus, where the antioxidant response element (ARE) promoter region activate multiple genes, including both phase II detoxification enzymes and several

antioxidant enzymes, among others, and induce cell cycle arrest and apoptosis (La Marca *et al.*, 2012).

Recent studies have shown that indole-3-carbinol (I3C) from indole GLS (4-hydroxyglucobrassicin, glucobrassicin, 4-methoxyglucobrassicin and neoglucobrassicin), plays important roles in apoptosis and arrest of cell growth in *in vitro* experiments with breast and prostate cancer cells (Wang *et al.*, 2015). The I3C showed a potential benefit in preventing obesity and metabolic disorders, involving multiple mechanisms including decreased adipogenesis and inflammation, along with activated thermogenesis (Choi *et al.*, 2012). It is well known that excess of adipose tissue produces inflammation inducing cytokines, increases oxidative damage and directly alters gene transcription. Research investigating the use of diet-derived chemoprevention compounds may have significant impact on qualifying or changing recommendations for high-risk cancer patients and thereby increase their survival through simple dietary choices with easily accessible foods.

Recently, other health benefits have been associated with SFN-rich broccoli sprouts, such as its capacity to reverse abnormalities that have been associated with autism disorders after 18 weeks of consumption, including oxidative stress and depressed glutathione synthesis, reduced mitochondrial function and oxidative phosphorylation, increased lipid peroxidation, and neuroinflammmation (Singh *et al.*, 2014). Through similar mechanisms, SFN-rich broccoli sprouts during the juvenile and adolescence may have therapeutic effects on cognitive impairment at adulthood (Shirai *et al.*, 2015). Further *in vitro* and *in vivo* assays to understand GLS bioavailability and ITC actions would encourage the use of cruciferous vegetables as

preventive and healthy foods in animal studies and human clinical trials to fight high prevalence and non-communicable chronic diseases.

6. PERSPECTIVES IN NATURAL FUNCTIONAL FOODS

Interest in functional foods has been growing over the last decades as consumers become increasingly aware with diet and nutrition related to health promotion. Scientist and food manufacturers are continuously developing strategies to improve phytochemical levels in vegetables. Elicitation in pre and post-harvest is a cost-effective strategy to enhance the amount of active compounds with nutraceutical or other functional properties without using genetic modifications. These investigations on increasing levels of *Brassicaceae* phytochemicals may have a potential for human intervention studies to investigate the effects of a specific compound on human health.

There is an increasing global market of foods and products enriched in cruciferous bioactives. In the last few years, pharmaceutical forms (pills, powders, capsules, etc.) containing GLS or ITC as food bioactive compounds (especially broccoli extracts that provide SFN, I3C and other phytochemicals) have appeared in the markets (Arai *et al*., 2015). Also minimally processed broccoli by-products (stalk or leaves among others) can be used as a source of bioactive ingredients, mainly GLS and phenolic compounds (Dominguez-Perles *et al.*, 2011a), to design new products, such as novel beverages.

A better understanding of these dietary bioactive compounds and their modes of action will help to elucidate mechanisms of prevention of diseases, as well as to improve human health. Incorporating these sprouts and health-promoting compounds

as natural foods, rich in functional ingredients (functional foods), may be an effective way to guard against many of today's most common diseases.

Development of safe and effective foods, for reducing the risk of cancer and other chronic diseases is a high priority of research in the connections between food, nutrition and health. In this aspect, sprouts may be naturally-functional fresh foods with a positive role in the future of personalized nutrition and global food for health trends.

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Chapter 2. Objectives

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The general objective of this Doctoral Thesis was to select and enrich cruciferous sprouts in health-promoting bioactive compounds as well as to validate their functionality to foster their applications as natural-healthy foods.

In order to achieve this general aim, the following specific objectives were established:

- Select suitable cruciferous sprouts varieties and appropriate germination conditions, to reach acceptable biomass and high contents of phytochemicals.

- Investigate the effect of exogenous application of elicitors and seed priming on cruciferous sprouts, to enhance their content in bioactive compounds.

- Ascertain the possible health-promoting effects of broccoli and radish sprouts, through investigation of the absorption and metabolism of their bioactive compounds, and their effects in cancer proliferation, glucose metabolism and antinociception.

- Evaluate the quality of broccoli and radish sprouts during shelf life, in order to obtain microbiologically safe foods maintaining their phytochemical composition.

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Chapter 3. General Materials and Methods

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This chapter describes materials and techniques used in the works carried out in this Doctoral Thesis. More specific methods are included in the publications of Chapter 4.

1. GERMINATION OF SPROUTS

Seeds of cruciferous sprouts used in this work (Table 3.1.) were of commercial quality of ready-for-sprouting lines and were provided by Intersemillas, S.A. (Valencia, Spain). Germination of the sprouts was carried out in the laboratories of CEBAS-CSIC, Murcia, Spain.

***Brassicaceae* species**	
Common name	**Scientific name**
Broccoli	*Brassica oleracea* var. *italica*
Radish	*Raphanus sativus* L.
Red Radish	*Raphanus sativus* cv. Rambo
China Rose Radish	*Raphanus sativus* var. *sativus*
Turnip	*Brassica rapa* L.
Garden cress	*Lepidium sativum* L.
Red cabbage	*Brassica oleracea* var. *capitata*
Kohlrabi	*Brassica oleracea* var. *gongyloides*
White mustard	*Sinapis alba* L.

Table 3.1*. Cruciferous species under study.*

Seeds were rinsed in distilled water, immersed in 5 $g \cdot L^{-1}$ sodium hypochlorite for 2 h, drained and placed in distilled water again under aeration overnight. After pouring off the soaking water, the seeds were weighed (day 0) and spread evenly on trays lined with cellulose growth pads of white viscose (CN Seeds Ltd, UK) and irrigated with 5 $g \cdot L^{-1}$ sodium hypochlorite in distilled water. The trays were transferred to a controlled environment chamber with a 16 h light/8 h dark cycle, with temperatures of 25 and 20 ºC and relative humidity 60 % and 80 %, respectively. Photosynthetically active radiation (PAR) of 400 $\mu mol \cdot m^{-2} \cdot s^{-1}$ was provided by a combination of fluorescent tubes (Philips TLD 36 W/83, Hamburg, Germany; Sylvania F36W/GRO, Danvers, Massachusetts, USA) and metal halide lamps (Osram HQI.T 400 W, Munich, Germany). Brassicaceae sprouts were allowed to grow for a maximum of 12 days depending on the experiment. Sprout samples were gently collected in the middle of the light period, taking three replicates (trays) for analysis. All samples were weighed (fresh mass), collected separately, flash frozen in liquid nitrogen, stored at −80 ºC and lyophilized prior to analyses.

2. TREATMENTS WITH ELICITORS

Treatments with different elicitors were carried out as exogenous spray during four or five days before harvest, depending on the experiments (Figure 3.1.). The elicitors used in the experiments were MeJA (S.A.F.C. St. Louis, USA), SA (Panreac, S.A., Barcelona, Spain), JA, sucrose and glucose (Sigma-Aldrich Co.,St. Louis, , USA) and methionine (Alfa Aesar GmbH & Co., Karlsruhe, Germany).

Figure 3.1. *Exogenous spraying of elicitors on the cotyledons of cruciferous sprouts.*

Sprouts were always treated with 10 mL of the elicitor per tray, at the correspondent concentration under study, and applied as exogenous spraying on the cotyledons (not as soaking or irrigation solution). Milli-Q water was used as control. Also priming of seeds by 100 % imbibition and aeration of the seeds for 24 h was performed, using MeJA and JA in a concentration of 250 µM and methionine at 10 Mm, respectively.

3. EXTRACTION AND DETERMINATION OF BIOACTIVE COMPOUNDS

In this work, glucosinolates and their hydrolysis products isothiocyanates and indoles; and phenolic compounds, quantified as flavonols, chlorogenic acid derivatives and sinapic or ferulic acid derivatives; as well as, individual

anthocyanins, from cruciferous sprouts, under study, have been analysed using advanced techniques of HPLC-DAD, HPLC-DAD-ESI-MS^n, and UHPLC-QqQ-MS/MS, as described ahead.

3.1. Glucosinolates and non-coloured phenolic compounds

Freeze-dried samples (50 mg) of sprouts were extracted with 1 mL of methanol 70% V/V, then were heated at 70 ºC for 30 min in a bath, shaking every 5 min, and centrifuged (17 500 × g, 5 min). The supernatants were collected and the extractant was removed using a rotary evaporator. The dry material obtained was re-dissolved in Milli-Q water and filtered (0.45 μm Millex-HV13 filter, Millipore, Billerica, MA, USA).

The quantitative analysis of glucosinolates and phenolic compounds was carried out simultaneously by a LC multipurpose method (Francisco *et al.*, 2009), in an HPLC-DAD (Agilent Technologies 1260 Infinity or Waters Chromatograph, depending the experiment), according to their UV spectra and order of elution already described for similar acquisition conditions, as well as by HPLC-DAD-ESI/MS^n (Figure 3.2.) analysis in order to identify, with higher confidence, the association of the UV/Vis spectra information with MS and MS^n spectra information of intact glucosinolates. Chromatograms were recorded at 227 nm for glucosinolates and at 330nm for phenolics.

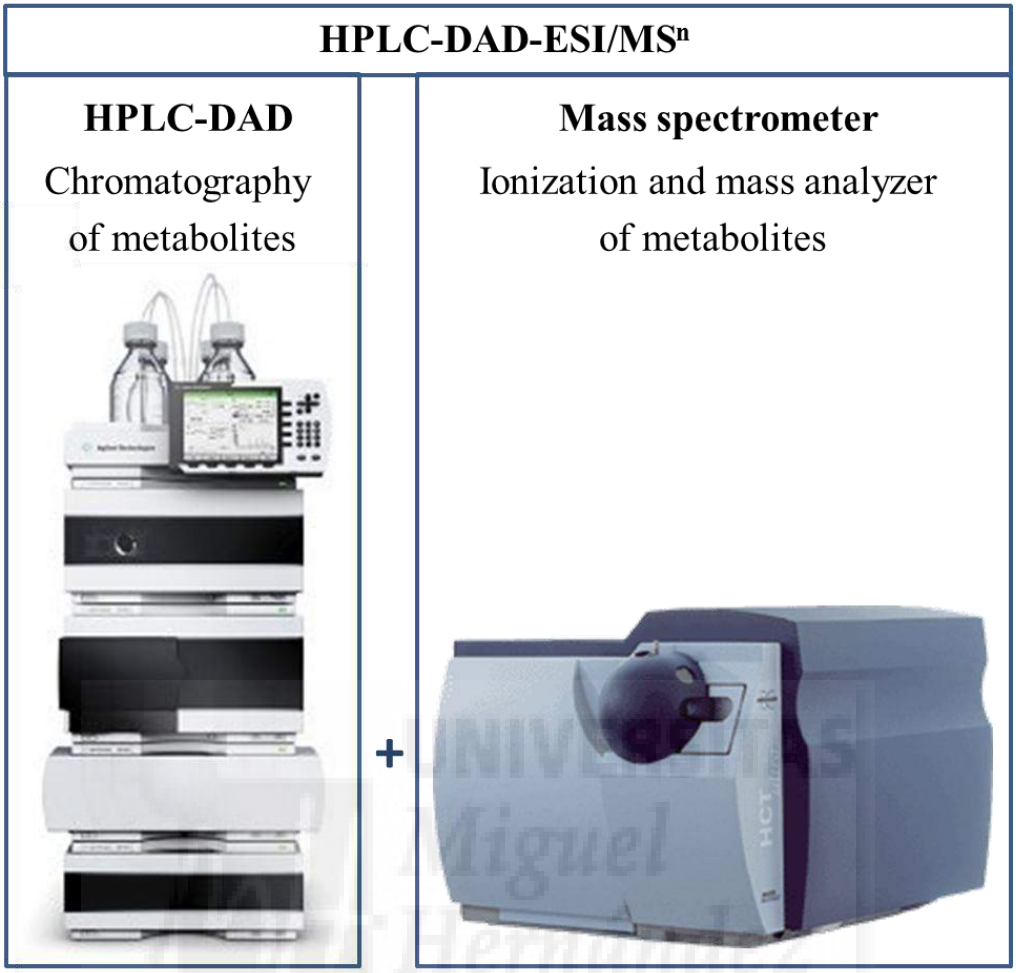


Figure 3.2. *HPLC-DAD-ESI/MS^n system consisting in HPLC-DAD (Agilent 1200) coupled to a mass spectrometer (Bruker Daltonics Ultra HCT-ESI Ion trap, Bremen, Germany).*

Glucosinolates were quantified using sinigrin and glucobrassicin as external standards of aliphatic and indole GLS, respectively (Phytoplan, Germany). Sinapic acid and ferulic acid derivatives were quantified as sinapinic acid; chlorogenic acid derivatives as chlorogenic acid and flavonols as rutin hydrate (Sigma-Aldrich Co., St. Louis, USA).

3.2. Isothiocyanates and indoles

Freeze-dried samples (50 mg) were extracted with 1.6 mL of Milli-Q water, shaken on a vortex mixer during 1 min and kept at room temperature for 24h. Then, samples were shaken again and centrifuged (17 500 × g, 5 min). The supernatants were collected and filtered (0.45 μm, PVDF filter, Millipore). Isothiocyanates were analyzed following their MRM transitions by UHPLC-QqQ-MS/MS (Figure 3.3.).

Figure 3.3. *Ultra-high performance liquid chromatography coupled with a 6460 tandem mass spectrometer with triple quadrupole technology (UHPLC-QqQ-MS/MS, Agilent Technologies, Waldbron, Germany).*

The analysis of GRA, SFN and its metabolites (SFN-GSH, SFN-CYS and SFN-NAC) was performed according to Dominguez-Perles *et al*. (2014). Also the optimization of the ITC sulforaphene (SFE) and iberin (IB); the indoles indole-3-carbinol (I3C) and 3,3-diindolylmethane (DIM), and the glucosinolates glucoraphenin (GRE), glucoiberin (GIB), glucobrassicin (GB), glucoraphasatin

(GPH), were carried out, assigning their retention times and preferential transitions (precursor and product ions) of the corresponding analytes. The MS fragmentation energy parameters: fragmentor (ion optics capillary exit voltage) (Frag) and collision energy (CE) were optimized for each compound to generate the most-abundant product ions for the MRM mode (Table 3.2). All ITC and indoles were obtained from Santa Cruz Biotech (Santa Cruz, CA).

Metabolite	Rt	Precursor Ion	Product ion	Frag (V)	CE (V)	Polarity
SFN	1.7	178	114	75	4	Positive
SFN-GSH	0.8	485	178	115	0	Positive
SFN-CYS	0.8	299	178	80	0	Positive
SFN-NAC	0.9	341	178	80	0	Positive
SFE	1.7	176.1	112	70	5	Positive
IB	1.5	164	105	90	6	Positive
I3C	1.7	130.1	77.1	70	25	Positive
DIM	0.4	247	105	60	5	Positive
GRA	0.7	438	196	90	4	Positive
GRE	0.7	434.1	97.1	80	20	Negative
GIB	0.7	421.9	357.7	100	0	Negative
GB	1.2	447.2	97	80	20	Negative
GPH	1.2	418.1	96.8	80	20	Negative

Rt: retention time, Frag: fragmentor, CE: collision energy.
SFN: sulforaphane, SFN-GSH: sulforaphane-glutathione, SFN-CYS: sulforaphane-cysteine, SFN-NAC: sulforaphane-N-acetylcysteine, SFE: sulforaphene, IB: iberin, I3C: indole-3-carbinol, DIM: 3,3-diindolylmethane, GRA: glucoraphanin, GRE: glucoraphenin, GIB: glucoiberin, GB: glucobrassicin, GPH: glucoraphasatin.

***Table 3.2**. Optimised MRM-ESI transitions for quantification and confirmation of the target analytes.*

3.3. Anthocyanins

Tandem mass spectrometry (MS/MS), which acquires mass spectra from the product ions produced from the fragmentation of a selected precursor ion, was used for identification and characterisation of anthocyanins. Freeze-dried samples (100 mg) were extracted with 1.5 mL of methanol/water/formic acid (25:24:1, v/v/v) (Moreno *et al.*, 2010). Briefly, samples were extracted in an ultrasonic bath for 60 min, then were kept at 4 °C overnight, and extracted again during 60 min before being centrifuged and filtered by 0.22 μm (PVDF filter, Millipore). Samples were first analysed by HPLC-DAD-ESI/MS^n for qualitative analysis and then, analysed by HPLC-DAD for quantification. Anthocyanins were quantified using cyanidin-3-glucoside-β-glucopyranoside (Polyphenols, Norway), as external standard. Chromatograms were recorded at 520 nm.

4. EVALUATION OF BIOAVAILABILITY AND FUNCTIONAL PROPERTIES OF SPROUTS

The absorption and metabolism, as well as different biological activities, of the predominant glucosinolates (GLS) and isothiocyanates (ITC) present in broccoli and radish cv. Rambo sprouts were evaluated using in vitro (cell cultures) and in vivo (Drosophila melanogaster and rodent) models. The following experiments were performed using an aqueous extract of broccoli or radish sprouts (Figure 3.4.).

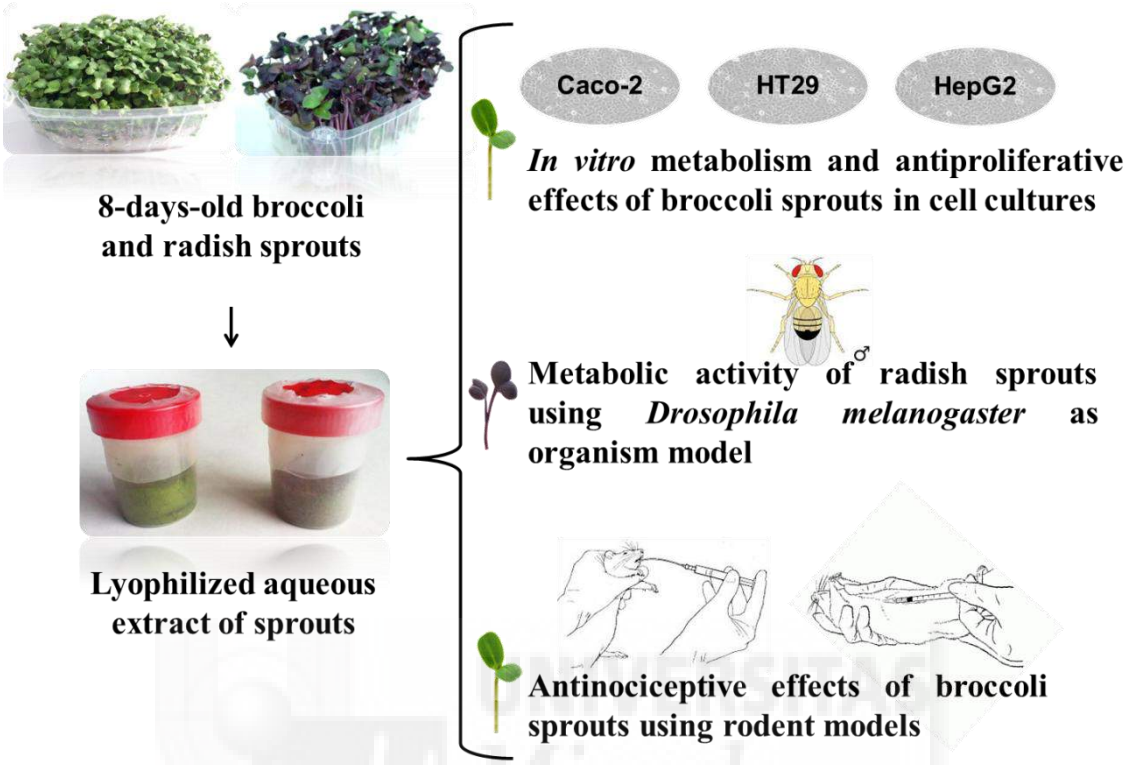


Figure 3.4. In vitro *and* in vivo *evaluations of biological activities of broccoli and radish sprouts*

4.1. Absorption and metabolism of GLS/ITC of broccoli sprouts and their antiproliferative activity using cell cultures as *in vitro* model

Experiments using human intestinal (Caco-2) and hepatic (HepG2) cells in order to evaluate the metabolism and antiproliferative effects of the ITC sulforaphane and broccoli sprouts were performed in the Department of Metabolism and Nutrition of the Institute of Food Science, Food Technology and Nutrition (ICTAN), CSIC, Madrid.

Sulforaphane's metabolites (e.g. SFN, SFN-GSH and SFN-CYS; from Santa Cruz Biotech, CA, USA) in both cell models of absorption and metabolism (Caco-2 and HepG2), during 3, 6, and 24h of treatment, were analyzed using a selective UHPLC-QqQ-MS/MS procedure (Dominguez-Perles *et al.*, 2014).

The antiproliferative activity of broccoli sprouts, glucoraphanin and sulforaphane was compared in Caco-2 and HT-29 human colorectal carcinoma cells, and HepG2 hepatocellular carcinoma cells, establishing the minimal concentration, of a given compound, to achieve half inhibition of the maximal cell growth (IC_{50}) by using a MTT assay.

4.2. Absorption and metabolic activity of radish sprouts using *Drosophila melanogaster* as *in vivo* model

The *in vivo* model system W^{1118} *D. melanogaster* was used in order to study absorption and metabolism of bioactive compounds of radish sprouts (*Raphanus sativus* cv. Rambo). This experiment was carried out in the Institute of Human Nutrition and Food Sciences of the University of Kiel, Germany.

Flies were subjected to a diet with sugar yeast medium supplemented with lyophilized radish sprouts at concentration 10.6 $g \cdot L^{-1}$, containing 50 $\mu mol \cdot L^{-1}$ of the health ITC sulforaphene, for 10 days, while the organisms were kept in a climate chamber under constant conditions of temperature (25 °C), humidity (60 %) and 12 h day/night cycle. The analyses performed in flies were the followings: gustatory assay (Linford *et al.*, 2013), to exclude differences in the food intake between control and treated flies; climbing assay by the negative geotaxis (RING)-assay method (Wagner

et al., 2015), considered an indicator of overall fitness of the flies; glucose analysis using the Fluitest®GLU (Analyticon Biotechnologies, Lichtenfels, Germany) (Piegholdt *et al.*, 2016); and real-time PCR for determining primer sequences of *D. melanogaster* spargel gene (srl) (Tinkerhess *et al.*, 2012), homologous of the mammalian PGC-1α involved in the regulation of glucose homeostasis and stimulation of mitochondrial biogenesis.

Finally, metabolites in fly homogenates were analyzed with UHPLC-QqQ-MS/MS in order to evaluate metabolism of isothiocyanates.

4.3. Antinociceptive effects of broccoli sprouts using rodents, as *in vivo* model

The antinociceptive effects of an aqueous extract obtained from broccoli sprouts in models of visceral and nociceptive pain was carried out in the Department of Neuroscience Research of the National Institute of Psychiatry Ramón de la Fuente Muñiz (INPRFM), City of México.

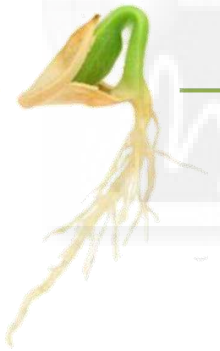
Broccoli sprouts aqueous extract was administered at 50, 100, 250 and 500 mg/kg via i.p., and at 500, 1000 and 2000 mg/kg orally, to Swiss albino mice and female Wistar rats, submitted to the writhing (Collier *et al.*, 1968) and formalin tests (Wheeler-Aceto *et al.*, 1990), respectively.

Gastric damage (Robert *et al.*, 1979) and sedative-like response (González-Trujano *et al.*, 1998), as possible adverse effect observed in the effect of anti-inflammatory non-steroidal and opioid analgesic drugs, respectively, were also explored in the effect of significant dosage (100 or 250 mg/kg BSE i.p.).

5. SHELF-LIFE QUALITY AND SAFETY OF BROCCOLI AND RADISH SPROUTS

Considering that broccoli and radish sprouts, collected at 8-day-old as optimum for consumption and commercialization, are fresh products that continue their plant metabolism during storage, the evaluation of changes in the phytochemical and microbial content was performed after 7 and 14 days of storage, at two different temperatures of refrigeration, 5 and 10 °C.

Microbiological determinations of pathogenic microorganisms, such as species of *Salmonella*, *Listeria*, *Staphylococcus*, *E. coli* and *Clostridium*, as well as *Enterobacteriaceae* organisms, aerobic mesophilic bacteria, aerobic psycrophilic bacteria, moulds and yeasts were carried out in the Food Engineering and Agricultural Equipment Department of the Technical University of Cartagena (UPTC), Spain. Glucosinolates, isothiocyanates and phenolic compounds were analyzed in CEBAS-CSIC by the methods described before.



Chapter 4. Publications

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1. SELECTING SPROUTS OF *BRASSICACEAE* FOR OPTIMUM PHYTOCHEMICAL COMPOSITION

(Publication 1)

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JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

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Selecting Sprouts of Brassicaceae for Optimum Phytochemical Composition

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ABSTRACT: Cruciferous foods (Brassicaceae spp.) are rich in nutrients and bioactive compounds. Edible sprouts are becoming popular fresh foods and, therefore, the phytochemical profiling of nine varieties of Brassicaceae (broccoli, kohlrabi, red cabbage, rutabaga, turnip, turnip greens, radish, garden cress, and white mustard) was evaluated for this purpose. The glucosinolates in seeds were significantly higher than in sprouts, and day 8 of germination was considered the optimum for consumption. The sprouts with higher concentrations of glucosinolates in 8-day-old sprouts were white mustard, turnip, and kohlrabi (~815, ~766, and ~653 mg 100 g^{-1} FW, respectively). Red cabbage and radish presented great total glucosinolates content (~516 and ~297 mg 100 g^{-1} FW, respectively, in 8-day-old sprouts) and also higher total phenolic contents, biomass, and antioxidant capacity. The selection of the best performers in terms of germination quality and phytochemical composition is the key to optimize new fresh foods enriched in health-bioactive compounds. Further research on the bioavailability of the bioactive compounds in *Brassica* foods will allow backing of recommendations for dietarily effective dosages for nutrition and health.

KEYWORDS: *germination, seeds, glucosinolates, phenolics, biomass, HPLC-PDA-ESI-MSn*

INTRODUCTION

Brassicaceae vegetables, or cruciferous foods, include a variety of horticultural crops with global economical relevance (oilseeds, forage, condiments, and vegetables). In Spain (Murcia), broccoli and cabbage (>190,000 tons) are a major agroeconomical activity.[1] Genomics studies of the U triangle[2] showed that *Brassica oleracea* (such as kale, cabbage, broccoli, and kohlrabi), *Brassica rapa* (such as turnip and Chinese cabbage), and *Brassica nigra* (black mustard) all originated from a common ancestral. Other species from this family are *Brassica napus* (such a rutabaga, rapeseed, and radicol), *Raphanus sativus* (radish), *Lepidium sativum* (garden cress), and *Sinapis alba* (white mustard). *Brassica* vegetables have received considerable research attention because of their association with health-promoting effects including improving the immune system, protection against allergies, antihypertensive properties, and reducing the risk for cardiovascular diseases and certain types of cancer.[3−5] Even if these vegetables are mainly recognized for their nitrogen−sulfur compounds, the glucosinolates, Brassicaceae foods are also rich in phenolic compounds, vitamins (A, C, E, and K), and minerals.[6] The content of bioactive compounds in Brassicaceae vegetables varies with genotype,[7,8] environmental stress,[9] growth conditions,[10] and storage, processing, and cooking methods.[11,12] Phenolic compounds and glucosinolates are present in high amounts in seeds and during the first days of germination, reaching a 10-fold increase compared to commercial adult plants.[13] Glucosinolates, nitrogen−sulfur compounds (β-D-thioglucoside-*N*-hydroxysulfates), are classified as aliphatic (the major group in almost all crucifer seeds and sprouts of *B. oleracea*, *B. napus*, *B. rapa*, and *R. sativus*), indolic (representing lower amounts in the glucosinolate profile), or aromatic (characteristic in *S. alba* and *L. sativum*.[14,15]) and have been extensively studied due to their hydrolysis compounds, the isothiocyanates (such as sulphoraphane[16] and benzyl isothiocyanate[17]) and indoles (indol-3-carbinol), which are associated with a reduced risk for particularly cancers of the gastrointestinal tract, lung, and prostate. In contrast, progoitrin, also present in crucifers, is an "undesirable" glucosinolate, because it is converted to the antithyroid goitrin after myrosinase hydrolysis.[18] The phenolic profile of sprouts is composed mostly of sinapic acid derivatives (hydroxycinnamic acids), a small portion of flavonoids (mainly quercetin and kaempferol commonly found as O-glycosides, and also isorhamnetin, characteristic of *B. rapa* species), and other hydroxycinnamic acids (chlorogenic, *p*-coumaric, and ferulic acids and their derivatives).[19,20] Brassicaceae sprouts are becoming popular health-food items and widely recommended by dieticians (highly nutritious, low-fat foods, rich in health-promoting phytochemicals, safe, and fresh); likewise, consumers are demanding foods to enjoy and promote wellness.[21]

The aim of the present work was to characterize nine varieties of Brassicaceae, highlighting their glucosinolate contents and natural antioxidants (phenolic compounds and in vitro antioxidant capacity) to foster their applications as naturally healthy foods.

MATERIALS AND METHODS

Plant Material and Experimental Conditions. Seeds provided by Intersemillas S.A. (Valencia, Spain) were of commercial quality of ready-for-sprouting lines. Nine varieties were used: broccoli (*B. oleracea* L. var. *italica*), kohlrabi (*B. oleracea* L. var. *gongylodes*), red cabbage (*B. oleracea* L. var. *capitata*), rutabaga (*B. napus* L. var. *napobrassica*), turnip greens ('Globo Blanco', WAM seeds, Galicia) and turnips (*B. rapa* L. subsp. *rapa*), radish (*R. sativus*), garden cress (*L. sativum*), and white mustard (*S. alba*). Seeds were rinsed in

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Table 1. Data of Biomass Increase Ratio (Sprouts vs Seeds) in Brassicaceae Sprouts[a]

variety	scientific name	% germination	day 4	day 8	day 12	ANOVA[b]	$LSD_{0.05}$[c]
broccoli	*Brassica oleracea* var. *italica*	>90	2.18a	3.17a	3.33a	ns	0.75
kohlrabi	*Brassica oleracea* var. *gongylodes*	7	0.28cdB	0.70cA	0.97bcA	**	0.22
red cabbage	*Brassica oleracea* var. *capitata*	5	0.79bcB	1.05bcB	1.90abA	***	0.11
rutabaga	*Brassica napus* var. *napobrassica*	8	1.21bB	2.73aAB	3.33aA	*	0.51
turnip green	*Brassica rapa* var. *rapa*	10	0.88bB	2.23abA	3.47aA	**	0.42
turnip	*Brassica rapa* var. *rapa*	2	0.29 cd	0.57c	0.87bc	ns	0.22
radish	*Raphanus sativus*	17	1.27bB	2.77aA	2.83aA	*	0.46
garden cress	*Lepidium sativum*	49	0.80dB	0.60cA	0.60cA	***	0.05
white mustard	*Sinapis alba*	18	0.32cdC	1.40bcB	2.37aA	***	0.14
$LSD_{0.05}$[c] (ANOVA $p < 0.001$)			0.15	0.37	0.25		

[a]Mean values ($n = 3$). Different lower case letters indicate statistically significant differences among varieties (for each sampling day). Different upper case letters indicate statistically significant differences between days (for each variety). [b]Levels of significance for each sampling day between species. Nonsignificant at $p > 0.05$ (ns); significant at $p < 0.05$ (*); significant at $p < 0.01$ (**); significant at $p < 0.001$ (***). [c]Least significant difference (LSD) for separating means in the respective column. The LSD was computed only after analysis of variance indicated a significant ($p < 0.05$) entry effect.

distilled water, immersed in 5 g L^{-1} sodium hypochlorite for 2 h, and drained and placed in distilled water under aeration overnight. After the soaking water had been poured off, the seeds were weighed (day 0) and spreaded evenly on trays (5 g per tray) lined with cellulose growth pad (CN Seeds, UK) and irrigated with Milli-Q water. Aliquots of 5 g of seeds were frozen in liquid nitrogen and stored at −80 °C pending phytochemical analysis.

The trays were transferred to a controlled environment chamber with a 16 h light/8 h dark cycle and air temperatures of 25 and 20 °C, respectively. The relative humidity (RH) was 60% (day) and 80% (night). Photosynthetically active radiation (PAR) of 400 μmol m^{-2} s^{-1} was provided by a combination of fluorescent tubes (Philips TLD 36 W/83, Hamburg, Germany; Sylvania F36W/GRO, Danvers, MA, USA) and metal halide lamps (Osram HQI.T 400 W, Munich, Germany). Brassicaceae sprouts were allowed to grow until they reached 12 days of age. Sprout samples (all sprouts from a single tray, germinated from 5 g of seeds) were collected at different time points after germination (days 4, 8, and 12). Three subsamples were rapidly and gently collected, always at 10 a.m., in the middle of the light period, taking three replicates for analysis. All samples were weighed (fresh mass), collected separately, flash frozen in liquid nitrogen, and stored at −80 °C prior to analyses.

Antioxidant Capacity Assay. The free radical-scavenging activity was determined using the free radical DPPH• as well as the ferric reducing antioxidant power (FRAP) assay in aqueous media according to the procedure of Mena et al.[21] Freeze-dried fine powdered samples (100 mg) were extracted with 10 mL of MeOH for 60 min in an ultrasonic bath (S5106-MTH, Danbury, CT, USA) and then were centrifuged at 10480*g* (model EBA 21, Hettich Zentrifugen) during 15 min at room temperature. Results were expressed as millimolar Trolox equivalents (TE) per 100 g FW.

Extraction and Determination of Glucosinolates and Phenolic Compounds. *Sample Extraction.* Freeze-dried samples (100 mg) were extracted with 1.5 mL of 700 g L^{-1} methanol in a sonicator bath for 10 min, then heated at 70 °C for 30 min in a heating bath, with shaking every 5 min using a vortex stirrer, and centrifuged (17500*g*, 30 min, 4 °C). The supernatants were collected, and methanol was completely removed using a rotary evaporator. The dry material obtained was dissolved in 1 mL of ultrapure water and filtered through a 0.45 μm Millex-HV13 membrane (Millipore Corp., Bedford, MA, USA). Freeze-dried powder samples (1g) were homogenized three times with 25 mL of 700 g L^{-1} methanol. The homogenates were filtered through cheesecloth and kept in ice. The homogenates were subsequently centrifuged (3600*g*, 5 min, 4 °C), and the supernatants were evaporated under vacuum at 50 °C to approximately 1 mL, diluted to 2 mL with water, and filtered through a 0.45 μm Millex-HV13 membrane (Millipore Corp.). Caffeoylquinic acid derivatives were quantified as chlorogenic acid (5-caffeoylquinic acid; Sigma, St. Louis, MO, USA), flavonoids as quercetin 3-rutinoside (Sigma), and sinapic acid and ferulic derivatives as sinapic acid (Sigma). The total analyte content of phenolic compounds in broccoli sprouts was expressed as milligrams per 100 g FW.

HPLC-PDA-ESI-MSn Qualitative and Quantitative Analysis of Glucosinolates and Phenolic Compounds. Glucosinolates and phenolic compounds were determined using a LC multipurpose method that simultaneously separates intact glucosinolates and phenolics, according to the procedure of Francisco et al.,[19] with slight modifications. The separated intact glucosinolates, hydroxycinnamic acids (chlorogenic acid derivatives and sinapic acid derivatives), and flavonols were identified following their MS2$[M - H]^-$ fragmentations (and also MS3 fragmentation of the major MS2 ions for hydroxycinnamic acids and flavonols), UV−visible spectra, and the order of elution previously described for similar acquisition conditions.[19,22]

Glucosinolates were quantified in the HPLC-PDA using sinigrin as standard (sinigrin monohydrate; Phytoplan Diehm & Neuberger, GmbH, Heidelberg, Germany). Caffeoylquinic acid derivatives were quantified as chlorogenic acid (5-caffeoylquinic acid, Sigma-Aldrich Chemie GmbH, Steinheim, Germany), flavonols (quercetin and kaempferol derivatives) as quercetin-3-rutinoside (Merck, Darmstadt, Germany), and sinapic acid derivatives as sinapic acid (Sigma).

Statistical Methods. All assays were conducted in triplicate. The data were processed using the SPSS 17.0 software package (LEAD Technologies, Inc., Chicago, IL, USA). A Student's *t* test was used to determine the significance of differences between means. A multifactorial analysis of variance (ANOVA) and Tukey's multiple-range test were carried out to determine significant differences at p values <0.05. Pearson correlation analyses were also performed to corroborate relationships between selected parameters.

■ RESULTS AND DISCUSSION

Biomass. The seeds used in the experiments were obtained of commercial quality for sprouting; therefore, the germination rate is usually lower than in the varieties used for plant production. Only broccoli seeds reached >90% of germination (Table 1), whereas garden cress seeds germinated 50%, and the rate was <20% for the remaining seeds of the different varieties (Table 1). Table 1 shows an increasing biomass ratio from day 0 to days 4, 8, and 12. Broccoli sprouts showed the highest values, increasing 2-fold at day 4 and 3-fold at day 12 and exhibiting the highest percentage of germination. The 8- and 12-day-old sprouts were more desirable for consumption and marketing than the 4-day-old ones (not ready for manipulation). At day 8 of the monitored period, broccoli, rutabaga, turnip greens, and radish had biomass ratios significantly higher than the rest (2−3-fold), consistent with the greater length

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Table 2. Glucosinolates Detected and Presented in Brassicaceae Seeds and Sprouts[a]

peak	code	compound	glucosinolate (gls) semisystematic name	class	t_R (min)	$[M - H]^-$ (*m/z*)	broccoli	kohlrabi	red cabbage	rutabaga	turnip greens	turnip	radish	garden cress	white mustard
1	GIB	glucoiberin	3-methylsulfinylpropyl-gls	aliphatic	6.5	422	+	+	+	0	0	0	0	+	+
2	PRO	progoitrin	(*R*)-2-hydroxy-3-butenyl-gls	aliphatic	7.1	388	+	+	+	+	+	+	0	+	0
3	GRE	glucoraphenin	4-methylsulfinyl-3-butenyl-gls	aliphatic	7.1	434	0	0	0	0	0	0	+	+	0
4	GRA	glucoraphasatin	4-methylsulfinylbutyl-gls	aliphatic	7.2	436	+	+	+	+	+	+	0	+	+
5	EPRO	epiprogoitrin	(*S*)-2-hydroxy-3-butenyl-gls	aliphatic	8.0	388	0	0	+	+	0	0	0	0	+
6	SIN	sinigrin	2-propenyl-gls	aliphatic	8.4	358	+	+	+	+	+	+	0	+	+
7	GAL	gluconapoleiferin	5-methylsulfinylpentyl-gls	aliphatic	11.7	450	+	+	+	+	+	+	+	+	0
8	GII	gluconapin	4-hydroxybenzyl-gls	aromatic	13.6	424	+	0	0	+	0	0	0	+	+
9	GNL	glucobrassicanapin	(*R*)-2-hydroxy-4-pentenyl-gls	aliphatic	14.3	402	+	+	+	+	+	+	0	+	0
10	GNA	glucobrassicin	3-butenyl-gls	aliphatic	17.5	373	+	+	+	+	+	+	+	+	+
11	GIV	glucoerucin	3-methylthiopropyl-gls	aliphatic	19.5	406	+	+	0	0	0	0	+	0	0
12	OHGRS	4-hydroxyglucobrassicin	4-hydroxy-3-indolylmethyl-gls	indolic	20.0	463	+	+	+	+	+	+	+	+	+
13		*n*-butyl	*n*-butyl-gls	aliphatic	20.9	374	0	0	+	+	+	+	0	0	0
14	GTP	glucotropaeolin	benzyl-gls	aromatic	20.9	408	0	0	0	0	0	0	0	+	+
15	GBN	glucobrassicanapin	4-pentenyl-gls	aliphatic	22.7	386	0	0	+	+	+	+	0	0	0
16	GER	glucoerucin	4-methylthiobutyl-gls	aliphatic	23.7	420	+	0	0	0	0	0	0	0	0
17	DER	deoxyerucin	4-methylthio-3-butenyl-gls	aliphatic	24.9	418	0	0	0	+	0	0	+	0	0
18		*n*-pentyl	*n*-pentyl-gls	aliphatic	26.0	388	+	+	+	0	+	+	0	0	0
19	GIS	glucoerysimin	3-indolylmethyl-gls	indolic	26.4	447	+	+	+	+	+	+	+	+	+
20	GST	glucoberteroin	5-methylthiopentyl-gls	aliphatic	28.2	434	+	+	0	+	0	0	+	+	+
21	GST	glucosativin	2-phenylethyl-gls	aromatic	28.3	422	+	+	+	+	+	+	0	+	+
22	MGBS	4-methoxyglucobrassicin	4-methoxy-3-indolylmethyl-gls	indolic	28.6	477	+	+	+	+	+	+	+	+	+
23		*n*-hexyl	*n*-hexyl-gls	indolic	31.4	402	+	+	+	+	+	+	0	0	0
24	NGBS	neoglucobrassicin	*N*-methoxy-3-indolylmethyl-gls	indolic	32.5	477	+	+	+	+	+	+	0	+	+

[a]Identification based on $[M - H]^-$ (*m/z*), retention time (t_R), and characteristic spectra. +, compound presence; 0, compound absence.

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(between 4 and 5 cm) (Table 1). On day 12, in addition to the above, red cabbage and white mustard reached significantly higher biomass values (2–3-fold) and greater growth (between 5 and 6 cm length). The higher values of biomass are indicative of better sprout growth (length) and better rate of fresh weight (FW) production. The biomass data of sprouts is not widely available in the literature. Gu et al.,[23] for example, recorded that broccoli sprouts grew rapidly after 36 h of germination, and similar sprout length as in our study was reported.[24] The early, noninvasive, and direct parameter of biomass is therefore a useful parameter to screen plant material for production of sprouts.

Glucosinolates. The main characteristic of the Brassicaceae wellness composition is their glucosinolate (GLS) profile;[8,15,26] therefore, the presence of individual intact GLSs was studied in seeds and sprouts (Table 2). The molecular ion $[M - H]^-$ (m/z)

Table 3. Data of Total Glucosinolates (mg 100 g^{-1} FW) Present in Brassicaceae Seeds and Sprouts[a]

	seeds	sprouts		
variety	D0	D4	D8	D12
broccoli	735.08e	209.32f	141.48e	117.48f
kohlrabi	1359.41c	994.40b	653.08b	450.54b
red cabbage	1307.78c	907.82c	516.43c	246.81c
rutabaga	2131.97b	951.88bc	386.84d	276.74c
turnip greens	1264.30c	736.66d	164.51e	119.44ef
turnip	1131.06d	938.63bc	765.07a	474.76b
radish	1350.76c	566.14e	296.77d	168.48de
garden cress	323.09f	194.94f	174.06e	176.32d
white mustard	2862.12a	2353.70a	815.10b	748.67a
$LSD_{0.05}$[b] (ANOVA $p < 0.001$)	37.40	26.96	24.47	14.24

[a]Mean values ($n = 3$). Different lower case letters indicate statistically significant differences among varieties (for each sampling day). [b]Least significant difference (LSD) for separating means in the respective column. The LSD was computed only after analysis of variance indicated a significant ($p < 0.05$) entry effect.

of GLSs, their fragmentation ion patterns, and retention times allowed the identification of 24 different compounds. The MS2 fragmentation of aglycone side chain produces the most consistent ion at m/z 259, and the MS3 fragmentation of this ion gives rise to fragments at m/z 97 (corresponding to the sulfate group) by the dissociation of GLSs in the ion trap mass spectrometer, constituting a very useful preliminary screening method for determining the presence of GLS in sprouts extracts.[22] Results showed significant differences of the characteristic GLS profiles among samples. All of the varieties studied contained common GLSs: gluconapin (**10**), 4-hydroxiglucobrassicin (**12**), glucobrassicin (**19**), 4-methoxyglucobrassicin (**22**), gluconasturtiin (**21**), neoglucobrassicin (**24**), and glucoraphanin (**4**), except for radish, which did not contain the last three compounds. In *B. oleracea* species, kohlrabi and broccoli, we found identical GLS profiles [glucoiberin (**1**), progoitrin (**2**), **4**, sinigrin (**6**), glucoalyssin (**7**), glucosinalbin (**8**), **10**, glucoiberverin (**11**), **12**, glucoerucin (**16**), *n*-pentyl (**18**), **19**, glucoibertaorin (**20**), **21**, **22**, *n*-hexyl-gls (**23**), and **24**]. By contrast, the red cabbage samples showed certain differences having epiprogoitrin (**5**), gluconapoleiferin (**9**), *n*-butyl-gls (**13**), and glucobrassicanapin (**15**), and not presenting compounds **7**, **11**, **16**, and **20**, being closely related to rutabaga, which differed in only five GLSs (containing **17** and **20** and not containing **1**, **5**, and **18**). The *B. rapa* samples of turnip greens and turnips showed also similar profiles (**2**, **4**, **6**–**10**, **12**, **13**, **15**, **18**, **19**, and **21**–**24**), but the glucosinalbin was not present in turnips, maybe due to their different origin of seeds. Garden cress and white mustard, which are closely related,[3] resulted also in similar GLS profiles. Radish presented **3**, **7**, **10**–**12**, **17**, **19**, **20**, and **22** GLS, quite different from the rest of the species.

Therefore, Brassicaceae sprouts showed characteristic GLSs according to species and their individual quantification (seeds; 4-, 8-, and 12-day-old sprouts; Tables 6–8). The general trend for the majority of the GLSs is decrease over germinated time,

Table 4. Data of Total Phenolic Compounds (mg 100 g^{-1} FW) Present in Brassicaceae Seeds and Sprouts[a]

	seeds	sprouts		
variety	D0	D4	D8	D12
broccoli	1773.44d	1167.87d	852.16d	628.53e
kohlrabi	1149.34e	870.32e	823.58d	765.55bc
red cabbage	2116.64c	1321.51c	1309.29ab	991.92a
rutabaga	1200.86bc	1429.29c	828.50d	661.99de
turnip greens	1283.88b	1844.55b	783.59d	620.78e
turnip	1792.65d	1343.15c	1236.41b	706.23 cd
radish	1778.82a	2123.57a	1076.42c	751.89bc
garden cress	491.96f	516.65f	507.34e	422.49f
white mustard	182.27g	799.96e	779.25d	797.96b
$LSD_{0.05}$[b] (ANOVA $p < 0.001$)	39.67	41.07	56.16	19.04

[a]Mean values ($n = 3$). Different lower case letters indicate statistically significant differences among varieties (for each sampling day). [b]Least significant difference (LSD) for separating means in the respective column. The LSD was computed only after analysis of variance indicated a significant ($p < 0.05$) entry effect.

having a greater amount of the compound until day 4 (Tables 6–8), followed by a marked decline between days 4 and 12 (in broccoli, rutabaga, turnip greens, and radish), corresponding to 50–90% loss of individual GLSs. Kohlrabi, red cabbage, turnip, garden cress, and white mustard showed the highest loss of individual GLSs from seeds to day 8 of germination. Consistent with their function in plant defense and nutrient reserve compounds, seeds have the largest amount of these metabolites, and the reduction in GLSs with germination upon a dilution effect of tissue expansion leads to an intermediate GLS profile between seeds and mature tissues.[9,27] Not all of the GLSs found in seeds are detected in sprouts, as happened to sinigrin, glucoberteroin, or gluconasturtiin in broccoli, glucobrassicin in turnip greens, and progoitrin and glucoalyssin in garden cress (Tables 6–8), because the GLS profile may vary significantly between tissues and organs.[28] On the other hand, some GLSs were present only in sprouting seeds, such as neoglucobrassicin in broccoli, red cabbage, and white mustard sprouts (Tables 6 and 8). Several reasons might justify this fact, such as the activation of secondary metabolism during germination,[29] interconversion between aliphatic GLSs, or the interference between GLSs[27] and fatty nutrients in the seeds during sample extraction. Some authors have avoided this interference by defatting the samples.[6]

The values were recorded from 8-day-old-sprouts, being considered the optimum for consumption (suitable germination time to allow manipulation and acceptable composition by panelists and consumers). At this stage, broccoli and kohlrabi showed glucoraphanin (sulforaphane GLS), as the major GLS

Table 5. Antioxidant Activity (mM Trolox 100 g^{-1} FW) of Brassicaceae Seeds and Sprouts Estimated by DPPH• Radical-Scavenging Assay and Ferric Reducing Antioxidant Assay (FRAP)[a]

	DPPH• assay					FRAP assay				
variety	seeds	D4	D8	D12	$LSD_{0.05}$[b]	seeds	D4	D8	D12	$LSD_{0.05}$[b]
broccoli	1.23bcA	0.47gB	0.28efC	0.21cdD	0.010	2.85cA	1.33fB	0.78cdC	0.63dD	0.053
kohlrabi	1.07deA	1.0aB	0.73cC	0.58aD	0.032	2.92bcA	2.46aB	1.61aC	1.18aD	0.044
red cabbage	1.51aA	0.98abB	0.77bC	0.40bD	0.035	3.43aA	2.08bB	1.61aC	1.00bD	0.066
rutabaga	1.12cdA	0.65eB	0.29fgC	0.21cdC	0.035	3.17bA	1.74cdB	0.81bcC	0.52eD	0.026
turnip greens	1.23bcA	0.70dB	0.24gC	0.18gD	0.017	2.75cdA	1.55deB	0.63deC	0.56deC	0.017
turnip	1.33bA	0.92bcB	0.71cC	0.46bD	0.032	2.51deA	1.84cB	1.57aC	0.96bD	0.057
radish	0.95eA	0.57fB	0.31eC	0.28eC	0.030	2.76cdA	1.42efB	0.98bC	0.76cD	0.068
garden cress	0.19gA	0.12hB	0.12hBC	0.09eD	0.010	0.52gA	0.49gA	0.43eB	0.32fC	0.012
white mustard	0.70 fA	0.64eB	0.36fC	0.24cdD	0.017	2.11fA	1.61deB	0.83bcC	0.76cC	0.060
$LSD_{0.05}$[b]	0.034	0.015	0.011	0.022		0.074	0.068	0.063	0.028	

[a]Mean values ($n = 3$). Different lower case letters indicate statistically significant differences between seeds and days (for each sampling day). Different upper case letters indicate statistically significant differences among varieties (for each variety). [b]Least significant difference (LSD) for separating means in the respective column. The LSD was computed only after analysis of variance indicated a significant ($p < 0.05$) entry effect. ANOVA values are significant at $p < 0.001$.

(35% of total) (Table 6). Broccoli also included other major GLSs, such as glucoerucin, 4-methoxyglucobrassicin, and neoglucobrassicin (15% of the total each), and kohlrabi showed glucoiberin (20% of the total) and glucoibervirin and 4-hydroxiglucobrassicin (10% of the total each). Red cabbage and rutabaga presented progoitrin, considered to be an antinutrient (goitrogenic effects), as the major GLS (35% of total). Red cabbage also included significant amounts of sinigrin (20% of the total) and glucoiberin and glucoibervirin (13% of the total each), and rutabaga presented glucoraspin and 4-hydroxyglucobrassicin (25% of the total each) (Table 7). *B. rapa* varieties, turnip greens and turnips, exhibited glucoraspin as characteristic GLS, with 75 and 50% of total, respectively (Table 7). Turnip greens presented <10% of the total for the rest of GLSs, and turnips also showed glucobrassicanapin (20% of the total) and 4-hydroxyglucobrassicin (14% of GLS). On the other hand, the also beneficial GLS glucoraphenin[24] was found to be dominant in radish (65% of total), showing also 4-hydroxyglucobrassicin as characteristic GLS (25% of the total) (Table 8). Finally, garden cress and white mustard presented a characteristic aromatic GLS, glucotropaeolin (80% of total) and glucosinalbin (87% of total), respectively, accounting for the rest of GLS, <10% of the total for both species. The total GLS content recorded in seeds (Table 3) was significantly higher and variable ($p < 0.001$) within species (from 2862.12 mg 100 g^{-1} FW in white mustard to 323.05 mg 100 g^{-1} FW in garden cress) than in sprouts. The GLSs recorded were higher during the first 4 days of germination, followed by a marked decrease over time, dropping from seeds to days 8 and 12 of germination, by 30 and 60%, respectively, in turnip; by 60 and 80%, respectively, in red cabbage; on average 60% in kohlrabi, garden cress, and white mustard sprouts; and around 80 and 85%, respectively, in broccoli, rutabaga, turnip greens, and radish sprouts (Table 3), also in agreement with previous results.[27] Our values are higher (2−15-fold) than those shown in studies with mature plants of broccoli, kohlrabi, or red cabbage[15] due to the physiological stage of sprouts. The differences also shown by other authors for broccoli, radish, and white mustard sprouts may be also due to the quality of the seeds used in the different works.[6,14,27] We also found similar results when comparing garden cress[29] and broccoli seeds of similar origin.[7]

Aliphatic GLSs (**1**−**7**, **9**−**11**, **13**, **15**−**18**, **20**, and **23** as shown in Tables 2 and 6−8), were the major GLSs in seeds and sprouts in all varieties (representing between 70 and 85%) (Figure 1), with values ranging from ∼1000 mg 100 g^{-1} FW in rutabaga seeds to ∼491 mg 100 g^{-1} FW in broccoli seeds, which decreased over the 12 day study period. Apart from glucoraphanin and glucoraphenin, the other predominant aliphatic GLS was sinigrin (which was mostly found in red cabbage, 91.83 ± 8.88 mg 100 g^{-1} FW, and kohlrabi, 28.99 ± 1.15 mg 100 g^{-1} FW), according to previous research (Tables 6−8).[14,29] Aliphatic GLSs are transformed by hydrolysis to isothiocyanates by specific myrosinases, which have been acknowledged as bioactive compounds with anticarcinogenic properties.[3,16] On the other hand, garden cress and white mustard exhibited a high content (90%) of aromatic GLSs (**8**, **14**, and **21** shown in Tables 2 and 6−8) in seeds (277 and ∼2749 mg 100 g^{-1} FW, respectively) and sprouts (Figure 1). Neither species showed any statistically significant difference among aromatic GLS concentration on 8- and 12-day-old sprouts (∼158 mg 100 g^{-1} FW in garden cress and ∼700 mg 100 g^{-1} FW in white mustard sprouts) (Table 8). The content of these GLSs provides a spicy taste, because the white mustard crop was bred for pungency as a condiment and now contains one of the highest concentrations reported of glucosinalbin in seeds (2749.53 mg 100 g^{-1} FW). For the rest of the cruciferous plants, aromatic GLSs accounted for <5% of the total GLSs. Indolic GLSs (**12**, **19**, **22**, and **24** shown in Table 2) in cruciferous seeds and sprouts showed values <30% of the total GLSs in all species except for garden cress and white mustard, which presented even much lower values (3.4 and 5%, respectively) (Figure 1). 4-Hydroxyglucobrassicin accounted for almost 90% of the indolic GLSs in all species. The process of germination resulted in a decreasing concentration of individual GLSs, except for the indole 4-methoxyglucobrassicin, which was found in trace amounts in all seeds, and some varieties presented high amounts of this GLS in growing sprouts (broccoli, red cabbage, rutabaga, turnip greens, radish, garden cress, and white mustard). In terms of biological effect, the expected breakdown product of the indole glucosinolate 4-methoxyglucobrassicin during ingestion, 4-methoxyindole-3-carbinol, has been studied because it might play a role in the cancer preventive effect by causing cell death and inhibiting cell proliferation of human colon cancer cells in vitro.[30]

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Table 6. Individual Glucosinolates (mg 100 g^{-1} FW) Detected and Present in *Brassica oleracea* Seeds and Sprouts[a]

			broccoli					kohlrabi					red cabbage				
peak	code	compound	D0	D4	D8	D12	LSD_{Tuk}[b]	D0	D4	D8	D12	LSD_{Tuk}	D0	D4	D8	D12	LSD_{Tuk}
1	GIB	gluconapin	241.03a	31.33b	20.02b	13.64b	8.72***	343.38a	187.65b	136.71c	103.57d	7.73***	265.02a	118.57b	73.46c	37.86d	5.17***
2	PRO	progoitrin	Tr	Tr	Tr	Tr		Tr	Tr	Tr	Tr		388.05a	458.44b	182.52c	99.15d	12.00***
3	GRE	glucoraphenin	nd	nd	nd	nd		nd	nd	nd	nd		nd	nd	nd	nd	
4	GRA	glucoraphanin	181.51a	80.65b	48.31c	42.39c	4.98***	522.13a	434.95b	286.57c	158.08d	9.43***	Tr	Tr	Tr	Tr	
5	EPRO	epiprogoitrin	nd	nd	nd	nd		nd	nd	nd	nd		Tr	Tr	Tr	Tr	
6	SIN	sinigrin	4.89	Tr	Tr	Tr		49.83a	46.12a	34.98b	21.87b	2.33***	224.43a	99.15b	91.85bc	44.54c	15.27***
7	GAL	glucoalyssin	Tr	Tr	Tr	Tr		Tr	Tr	Tr	Tr		Tr	Tr	Tr	Tr	
8	GST	gluconasturtiin	nd	Tr	Tr	Tr		Tr	nd	nd	nd		nd	nd	nd	nd	
9	GNL	glucoerysolin	nd	nd	nd	nd		nd	nd	nd	nd		nd	nd	nd	nd	
10	GNA	gluconapoleiferin	5.82a	1.17b	1.07b	0.97b	0.87***	55.79a	51.66a	35.09b	14.88c	2.44***	114.68a	70.85b	46.98c	17.89d	4.61***
11	GIV	glucobrevicaulin	Tr	Tr	Tr	Tr		223.10a	89.95b	65.48b	47.13b	6.83***	41.09a	67.05a	63.47a	38.31c	3.79***
12	OHGBS	4-hydroxyglucobrassicin	228.20a	9.53b	Tr	Tr	7.87***	181.68a	177.97a	120.39b	41.87c	8.10***	330.45a	144.98b	96.38c	17.87d	7.63***
13		n-butyl	nd	nd	nd	nd		nd	nd	nd	nd		nd	nd	nd	nd	
14	GTP	glucotropaeolin	nd	nd	nd	nd		nd	nd	nd	nd		nd	nd	nd	nd	
15	GBN	glucobrassicanapin	nd	nd	nd	nd		nd	nd	nd	nd		Tr	Tr	Tr	Tr	
16	GER	gluconeoliferin	48.61a	19.84b	18.39b	21.18b	3.54***	Tr	Tr	Tr	Tr		Tr	Tr	Tr	Tr	
17	BER	4MSOB	nd	nd	nd	nd		nd	nd	nd	nd		0	0	0	0	
18		n-pentyl	Tr	Tr	Tr	Tr		Tr	Tr	Tr	Tr		Tr	Tr	Tr	Tr	
19	GRS	glucoerucin	14.62a	11.43ab	8.67b	7.96b	1.73**	35.13a	26.13a	26.45a	25.57a	1.06*	Tr	5.89b	2.78b	3.44b	0.25***
20	GBT	glucobrassicanapin	4.82	Tr	Tr	Tr		Tr	Tr	Tr	Tr		nd	nd	nd	nd	
21	GST	gluconasturtiin	3.89	Tr	Tr	Tr		38.96a	12.60b	10.08c	5.11c	2.39***	18.05a	11.80b	Tr	Tr	0.86***
22	MGBS	4-methoxyglucobrassicin	Tr	36.43a	18.64b	7.45c	3.01***	Tr	Tr	Tr	Tr		Tr	Tr	Tr	Tr	
23		n-hexyl	3.67a	4.57a	4.73a	4.74a	0.64*	16.41a	7.35b	5.26c	3.16d	0.31***	Tr	Tr	Tr	Tr	
24	NGBS	neoglucobrassicin	Tr	24.87a	21.15b	20.59b	0.73***	Tr	Tr	Tr	Tr		Tr	12.89a	9.00b	8.45b	0.89***

[a]Mean values ($n = 3$). Different lower case letters mean statistically significant differences between seeds and days (for each varieties). Tr, traces, not quantified (<0.5 mg 100 g^{-1} FW); nd, not detected (<0.1 mg 100 g^{-1} FW). [b]Least significant difference (LSD) for separating means in the respective column. The LSD was computed only after analysis of variance indicated a significant ($p < 0.05$) entry effect. ANOVA p value: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.0001$; ns, $p > 0.05$.

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Table 7. Individual Glucosinolates (mg 100 g^{-1} FW) Detected and Present in *Brassica napus* (Rutabaga) and *Brassica rapa* (Turnip Greens and Turnip) Seeds and Sprouts[a]

			rutabaga					turnip greens				turnip				
peak	code	compound	D0	D4	D8	D12	LSD$_{tab}$[b]	D0	D4	D8	D12	LSD$_{tab}$	D0	D4	D8	D12	LSD$_{tab}$
1	GBS	gluconapin	nd	nd	nd	nd		nd	nd	nd	nd		nd	nd	nd	nd	
2	PRO	progoitrin	871.36b	241.04b	124.34c	98.46c	26.19***	85.06a	24.42b	13.06c	10.46c	2.69***	33.32a	28.67b	26.76b	23.07c	2.93***
3	GRS	gluconapoleiferin	nd	nd	nd	nd		nd	nd	nd	nd		nd	nd	nd	nd	
4	GBA	gluconapoleiferin	Tr	Tr	Tr	Tr		Tr	Tr	Tr	Tr		Tr	Tr	Tr	Tr	
5	EPRO	epiprogoitrin	nd	nd	nd	nd		nd	nd	nd	nd		nd	nd	nd	nd	
6	SIN	sinigrin	Tr	Tr	Tr	Tr		Tr	Tr	Tr	Tr		nd	nd	nd	nd	
7	GAL	gluconapin	132.46b	48.93b	8.76c	8.48c	7.77***	Tr	Tr	Tr	Tr		Tr	32.59a	33.86b	33.50c	2.43***
8	GES	gluconasturtiin	nd	nd	nd	nd		Tr	nd	nd	nd		nd	nd	nd	nd	
9	GNL	gluconapoleiferin	113.25a	93.86a	31.38c	31.48c	3.18***	Tr	nd	nd	nd		nd	nd	nd	nd	
10	GNA	gluconapin	684.75a	186.64b	88.57c	58.37d	10.05***	986.10a	542.39b	234.01c	99.64d	41.1***	595.97a	457.23b	396.78b	278.19c	19.88***
11	GBN	gluconapoleiferin	nd	nd	nd	nd		nd	nd	nd	nd		nd	nd	nd	nd	
12	OHGBS	4-hydroxyglucobrassicin	454.75a	184.35b	78.58c	38.25c	9.91***	340.84a	128.91b	14.25c	6.38c	8.5****	183.63a	156.27b	117.51c	61.59d	6.37***
13		*n*-octyl	Tr	Tr	Tr	Tr		Tr	Tr	Tr	Tr		nd	nd	nd	nd	
14	GTF	gluconapoleiferin	nd	nd	nd	nd		nd	nd	nd	nd		nd	nd	nd	nd	
15	GBN	gluconasturtiinpt	14.63a	7.16b	5.53c	2.95d	0.51***	13.94a	9.48b	5.17c	2.95d	0.86***	39.52a	223.87b	161.34c	95.81d	7.85***
16	GBS	gluconapin	nd	nd	nd	nd		nd	nd	nd	nd		nd	nd	nd	nd	
17	DHB	dehydroerucin	nd	nd	nd	nd		nd	nd	nd	nd		nd	nd	nd	nd	
18		*n*-pentyl	nd	nd	nd	nd		Tr	Tr	Tr	Tr		Tr	nd	nd	nd	
19	GBS	gluconapoleiferin	10.99a	5.83b	2.66c	2.44c	0.38***	2.73	Tr	Tr	Tr		Tr	Tr	Tr	Tr	
20	GBT	gluconapoleiferin	Tr	Tr	Tr	Tr		nd	nd	nd	nd		nd	nd	nd	nd	
21	GST	gluconasturtiin	49.79a	25.88b	23.76b	29.93b	4.81*	55.89a	10.09b	Tr	Tr	3.14****	41.68a	40.10a	30.84a	23.53b	4.72**
22	3MSB	4-methoxyglucobrassicin	nd	8.17a	17.38a	12.33b	0.91***	33.67a	20.72b	Tr	Tr	0.80***	Tr	Tr	Tr	Tr	
23		*n*-heptyl	Tr	Tr	Tr	Tr		Tr	Tr	Tr	Tr		Tr	Tr	Tr	Tr	
24	3MSB	neoglucobrassicin	Tr	Tr	Tr	Tr		Tr	Tr	Tr	Tr		Tr	Tr	Tr	Tr	

[a]Mean values (*n* = 3). Tr, traces, not quantified (<0.5 mg 100 g^{-1} FW); nd, not detected (<0.1 mg 100 g^{-1} FW). Different lower case letters indicate statistically significant differences between seeds and days (for each variety). [b]Least significant difference (LSD) for separating means in the respective columns. The LSD was computed only after analysis of variance indicated a significant ($p < 0.05$) entry effect. ANOVA *p* value: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ns $p \geq 0.05$.

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Table 8. Glucosinolates (mg 100 g^{-1} FW) Detected and Present in *Raphanus sativus* (Radish), *Lepidium sativum* (Garden Cress), and *Sinapis alba* (White Mustard) Sprouts[a]

			radish					garden cress					white mustard				
peak	code	compound	D0	D4	D8	D12	$LSD_{0.05}$[b]	D0	D4	D8	D12	$LSD_{0.05}$	D0	D4	D8	D12	$LSD_{0.05}$
1	GLB	glucobrassicin	nd	nd	nd	nd		Tr	nd	nd	nd		Tr	Tr	Tr	Tr	
2	PRO	progoitrin	nd	nd	nd	nd		27.45	Tr	Tr	Tr		nd	nd	nd	nd	
3	GRE	gluconapoleiferin	883.75a	394.37b	197.34c	119.24d	8.97***	Tr	nd	nd	nd		nd	nd	nd	nd	
4	GRA	gluconapoleiferin	nd	nd	nd	nd		Tr	nd	nd	nd		Tr	Tr	Tr	Tr	
5	EPRO	epiprogoitrin	nd	nd	nd	nd		nd	nd	nd	nd		64.06	63.97a	25.49b	20.55b	1.61***
6	SIN	sinigrin	nd	nd	nd	nd		Tr	Tr	Tr	Tr		Tr	Tr	Tr	Tr	
7	GAL	gluconapin	Tr	Tr	Tr	Tr		2.39	Tr	Tr	Tr		nd	nd	nd	nd	
8	GLI	glucoiberin	nd	nd	nd	nd		11.34c	18.30a	15.80b	15.99b	0.61***	2749.33a	2350.42b	718.02c	680.66c	45.73***
9	GNL	gluconapoleiferin	nd	nd	nd	nd		Tr	nd	nd	nd		nd	nd	nd	nd	
10	GNA	glucoiberin	Tr	Tr	5.88a	5.73a	0.68 **	22.27a	10.23b	7.56c	7.47c	0.52***	nd	Tr	Tr	Tr	
11	GIV	glucoberteroin	Tr	Tr	Tr	Tr		nd	nd	nd	nd		nd	nd	nd	nd	
12	OHGBS	4-hydroxyglucobrassicin	426.16a	164.62b	74.03c	30.92d	5.72***	4.34	Tr	Tr	Tr		39.31a	27.58b	19.73c	19.31c	0.66***
13		4-acetyl	nd	nd	nd	nd		nd	nd	nd	nd		nd	nd	nd	nd	
14	GTP	glucotropaeolin	nd	nd	nd	nd		369.08a	165.97b	141.61b	142.36b	8.81***	nd	6.16a	2.99b	2.36b	0.22***
15	GBN	glucobrassicanapin	nd	nd	nd	nd		nd	nd	nd	nd		nd	nd	nd	nd	
16	GER	glucoerucin	nd	nd	nd	nd		nd	nd	nd	nd		nd	nd	nd	nd	
17	DER	dehydroerucin	Tr	Tr	Tr	Tr		nd	nd	nd	nd		nd	nd	nd	nd	
18		4-pentyl	nd	nd	nd	nd		nd	nd	nd	nd		nd	nd	nd	nd	
19	GBS	glucobrassicin	Tr	Tr	Tr	Tr		Tr	Tr	Tr	Tr		9.01a	5.72b	5.16c	2.89c	0.52***
20	GST	glucohesperin	Tr	Tr	Tr	Tr		Tr	Tr	Tr	Tr		Tr	Tr	Tr	Tr	
21	GST	glucomastartin	nd	nd	nd	nd		Tr	nd	nd	nd		nd	17.88a	17.14ab	16.54b	0.30***
22	3OHB	4-methoxyglucobrassicin	40.45a	7.15d	29.96b	12.59c	0.89***	Tr	3.44c	9.07b	10.50a	0.06***	nd	17.57b	21.84b	31.34a	1.15***
23		4-hexyl	nd	nd	nd	nd		nd	nd	nd	nd		nd	nd	nd	nd	
24	NGBS	neoglucobrassicin	nd	nd	nd	nd		Tr	Tr	Tr	Tr		nd	34.38a	7.51b	5.61b	0.84***

[a]Mean values (n = 3). Different lower case letters indicate statistically significant differences between seeds and days (for each variety). Tr, traces, not quantified (<0.5 mg 100 g^{-1} FW); nd, not detected (<0.1 mg 100 g^{-1} FW). [b]Least significant difference (LSD) for separating means in the respective columns. The LSD was computed only after analysis of variance indicated a significant ($p < 0.05$) entry effect. ANOVA p value: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ns, $p > 0.05$.

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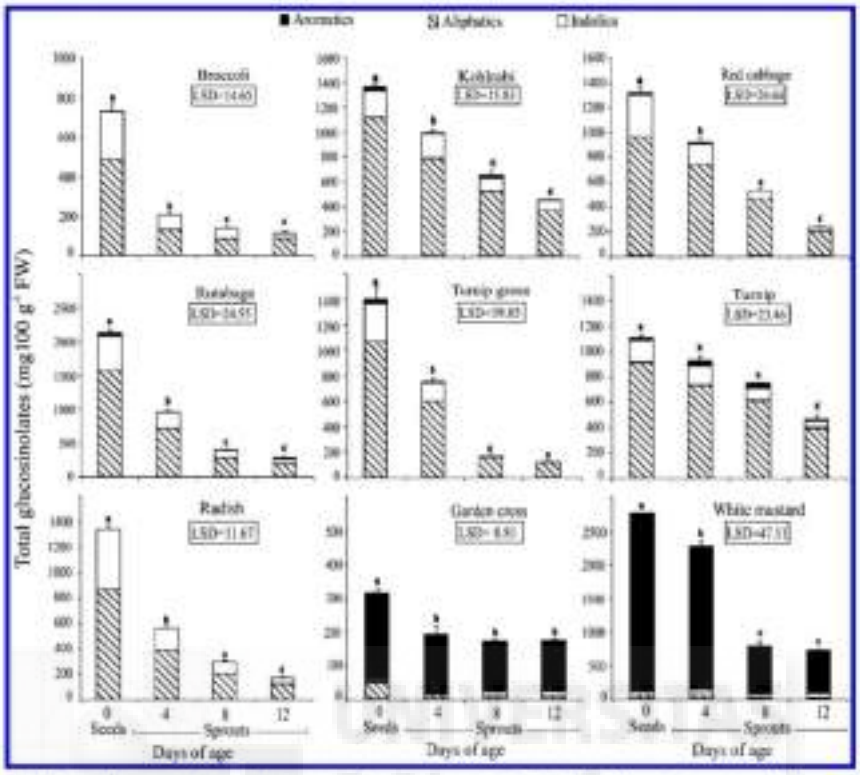

Figure 1. Aliphatic, indolic, and aromatic glucosinolates in cruciferous sprouts at 0, 4, 8, and 12 days after sowing. Values are the mean of three replicates representing mg 100 g^{-1} FW. Bars represent ±SD, and different symbols indicate significant differences between groups in the same parameter ($p < 0.05$).

In radish, the decreases of glucoraphenin and the increases of 4-methoxyglucobrassicin are convergent with data obtained by Ciska et al.[27] The age effect on the aliphatic, indolic, and aromatic GLSs showed little differences among varieties (Figure 1); because all species were cultivated under the same conditions, the observed variation in the level of total GLSs is expected to be mainly due to the genetic variation, as found by other authors,[1,5] as well as differences in characteristic individual GLSs in each species. Kohlrabi, red cabbage, turnip, and white mustard sprouts showed the highest amount of GLSs on days 8 and 12 of the germination period. The importance of GLSs, and more specially their hydrolysis products, in human health has been demonstrated by many researchers.[6,17,29] By selection of cruciferous crops, the level of desirable glucosinolates (i.e., glucoraphanin) can be enhanced considerably, which can lead to a substantial increase of the intake of health-promoting glucosinolates even without increasing the overall vegetable consumption. By contrast, a reduction of detrimental glucosinolates (progoitrin) has been carried out as a potential application for producing improved Brassicaceae vegetable breeding.[33] To reach this goal also the critical points in the finally consumed product (industrial processing and consumer preparation) have to be optimized and controlled.[18]

Phenolic Compounds. The phenolic composition of Brassicaceae vegetables has been recently investigated and, nowadays, the profile of the *Brassica* species is well established. The main classes of phenolic compounds found in crucifers were flavonols (mainly quercetin and kaempferol, but also isorhamnetin in some species) and hydroxycinnamic acids (specifically sinapic acid and chlorogenic acid derivatives). Phenolic compounds in seeds were significantly higher in content (Table 4) and variability ($p < 0.001$) than in sprouts (from ~3778 mg 100 g^{-1} FW in radish and ~1149 mg 100 g^{-1} FW in kohlrabi) except for garden cress and white mustard, which had lower values (~ 492 and 182 mg 100 g^{-1} FW, respectively). These results in seeds showed differences among varieties and species suggesting the genotype as the main factor of variation. A decrease of phenolic compounds with growth was observed, although in terms of total contents, from seeds to days 8 and 12 of germination, by approximately 50 and 65%, respectively, in broccoli; by 30% in kohlrabi; by 35 and 55%, respectively, in red cabbage and turnip; and 70, 75, and 75% in rutabaga, turnip greens, and radish, respectively (Table 4). Garden cress showed similar values from seeds to 8-day-old sprouts (~505 mg 100 g^{-1} FW), recording a decrease by 15% in 12-day-old sprouts. White mustard presented a 75% increase in total phenolics after sprouting. The sprouting seeds, due to their physiological stage,[35] showed higher values of total phenolics than commercial mature plants. The main phenolic compound group is the sinapic acid derivatives in seeds and sprouts.[33] These compounds accounted for >98% of the total phenolics, whereas flavonols and chlorogenic acid derivatives were <2% (Figure 2). In our study, sinapic acid derivatives accounted for approximately 70 and 80% in *B. rapa* seeds and sprouts, respectively, but showed higher values of flavonols in seeds (25−30% of total phenolics) and sprouts (21% in 8-day-old

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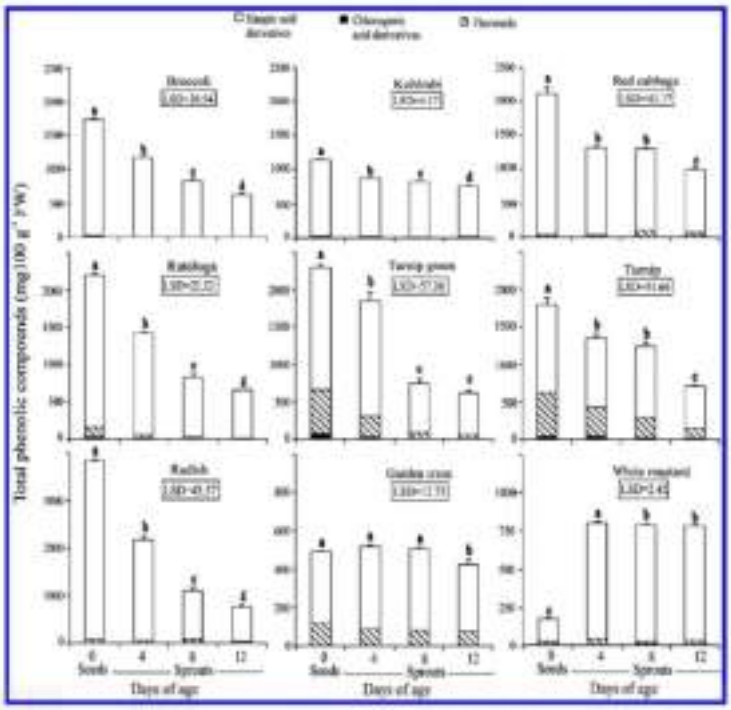

Figure 2. Sinapic acid derivatives, flavonols, and chlorogenic acid derivatives in cruciferous sprouts at 0, 4, 8, and 12 days after sowing. Values are the mean of three replicates representing mg 100 g^{-1} FW. Bars represent ±SD, and different symbols indicate significant differences between groups in the same parameter ($p < 0.05$).

sprouts of turnip; 11% in 8-day-old sprouts of turnip greens). These values may be associated with the presence of isorhamnetin, a flavonol that is almost absent in *B. oleraceae*.[32] Chlorogenic acid derivatives were reported between 1 and 4% in turnip crops, recording higher values in seeds. Rutabaga, radish, and white mustard also registered high values of sinapic acid derivatives (~90%), as well as flavonols (from 2 to 6% in seeds and from 2 to 7% in 8-day-old sprouts) and chlorogenic acid derivatives (~1%). Garden cress seeds and sprouts recorded between 75 and 80% of sinapic acid derivatives and between 15 and 20% of flavonols, containing traces of chlorogenic acid derivatives (Figure 2). According to earlier works,[33] red cabbage seems to be a very good source of phenolics among *B. oleraceae*, with values similar to those found in turnips on day 8 of germination (~1300 mg 100 g^{-1} FW total phenolics). After 12 days of sprouting, also radish (752 mg 100 g^{-1} FW) and kohlrabi (766 mg 100 g^{-1} FW) showed high values of total phenolics. In our results, and according to other authors,[20,26,33−35] hydroxycinnamic acids are predominant phenolics, and flavonols were found in lower concentrations. The distribution of phenolic compounds was variable according to the variety evaluated.

In Vitro Antioxidant Capacity. Comparison of antioxidant capacity between varieties was used as a comparison criterion in the study, and it is also useful to correlate with the phenolic compounds in the sprouts and seeds. Similarly to what was previously found, the antioxidant activity of the vegetables largely depends on growth conditions.[35,36] Two different methods to evaluate the antioxidant capacity were used: a free radical scavenging method (DPPH• assay) and a ferric reducing antioxidant potential (FRAP) assay (Table 5). These methods have been widely used because they require relatively standard equipment and provide rapid and reproducible results. Antioxidant activity values obtained with the FRAP assay were higher than those obtained with the DPPH• assay (Table 5), coinciding with Ali et al.[37] All species tested showed a decrease of the antioxidant capacity during the germination period. The activity expressed on a fresh weight basis (FW) may be influenced by the dilution effect. Statistically significant variations among species for seeds and sprouts for the DPPH• assay were found, with values ranging from 1.51 to 0.19 mM Trolox g^{-1} FW in seeds, and for the FRAP assay, these values ranged from 2.08 to 0.49 mM Trolox g^{-1} FW (Table 5). These values are similar to those previously reported for broccoli sprouts,[7] cabbage,[36] and radish.[38] As for germinating seeds, 4-day-old sprouts provided the highest values of antioxidant capacity (from 1.00 to 0.12 mM Trolox g^{-1} FW in the DPPH• assay and from 2.46 to 0.49 mM Trolox g^{-1} FW in the FRAP assay). Because a minimum of 8 days of growth is necessary to provide commercial edible sprouts, at this point red cabbage, turnip, and kohlrabi were the varieties with the highest values of antioxidant capacity, around 0.75 and 1.60 mM Trolox g^{-1} FW on day 8 and 0.50 and 1.00 mM Trolox g^{-1} FW on day 12, obtained by the DPPH• and FRAP assays, respectively. Results exhibited relatively significant ($p < 0.01$) correlation between values of total phenolics and antioxidant capacity ($r^2 = 0.686$ for the DPPH• assay and $r^2 = 0.712$ for the FRAP assay). Because sinapic acid derivatives were the predominant group of phenolic compounds analyzed, similar values for correlation with antioxidant capacity were found. The trend for both assays of the nine sprout varieties tested did not vary markedly, and a significant correlation

($p < 0.001$) between methods ($r^2 = 0.965$) was found, in agreement with Dudonné et al.,[39] who reported $r^2 = 0.822$. These values of antioxidant capacity of sprouts reached a 10-fold increase compared to commercial adult plants studied by different authors.[38,39] Some previously published results indicated similar values in broccoli sprouts[7] and radish sprouts.[38] In agreement with Podsedek et al.,[34] red cabbage belongs to the group of *Brassica* species with higher antioxidant capacity. Phenolic compounds are the major natural antioxidants of crucifers, and in broccoli,[40] it was reported they were responsible for 80−95% of the total antioxidant capacity.

To summarize, Brassicaceae sprouts are foods rich in glucosinolates and natural antioxidants. The differences observed in GLS profiling among genotypes are both qualitative and quantitative, finding characteristic GLSs in different species. The phenolic compounds also showed significant differences between varieties in accordance with previous results.[32,33] The sprouts with better biomass ratio should be selected (i.e., red cabbage and radish) also with higher glucosinolates, phenolics, and antioxidant capacity. On the other hand, white mustard, turnips, or kohlrabi, having the highest concentrations of glucosinolates, showed lower values of biomass.

The selection of suitable varieties and the germination time, 8- and 12-day-old sprouts, for biomass and size is important to maximize the health-promoting properties of the sprouts, even without increasing the overall vegetable consumption. To reach this goal, also critical points of industrial processing and consumer preparation need to be optimized.[15] Further research is guaranteed for the understanding of the bioavailability and metabolism of these phytochemicals to allow scientifically backed statements and recommendations for dietary intake, effective dosages, and dietary guidelines for nutrition and health.

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Notes

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2. USING ELICITATION TO ENHANCE THE CONTENT OF BIOACTIVE COMPOUNDS IN CRUCIFEROUS SPROUTS

(Publications 2, 3 and 4)

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Biotic Elicitors Effectively Increase the Glucosinolates Content in *Brassicaceae* Sprouts

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Supporting Information

ABSTRACT: Several biotic elicitors have been used in *Brassicaceae* species to enhance their phytochemical quality. However, there is no comparison between elicitors under controlled growth conditions. In order to draw general conclusions about the use of elicitors to enrich ready-to-eat sprouts in health-promoting glucosinolates, the aim of this study was to unveil the effect of the phytohormones methyl jasmonate (25 μM), jasmonic acid (150 μM), and salicylic acid (100 μM), the oligosaccharides glucose (277 mM) and sucrose (146 mM), and the amino acid DL-methionine (5 mM) as elicitors over 8-day sprouting *Brassica oleracea* (broccoli), *Brassica napus* (rutabaga cabbage), *Brassica rapa* (turnip), and *Raphanus sativus* (China rose radish and red radish), representative species high in glucosinolates previously studied. Results indicated that the phytohormones methyl jasmonate and jasmonic acid and the sugars acted as effective elicitors, increasing the total glucosinolate contents of the sprouts, particularly, glucoraphanin (from 183 to 294 mg·100 g^{-1} in MeJA-treated broccoli sprouts), glucoraphenin (from 33 to 124 mg·100 g^{-1} and from 167 to 227 mg·100 g^{-1} in MeJA-treated China rose radish and red radish, respectively), and glucobrassicin (from 23.4 to 91.0 mg·100 g^{-1} and from 29.6 to 186 mg·100 g^{-1} in MeJA-treated turnip and rutabaga sprouts, respectively).

KEYWORDS: *germinating seeds, Brassicaceae, elicitation, healthy edible sprouts, glucosinolates*

INTRODUCTION

Brassicaceae (cruciferous) sprouts are a good source of vitamin C, vitamin A, folic acid, dietary fiber, and minerals, which have higher levels of phytochemicals, glucosinolates (GLSs), and phenolic compounds compared to adult plants because of their physiological state.[1,2] As the phytochemical content of the sprouts decreases over the germination period due to a dilution effect of tissue expansion, 8-day-old sprouts were considered optimum for consumption, biomass, and size in order to deliver their health-promoting properties.[3]

Cruciferous vegetables have been widely investigated because of their economic importance and content of health-promoting phytochemicals with a positive effect against various pathologies and chronic diseases.[4] In particular, interest has been focused on GLSs, nitrogen- and sulfur-containing secondary metabolites mainly found in *Brassicaceae*, the precursors of bioactive isothiocyanates (ITCs), which are released by myrosinase (β-thioglucoside glucohydrolase; E.C. 3.2.1.147) hydrolysis upon chewing, cutting, or other mechanical disruption or by the intestinal microflora upon intake of vegetables tissues.[5] *Brassica oleracea* is the mainly harvested species of this family, such as broccoli and cauliflower, and a variety of horticultural crops, such as *Brassica napus* (rutabaga), *Brassica rapa* (turnip and rapini), and *Raphanus sativus* (radishes). The differences in the phytochemical profiling among species are both qualitative and quantitative, finding characteristic GLSs in different species.[5,6] Broccoli sprouts have been intensively studied due to their high concentration of glucoraphanin and its hydrolysis product sulforaphane (4-methylsulfinylbutyl ITC). Also, the ITC iberin (3-methylsulfinylpropyl ITC) from its GLS glucoiberin has shown properties as inducer carcinogen detoxification (phase II enzymes).[7] Radish sprouts contain beneficial GLSs as well, such as dehydroerucin, also called glucoraphasatin, and glucoraphenin, which breakdown products, raphasatin (4-methylsulfanyl-3-butenyl ITC) and sulforaphene (4-methylsulfinyl-3-butenyl ITC), respectively, and show selective cytotoxic/apoptotic activity on three human colon carcinoma cell lines.[8] Indolic GLSs (glucobrassicin, 4-methoxyglucobrassicin, and neoglucobrassicin GLS) are present in *B. oleraceae*, *B. rapa*, *B. napus*, and *R. sativus* species, and their hydrolysis products, indoles, have also exhibited protective activities against many types of cancer.[9]

Elicitors are substances which induce physiological changes in the plant. Biotic elicitors have biological origin and are commonly applied to enhance the phytochemical composition of plants.[10,11] Depending on the type of compound, the plant activates different signaling pathways to synthesize an optimal mixture of defensive metabolites. The phytohormones salicylic acid (SA) and jasmonic acid (JA) play key roles in this signal interplay for defense gene expression, being accumulated following pathogenic or environmental stresses. Moreover, addition of exogenous JA and its methyl ester, methyl jasmonate (MeJA), or SA can also simulate pathogen-induced plant defense responses and lead to production of bioactive secondary metabolites though several mechanisms.[11,12] Sugars, such as glucose and sucrose, are also recognized as effective signaling molecules throughout plant life, modulating many developmental and metabolic processes including ROS-

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scavenging functions, germination, development, photosynthesis, carbon and nitrogen metabolism, flowering, stress responses, and senescence.[13] Finally, previous experiments demonstrated that also application of the amino acid methionine, as a biosynthetic precursor, led to enhanced GLSs contents in radish as well as in broccoli heads.[14,15]

The aim of this study was to investigate the effect of the most active elicitors found in the literature, the JA,[16] methyl jasmonate,[17,18] salicylic acid,[18] glucose,[19] sucrose,[20,21] and D,L-methionine,[14] using 5 days of treatment from 3- to 8-day of sprouting under controlled growth conditions of *B. oleracea* (broccoli), *B. napus* (rutabaga), *B. rapa* (turnip), and *R. sativus* (China rose and red radishes), rich in aliphatic and indolic GLSs because their young physiological state, in order to provide fresh, safe, and ready-to-eat sprouts, maximizing their health-promoting compounds.

MATERIAL AND METHODS

Chemicals. Jasmonic acid, sucrose, and glucose were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA); methyl jasmonate was purchased from SAFC (St. Louis, MO, USA); salycilic acid and ethanol absolute were obtained from Panreac S.A. (Barcelona, Spain). D,L-Methionine was from Alfa Aesar GmbH & Co. (Karlsruhe, Germany). Formic acid (98−100%) for analysis was obtained from EMSURE, ACS, Reag. Ph Eur, Merck, KGaA (Darmstadt, Germany). Trifluoroacetic acid for optima LC/MS was purchased from Fisher Scientific Co. (New Jersey, USA). Methanol and acetonitrile were LC-MS grade from HiPerSolv Chromanorm, BDH Prolabo (Leuven, Belgium). Sinigrin monohydrate was obtained from Phytoplan (Germany).

Plant Material and Germination Conditions. Seeds provided by Intersemillas S.A. (Valencia, Spain) were of commercial quality for ready-for-sprouting lines. Five varieties from the *Brassicaceae* family were used: broccoli (*B. oleracea* L. var. *italica*), rutabaga (*B. napus* L. var. *napobrassica*), turnip (*B. rapa* L. subsp. *rapa*), China rose radish (*R. sativus* L. cv. China rose), and red radish (*R. sativus* L. cv. Rambo). Seeds were rinsed in distilled water and immersed in 5 $g \cdot L^{-1}$ sodium hypochlorite under aeration for 24 h. After pouring off the soaking water, the seeds were weighed (day 0) and spread evenly on trays (5 g per tray) lined with cellulose growth pad (CN Seeds, U.K.) and irrigated everyday with Milli-Q water with 5 $g \cdot L^{-1}$ sodium hypochlorite. Aliquots of 5 g of seeds were frozen in liquid nitrogen and stored at −80 °C pending phytochemical analysis.

The three replicates (trays) per sample were germinated for 2 days in a controlled dark chamber at 28 °C, for increasing the stem elongation of sprouts. Then, trays were transferred to a controlled environment chamber with a 16 h light/8 h dark cycle and air temperatures of 25 and 20 °C, respectively. The relative humidity (RH) was 60% (*day*) and 80% (*night*). Photosynthetically active radiation (PAR) of 400 $\mu mol\ m^{-2}\ s^{-1}$ was provided by a combination of fluorescent tubes (Philips TLD 36 W/83, Hamburg, Germany; Sylvania F36W/GRO, Danvers, MA, USA) and metal halide lamps (Osram HQI.T 400 W, Munich, Germany). Three replicates per treatment of *Brassicaceae* sprouts samples were rapidly and gently collected at day 8 after germination, in the middle of the light period, for analysis. All samples were weighed (fresh mass), collected separately, flash frozen in liquid nitrogen, and stored at −80 °C prior to analyses.

Treatments with Elicitors. The phytohormones jasmonic acid (JA) (150 μM), methyl jasmonate (MeJA) (25 μM), and salicylic acid (SA) (100 μM), the oligosaccharides glucose (277 mM) and sucrose (146 mM), and the amino acid D,L-methionine (5 mM) were selected as elicitors according to a literature review. JA, MeJA, and SA were dissolved in 0.2% ethanol in Milli-Q water. Sucrose and glucose were also dissolved in Milli-Q water. D,L-Methionine was dissolved in 0.04% ethanol in Milli-Q water. Elicitors were applied as exogenous spraying on the cotyledons (not as soaking or irrigation solution) with 30 mL of test solution per sample (10 mL per tray) from day 3 to day 7 of sprouting (5 days of treatment) using Milli-Q water as control.

Extraction and Determination of Glucosinolates. *Sample Extraction.* Freeze-dried samples (100 mg) were extracted with 1.5 mL of methanol 70% V/V in a US bath for 10 min, then heated at 70 °C for 30 min in a heating bath, with shaking every 5 min using a vortex stirrer, and centrifuged (17 500 × *g*, 15 min, 4 °C). Supernatants were collected, and methanol was completely removed using a rotary evaporator. The dry material obtained was redissolved in 1 mL of ultrapure water and filtered through a 0.45 μm Millex-HV13 filter (Millipore, Billerica, MA, USA).

HPLC-DAD-ESI-MS^n Qualitative and Quantitative Analysis of Glucosinolates. First, the separate intact GLSs were identified from the extracted samples following their MS^2 $[M - H]^-$ fragmentations in HPLC-DAD-ESI-MS^n, carried out on a Luna C18 100Å column (150 × 1.0 mm, 3 μm particle size; Phenomenex, Macclesfield, U.K.). Water:formic acid (99:1, v/v) and acetonitrile were used as mobile phases A and B, respectively, with a flow rate of 20 μL/min. The linear gradient started with 1% of solvent B, reaching 17% solvent B at 15 min up to 17 min, 25% at 22 min, 35% at 30 min, 50% at 35 min, which was maintained up to 45 min. The injection volume was 3 μL. Chromatograms were recorded at 227 nm. HPLC-DAD-ESI/MS^n analyses were carried out in an Agilent HPLC 1200 (Agilent Technologies, Waldbronn, Germany) and coupled to a mass detector in series. The HPLC system consisted of a binary capillary pump (model G1376A), an autosampler (model G1377A), a degasser (model G1379B), a sample cooler (model G1330B), and a photodiode array detector (model G1315D) and controlled by ChemStation software (v.B.0103-SR2). The mass detector was a Bruker, model UltraHCT (Bremen, Germany), ion trap spectrometer equipped with an electrospray ionization interface (ESI) and controlled by Bruker Daltonic Esquire software (v.6.1). Ionization conditions were adjusted at 350 °C and 4 kV for capillary temperature and voltage, respectively. The nebulizer pressure and flow rate of nitrogen were 65.0 psi and 11 L/min, respectively. The full-scan mass covered the range from *m/z* 50 to 600. Collision-induced fragmentation experiments were performed in the ion trap using helium as the collision gas with voltage ramping cycles from 0.3 up to 2 V. Mass spectrometry data were acquired in the negative ionization mode for glucosinolates. MS^n was carried out in the automatic mode on the more abundant fragment ion in $MS^{[n-1]}$. Then, the extracted samples (10 μL) were analyzed and quantified in a Water HPLC-DAD system (Waters Cromatografía S.A., Barcelona, Spain) as previously described by Pérez-Balibrea et al.[22] Intact GLSs were identified following their UV spectra and the order of elution previously described for the acquisition conditions. Glucosinolates were quantified using sinigrin as external standard, because of the similar structure to the glucosinolates in the sample.[23,24]

Statistical Methods. All assays were conducted by triplicate. Data were processed using the SPSS 15.0 software package (LEAD Technologies, Inc., Chicago, IL, USA). We carried out a multifactorial analysis of variance (ANOVA) and the Ducan's Multiple Range Test to determine significant differences at P values < 0.05.

RESULTS AND DISCUSSION

Biomass. The weight of seeds and sprouts was collected on day 0 (embebed seeds) and day 8. The ratio of fresh weight between sprouts and seeds as an indication of biomass production (Table 1) showed the expected increase in weight over sprouting and served as a quality index to select species with higher biomass production. Growing plants are exposed to a range of genetic, environmental, biotic, and abiotic factors which affect their growth and yield.[24] The biomass of the *Brassicaceae* sprouts treated with sucrose increased significantly over other treatments, ranging from about 15% in turnip and China rose radish to 80% in Red radish (Table 1), in agreement with results of Guo et al.[25] using a 146 mM sucrose treatment. Stewart et al.[26] explained that sucrose (88 mM) alters the growth rate and causes a dramatic increase in hypocotyl length.

Table 1. Biomass of Sprouts:Seeds Ratio in Cruciferous Edible Sprouts on a Fresh Weight Basis

species	broccoli	rutabaga	turnip	china rose radish	red radish
control	2.67[a]c	4.34b	2.89c	3.29b	1.45b
methyl jasmonate	2.24 cd	5.39a	2.71 cd	3.78b	1.61b
jasmonic acid	1.55e	3.15c	1.37e	2.14c	2.28a
salicylic acid	1.50de	4.24b	1.54de	2.50c	1.39b
glucose	0.95e	3.94bc	1.03e	2.38c	1.92a
sucrose	4.21a	6.43a	5.17a	5.76b	2.65a
DL-methionine	3.67b	4.18b	2.81b	5.13a	2.34a
$LSD_{0.05}$ [b](ANOVA $P < 0.001$)	0.22[b]***	0.27**	0.47*	0.15***	0.21***

[a]Mean values ($n = 3$) comparing species for each elicitor treatment, followed by different lowercase letters are significantly different at $P < 0.05$. a−h, Different lowercase letters mean statistically significant differences among elicitor treatments ($p < 0.05$). [b]Least significant difference (LSD) for separating means in the respective column. The LSD was computed only after analysis of variance indicating a significant ($p < 0.05$) entry effect. Levels of significance for each sampling day between species. Nonsignificant at $P > 0.05$ (n.s.); significant at $P < 0.05$ (*); significant at $P < 0.01$ (**); significant at $p < 0.001$ (***).

Sucrose could supply a balanced carbon source for cell growth by hydrolysis of invertase and sucrose synthase, with the resulting hexose directly participating in the glycolytic and pentose phosphate pathway (required for cells to synthesize nucleic acids and quickly replicate).[27] Stressful conditions such as starvation or hypoxia result in low energy status in the cell; Smeekens et al.[28] showed that sugars repressed the bZIP growth regulatory system activity in a concentration-dependent manner; therefore, our employed dose (5g/100 mL) was appropriate for biomass increase in sucrose treatment sprouts but not in the case of glucose, also in accord with Mirnezhad.[29]

DL-Methionine also showed a positive effect, increasing the fresh weight of sprouts in almost all varieties, 30% in broccoli, 4% in turnip, 57% in China rose radish, and 75% in red radish, except for rutabaga, agreeing with previous reports.[30,31] Gigolashvili et al.[32] reported a relationship between the overexpression of the HAG1/MYB28 gene, specific for methionine-derived GLSs (aliphatics), and strongest growth phenotype in *Arabidopsis thaliana*. On the other hand, glucose and the phytohormones (JA, MeJA, and SA) did not increase the fresh weight of sprouts and even reduced the size as for the control, as happened in broccoli, turnip, and China rose radish, founding a decrease around 60% in JA- and SA-treated sprouts, as also found by Kastell et al.[33] MeJA and SA regulate the overexpression of the OBP2 transcription factor involved in GLS biosynthesis, which altered the phenotype of *A. thaliana*, with smaller leaves,[34] supporting our result. In red radish sprouts, nonsignificant differences were found between the glucose- and phytohormones-treated sprouts and the controls. Higher values of biomass ratio not only means better growth (data not shown) but also higher fresh weight, making the sprouts more palatable. Concentration of elicitor and interval between treatment and harvest induce different responses characteristic of plant species, making it necessary to find the required effective dose and time empirically.[35]

Glucosinolate Profiles of *Brassicaceae* Sprouts. Identification and quantification of individual GLSs in seeds and 8-day-old sprouts of the five *Brassicaceae* cultivars are presented in Tables 2−5. The molecular ion $[M - H]^-$ (m/z) of GLSs, their fragment ion pattern, and retention times allowed identification of 16 different compounds.[5] The MS^2 fragmentation of aglycone side chain produces the most consistent ion at m/z 259, and the MS^3 fragmentation of this ion gives rise to fragments at m/z 97 (corresponding to the sulfate group) by dissociation of GLSs in the ion trap mass spectrometer, constituting a very useful preliminary screening method for determining the presence of GLSs in sprouts.[36] Sixteen GLSs, belonging to the aliphatic, indolic, and aromatic classes based on their different side chain structure, were detected. Results showed significant differences of the characteristic GLSs profile among cruciferous seeds and sprouts (Tables 2−5). The aliphatic GLSs were the major group in *B. oleracea*, *B. napus*, and *R. sativus* sprouts, corresponding to 60% in *Brassica* and 90% in *Raphanus* varieties. In contrast, *B. rapa* sprouts showed higher amount of indolic GLSs, corresponding to 65% of the total (Table 4). Seeds exhibited the largest amount of GLSs being the nutrient reservoir organ, containing ranging concentrations from 563.79 to 1731.32 mg·100 g^{-1} F.W. in turnip and broccoli, respectively (Table 2), of interest for the composition of the sprouts during germination. According to Pérez-Balibrea et al.,[18,22] the major source of glucoraphanin are broccoli seeds and sprouts (987.02 and 182.46 mg·100 g^{-1} F.W., respectively) (Tables 2 and 3), which has been intensively studied because of its derived product sulforaphane, a potential chemopreventive beneficial compound against cancer, cardiovascular, and neurological diseases.[4] Turnip and rutabaga seeds and sprouts showed the antinutrient progoitrin as the major GLS, and glucoraphanin and gluconasturtiin were absent in the sprouts, probably degraded or diluted during germination.[22] In radish cultivars, specific GLSs in seeds were found as well (traces of the aromatic glucoberteroin). The major characteristic GLS in this species is glucoraphenin, containing 1051.88 and 32.78 mg·100 g^{-1} F.W. in China rose radish and 887.20 and 166.93 mg·100 g^{-1} F.W. in red radish in seeds and sprouts, respectively (Table 5). The bioactive sulforaphene, like sulforaphane, is a potential anticancer agent.[4] In *Brassica* species, in addition to the parent indole GLS glucobrassicin, 4-hydroxyglucobrassicin, 4-methoxyglucobrassicin, and neoglucobrassicin were also detected in the samples (Tables 2−5). Only the indole 4-hydroxiglucobrassicin GLS was present in all species, being also one of the major compounds in seeds (from 152.49 to 358.34 mg·100 g^{-1} F.W. in China rose radish and broccoli, respectively). On the contrary, in *Raphanus* sprouts only 4-hydroxiglucobrassicin and 4-methoxyglucobrassicin were detected.[6]

Phytohormones as Elicitors. The jasmonates are signal compounds in the elicitation process leading to de novo transcription, translation, and, ultimately, biosynthesis of secondary metabolites in plant cell cultures. Methyl jasmonate (MeJA) is believed to be, at least, partially hydrolyzed by endogenous esterases to free jasmonic acid (JA) within the plant tissue.[12] MeJA elicitor (25 μM) was found highly effective for almost all the 8-day-old *Brassicaceae* sprouts, increasing by 84%, 50%, 123%, 25%, and 23% the total GLSs amount in broccoli, turnip, rutabaga, China rose radish, and red radish, respectively, increasing the indoles more than the aliphatic GLSs (Tables 3−5). After MeJA treatments, the broccoli sprouts showed significantly much more glucoraphanin, glucobrassicin, 4-methoxyglucobrassicin, and neoglucobrassicin by 60%, 241%, 48%, and 247%, respectively, associated with

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Table 2. List of Individual Glucosinolates (mg 100 g^{-1} F.W.) Detected in the Seeds of *Brassicaceae* Varieties

				seeds				
peak	compound	GLS systematic name	class	broccoli	cabbage	turnip	china rose radish	red radish
1	gluconapin	3-methylsulfinylpropyl-gls	aliphatic	26.0 ± 3.45[a]	n.d.	n.d.	n.d.	n.d.
2	progoitrin	(*R*)-2-hydroxy-3-butenyl-gls	aliphatic	n.d.	127.8 ± 89.5	27.7 ± 0.56	n.d.	n.d.
3	gluconapoleiferin	4-methylsulfinyl-3-butenyl-gls	aliphatic	n.d.	n.d.	n.d.	1052 ± 36.8	887 ± 49.6
4	gluconapin	4-methylsulfinylbutyl-gls	aliphatic	987 ± 51.4	48.6 ± 3.66	28.7 ± 1.42	n.d.	n.d.
5	glucoiberin	5-methylsulfinylpentyl-gls	aliphatic	10.1 ± 12.1	n.d.	2.30 ± 1.02	n.d.	n.d.
6	glucoraphenin	(*R*)-2-hydroxy-4-pentenyl-gls	aliphatic	n.d.	11.9 ± 2.32	Tr	n.d.	n.d.
7	glucoraphin	3-butenyl-gls	aliphatic	n.d.	95.8 ± 4.62	44.9 ± 10.9	n.d.	n.d.
8	4-hydroxyglucobrassicin	4-hydroxy-3-indolylmethyl-gls	indolic	358 ± 46.2	239 ± 23.5	280 ± 24.5	152.5 ± 19.4	223 ± 8.60
9	glucobrassicanapin	4-pentenyl-gls	aliphatic	n.d.	Tr	Tr	n.d.	n.d.
10	glucoerucin	4-methylthiobutyl-gls	aliphatic	224 ± 43.7	n.d.	n.d.	3.96 ± 0.36	n.d.
11	dehydroerucin	4-methylsulfinyl-3-butenyl-gls	aliphatic	n.d.	n.d.	n.d.	85.1 ± 5.18	31.3 ± 3.33
12	glucobrassicin	3-indolylmethyl-gls	aliphatic	34.9 ± 4.61	1.79 ± 0.05	24.5 ± 7.57	n.d.	n.d.
13	gluconasturtiin	2-phenylethyl-gls	aromatic	Tr	Tr	Tr	n.d.	n.d.
14	4-methoxyglucobrassicin	4-methoxy-3-indolylmethyl-gls	indolic	6.23 ± 4.36	Tr	Tr	n.d.	n.d.
15	n-butyl	n-butyl-gls	aliphatic	Tr	n.d.	n.d.	n.d.	n.d.
16	neoglucobrassicin	*N*-methoxy-3-indolylmethyl-gls	indolic	7.36 ± 1.47	Tr	7.14 ± 3.58	n.d.	n.d.
		total		1753a	1666a	564d	1393b	1143c

^{a}Mean values (*n* = 3 ± SD). Tr, traces, not quantified; n.d., not detected. a–d, Different lowercase letters mean statistically significant differences in the total glucosinolates content between species. ^{b}Least significant difference (LSD) for separating means in the respective row. The LSD was computed only after analysis of variance indicated a significant ($p < 0.05$) entry effect. ANOVA p value. ^{c}(*) $p < 0.05$. ^{d}(**) $p < 0.01$. ^{e}(***) $p < 0.001$; n.s. $p > 0.05$.

Table 3. List of Individual and Total Glucosinolates (mg 100 g^{-1} F.W.) in Broccoli (*B. oleracea*) Sprouts under Elicitor Treatments

		broccoli							
peak	compound	control	MeJA	JA	SA	glucose	sucrose	DL-methionine	LSD$_{0.05}$[b]
1	glucoiberin	10.8[a]a	7.68ab	9.33a	3.41b	6.35ab	11.9a	11.5a	3.86[c]
2	progoitrin	Tr	Tr	Tr	Tr	Tr	Tr	Tr	
3	glucoerysolin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	
4	glucoerysolin	183c	284a	265ab	288ab	297a	253b	208c	13.2[d]
5	glucoalyssin	0.46b	Tr	0.70a	Tr	Tr	Tr	Tr	0.16[c]
6	glucoraphenin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	
7	gluconapin	Tr	Tr	Tr	Tr	Tr	Tr	Tr	
8	4-hydroxyglucobrassicin	39.9bc	40.1bc	52.4a	42.4b	58.7a	44.7b	55.8a	3.09[c]
9	glucobrassicanapin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	
10	glucoerucin	39.1	31.7	36.5	39.1	36.8	41.0	37.8	4.35ns
11	dehydroerucin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	
12	glucobrassicin	55.2 cd	188.5a	86.4b	41.0d	53.8 cd	93.3b	74.2bc	8.66[c]
13	glucoraphanin	Tr	Tr	Tr	Tr	Tr	Tr	Tr	
14	4-methoxyglucobrassicin	28.7c	42.7ab	49.2a	39.8b	45.4ab	45.4ab	40.2b	2.47[c]
15	4-benzyl	Tr	Tr	Tr	Tr	Tr	Tr	Tr	
16	neoglucobrassicin	30.9c	107.0a	95.6a	25.5c	45.1bc	61.2b	43.1bc	6.69[c]
	total	388a	712a	575b	481 cd	536bc	540b	463d	21.1[c]

[a]Mean values ($n = 3$). Tr, traces, not quantified. n.d., not detected. a–d, Different lowercase letters mean statistically significant differences between treatments (for each variety). [b]Least significant difference (LSD) for separating means in the respective row. The LSD was computed only after analysis of variance indicated a significant ($p < 0.05$) entry effect. ANOVA p value. [c](*) $p < 0.05$. [d](**) $p < 0.01$. [e](***) $p < 0.001$; n.s. $p > 0.05$.

potential health benefits due to the biological activity of their products.[4] The enhancement of the aliphatic GLS glucoraphenin in China rose and red radish sprouts after MeJA treatment was 278% and 35%, respectively. Indole GLSs in turnip, rutabaga, red radish, and China rose radish sprouts were also higher than the controls and increased by 109%, 223%, 54%, and 200%, respectively. The JA (150 μM) also produced an increase of total GLSs, especially in broccoli (by 50%), rutabaga (by 95%), and turnip (by 24%), having a higher effect on the indoles than on the aliphatic GLSs (Tables 3 and 4). In contrast, scarce differences were found in total GLSs in the treated radish sprouts compared to control samples (Table 5). Salicylic acid (SA) caused an increase of 20% in total GLSs in broccoli and radish sprouts, with aliphatic GLSs being the most affected (Tables 3 and 5), and no effects were found in turnip or rutabaga sprouts (Table 4). This phytohormone produced an increase in glucoraphanin in broccoli (by 58%) as well as in glucoraphenin (by 50% and 14%) and dehydroerucin (by 18% and 29%) in China rose and red radish sprouts, respectively (Tables 3 and 5).

Biosynthesis of glucosinolates can be drastically induced by wounding, hormone application, and pathogen or herbivore attack. Berger[51] demonstrated the induction of several pathway genes after phytohormones spraying application in *A. thaliana*, where IQD1 protein, OBP2 transcription factor, and ATR1/MYB34 and HIG1/MYB51 genes were overexpressed and regarded as a regulator with respect to increased concentrations of major indole GLSs. Nevertheless, the genes respond differently to biotic stress conditions in time and the site of metabolites accumulation in the plant.[34] These treatments increased the concentration of individual health-promoting glucosinolates (such as glucoraphanin, glucoraphenin, dehydroerucin, and indole GLSs) and also of great interest had not effect or even decreased the concentrations of the antinutrient progoitrin by JA and MeJA, present in rutabaga and turnip sprouts (Tables 3–5). Similar induction of GLSs by exogenous application of phytohormones as elicitors has been previously found by different authors, particularly, increased indole-GLSs.[16−18,53,54] Consistent with Brader et al.,[57] MeJA is able to trigger accumulation of the indole GLSs by inducting the tryptophan biosynthesis as demonstrated in *A. thaliana*, in contrast to SA, which seems to play a minor role in this response. The above-mentioned treatments, particularly, JA and its ester MeJA, were highly effective elicitors in *Brassica* sprouts. On the other hand, SA was more effective in radish sprouts than JA, with the MeJA solution being an interesting common elicitor to enrich in GLSs all species studied.

Sugars as Elicitors. Nonstructural carbohydrates, both sucrose and glucose, used as elicitors, enhanced the total GLSs amount in all sprouts under study, in accordance with some studies on broccoli, cabbage, and radish sprouts.[19,20] Sucrose (146 mM) showed higher effects in *Brassica* species, increasing by 42%, 31%, and 159% the total GLSs in broccoli, turnip, and rutabaga, respectively (Tables 3 and 4). By contrast, total GLSs in radish sprouts were increased higher after glucose treatment (277 mM) by 22% and 26% in China rose and red radish, respectively (Table 5). It must be emphasized the elicitation effect observed in broccoli sprouts, where glucoraphanin was increased by 40% and 60% under sucrose and glucose treatments, respectively (Table 3). Glucoraphanin was enhanced as well, by 50% and 30%, under both sucrose and glucose spray in China rose and red radish, respectively (Table 5). The other major aliphatic GLS from radish, dehydroerucin, was increased by the glucose treatment by 22% and 33% in China rose and red radish, respectively. In contrast to what was found by Wei et al.,[19] who showed a decrease in this compound. These results were consistent with those previously reported by Guo et al.,[25] indicating that the *Bo-Elong* gene involved in the aliphatic GLSs pathway was up-regulated by sucrose. Gigolashvili et al.[32] described glucose as an important signaling molecule that may induce transcriptional regulatory mechanisms, integrating carbohydrate availability and hormone action, regulating this class of GLSs by the HAG1/MYB28 gene, in response to carbohydrate availability, in *A. thaliana*.

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Table 4. List of Individual and Total Glucosinolates (mg 100 g^{-1} F.W.) in Turnip (*B. rapa*) and Rutabaga (*B. napus*) Sprouts under Elicitor Treatments

		turnip								rutabaga							
peak	compound	control	MeJA	JA	SA	gluconapin	4-hydroxyglucobrassicin	glucobrassicanapin	LSD$_{0.05}$[a]	control	MeJA	JA	SA	gluconapin	4-hydroxyglucobrassicin	glucobrassicanapin	LSD$_{0.05}$[a]
1	glucoiberin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.		n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	
2	progoitrin	41.9^{fg}	27.5c	3.69d	40.5fg	63.8a	43.5b	4.95^{g}	4.68^{g}	1.85e	29.9c	2.40d	1.98e	25.3d	44.8a	34.5b	8.64^{g}
3	glucoraphanin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.			n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	
4	gluconapin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.			n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	
5	glucoerysolin	1.09	0.98	Tr	Tr	Tr	1.27	0.25n.d		n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	
6	glucoerucin	Tr	Tr	Tr	Tr	Tr	Tr			Tr	Tr	Tr	Tr	Tr	Tr	Tr	
7	glucoiberin	7.81	0.38	1.50	5.19	1.06	0.80	1.41	2.14 $^{n.s}$	15.8b	17.8ab	5.36 cd	1.22d	Tr	11.8bc	24.0a	2.51^{g}
8	4-hydroxyglucobrassicin	23.6bc	25.5ab	31.9a	17.8c	33.0a	36.5ab	23.8bc	3.28^{g}	17.1c	21.5b	22.9b	17.0c	23.8b	28.7a	14.8c	1.26^{g}
9	glucobrassicanapin	Tr	Tr	Tr	Tr	Tr	Tr	Tr		Tr	Tr	Tr	Tr	Tr	Tr	Tr	
10	glucoraphin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.		n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	
11	dehydroerucin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.		n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	
12	glucobrassicin	33.4c	91.0a	41.1b	18.4c	26.1c	37.7b	25.0c	3.26^{g}	29.6c	18.8e	1.58h	34.3c	46.4b	113a	94.6d	8.38^{g}
13	gluconasturtiin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.			n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	
14	4-methoxyglucobrassicin	22.4b	22.3b	42.8a	23.0b	25.9b	23.9b	38.5b	2.05^{g}	37.8de	61.9bc	33.6de	23.2e	48.3 cd	99.6a	73.5b	5.05^{g}
15	n-benzyl	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.		n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	
16	neoglucobrassicin	34.2 cd	48.8a	36.7a	28.0cd	41.4ab	32.4bc	22.7d	6.22^{g}	33.9d	131b	16.2a	47.9d	75.8c	116b	38.4d	3.53^{g}
	total	150 cd	218a	180b	133d	176bc	189b	126d	9.13^{g}	319e	710b	631c	314e	445d	826a	388e	12.5^{g}

[a]Mean values ($n = 3$). Tr, traces, not quantified; n.d., not detected; a–d, Different lowercase letters mean statistically significant differences between treatments (for each variety). [b]Least significant difference (LSD) for separating means in the respective row. The LSD was computed only after analysis of variance indicated a significant ($p < 0.05$) entry effect. ANOVA p value: *($p < 0.05$; **) $p < 0.01$; ***) $p < 0.001$; n.s. $p > 0.05$.

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Table 5. List of Individual and Total Glucosinolates (mg 100 g^{-1} F.W.) in China Rose Radish and Red Radish (*R. sativus*) Sprouts under Elicitor Treatments

		China rose radish								red radish							
peak	compound	control	MeJA	JA	SA	glucose	sucrose	D-methionine	LSD_{tuk}[a]	control	MeJA	JA	SA	glucose	sucrose	2L-methionine	LSD_{tuk}[a]
1	glucoiberin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.		n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	
2	progoitrin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.		n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	
3	glucoraphanin	32.8d	134a	88.7b	50.8c	51.4c	50.6c	31.6d	3.18[b]	187 cd	237a	185bc	191ab	210ab	225a	146d	12.8[b]
4	gluconapin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.		n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	
5	glucobrassicanapin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.		n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	
6	glucoerucin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.		n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	
7	glucoraphi	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.		n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	
8	4-hydroxyglucobrassicin	13.2b	22.6a	22.5a	16.1b	16.1b	12.7b	12.8b	1.37[b]	37.3b	44.2a	31.1b	21.1c	22.2c	41.7a	27.0b	3.42[b]
9	glucobrassicanapin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.		n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	
10	glucoerucin	1.95b	10.9a	0.59b	1.61b	0.98b	1.73b	1.95b	0.67[b]	Tr	Tr	Tr	Tr	Tr	Tr	Tr	
11	dehydroerucin	4.11bc	45.4b	3.70c	4.89b	5.02a	4.89b	4.03c	0.53[b]	17.2c	193bc	190bc	223ab	229a	183bc	156c	14.4[b]
12	glucobrassicin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.		n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	
13	gluconasturtiin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.		n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	
14	4-methoxyglucobrassicin	37.2ab	19.8c	15.5d	20.8a	25.0b	17.8 cd	29.0ab	1.51[b]	Tr	Tr	Tr	Tr	Tr	Tr	Tr	
15	4-deoxy	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.		n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	
16	neoglucobrassicin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.		n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	
	total	488bc	611a	498bc	588a	596a	520b	478c	12.1[b]	346cd	452ab	386c	431b	461a	453ab	333e	8.04[b]

[a]Mean values ($n = 3$). Tr, traces, not quantified; n.d., not detected. a–d, Different lowercase letters mean statistically significant differences between treatments (for each variety). [b]Least significant difference (LSD) for separating means in the respective row. The LSD was computed only after analysis of variance indicated a significant ($p < 0.05$) entry effect. ANOVA p value, "(*) $p < 0.05$; "(**) $p < 0.01$; "(***) $p < 0.001$; n.s. $p > 0.05$.

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As for the indole-GLSs, no effects were found on radish sprouts, while both sucrose and glucose highly and significantly enhanced 4-hydroxiglucobrassicin, 4-methoxyglucobrassicin, and neoglucobrassicin in *Brassica* species, with the glucobrassicin being mainly increased by sucrose (Tables 3 and 4). Sivanandhan et al.[39] showed that the type and concentration of carbon source induces profound effects on growth and quality of the metabolites produced. Sugars serve as the carbon and energy source and also affect the osmotic pressure of the medium, which stimulates mitochondrial activity and, hence, energy production for metabolites synthesis.[39] The secondary product formation after sugar application could be attributed to a certain level of osmotic stress, which initiated the signal perception through a receptor in the cell membrane to activate the signal transduction network. This activates the transcription factors, which regulates gene expression involved in biosynthesis of the target metabolites.[16,18]

D,L-Methionine as Elicitor. Aliphatic GLSs, such as glucoraphanin and glucoiberin, are secondary metabolites derived from amino acids, mainly methionines.[31] The effect of the exogenous spray application of this amino acid (5 mM) has been studied in order to increase the amount of GLSs in sprouts, mainly aliphatic ones.[17,18] In the biosynthesis of glucosinolates, first, methionine is transaminated to the corresponding α-keto-acids, and subsequently, the side-chain elongation of the amino acid is produced, followed by formation of the GLS core structure mediated by cytochrome P450 mono-oxygenase.[32] Only in broccoli and rutabaga sprouts a significant effect after application of this amino acid was found, where the total GLSs were increased by 19% and 85%, respectively (Tables 3 and 4). China rose radish sprouts remained without changes in GLSs contents, while turnip and red radish sprouts showed a small decrease in total GLSs after the D,L-methionine applications (Tables 4 and 5). Opposite to our first hypothesis, aliphatic GLSs were not affected to a higher degree than indole GLSs upon D,L-methionine treatment, probably resulting from expression of HAG1/MYB28 in young sprouts, reported by Gigolashvili et al.[32] Broccoli-treated sprouts showed a weak increase of 7% and 28% in aliphatic (glucoiberin and glucoraphanin) and indole GLSs (4-hydroxiglucobrassicin, glucobrassicin, 4-methoxyglucobrassicin), respectively. Rutabaga sprouts registered a significant increase (by 85%) in both aliphatic (progoitrin and gluconapin) and indole GLSs (glucobrassicin and 4-methoxyglucobrassicin). Some authors reported that application of methionine to growing broccoli plants increased not only their aliphatic GLSs content but also the indolic GLSs.[41] Few reports on the effect of methionine elicitor have been found, and based on our results we may conclude that low concentrations of methionine, such as 5 and 10 mM applied by Pérez-Balibrea et al.,[18] allowed a certain increase of total GLSs (23% and 21% respectively) than higher concentrations, such as 200 mM applied by Schreiner et al.,[41] where a similar increase by 28% was found in broccoli at the time of head formation, while no significant impact on total GLSs was found in broccoli heads or radish hypocotyls.

All elicitors promoted the accumulation of GLSs in *Brassicaceae* sprouts. Detected differences in the quantified total and individual GLSs between controls and treated sprouts were not only due to cultivar differences but also due to the specific elicitor nature used. Indole GLSs in all species were found to either increase or remain stable after elicitor treatments. The total GLSs performed in similar way. Major desirable aliphatic GLSs, such as glucoraphanin, glucoraphenin, and dehydroerucin, were increased by elicitors, except with D,L-methionine. Only undesirable aliphatic progoitrin and the glucoiberin decreased after the treatments, and minor GLSs, such as glucoerucin or gluconapin, were not affected. Elicitation practices, particularly using MeJA, could be established as an effective treatment to enrich in health-promoting GLSs cruciferous sprouts, for natural functional foods, a source of bioactive ingredients. The increase in the production of desirable healthy GLSs (glucoraphanin, glucoraphenin, dehydroerucin, and indole-GLSs) is important in order to enhance the intake of beneficial phytochemicals on a daily basis. Understanding the changes in the metabolism of sprouts is crucial to design strategies that would enhance the biosynthesis of secondary metabolites as novel cost-effective tools for nutrition and health applications that guarantee further research.

■ ASSOCIATED CONTENT

Supporting Information

Intact glucosinolates identified by HPLC-DAD-ESI-MSn in the Brassicaceae sprouts under study. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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Radish sprouts—Characterization and elicitation of novel varieties rich in anthocyanins

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ABSTRACT

The anthocyanin profile of two varieties of red radish sprouts (*Raphanus sativus*), cv. China rose and Rambo, were studied using HPLC-DAD-ESI-MS^n and HPLC-DAD. The most abundant type of anthocyanin was cyanidin and its derivatives, with one or two acylating groups, with qualitative and quantitative differences among varieties. Some compounds were identified for the first time in both varieties, to the best of our knowledge. Radish sprouts were treated during germination (days 3 to 8) using methyl jasmonate, jasmonic acid, salicylic acid, sucrose and glucose as elicitors in order to enrich their total anthocyanin content (TAC). An increase in TAC was achieved by 50% in China rose radish sprouts and by 30% in Rambo red radish after glucose treatment. Methyl jasmonate and sucrose also contribute to enhance TAC. Enriching natural food in anthocyanins may contribute to sustaining their regular intake with preventive and therapeutic roles in a number of human diseases.

1. Introduction

Promising results regarding nutrition and health benefits have been found when eating cruciferous sprouts containing significantly greater concentrations of bioactive compounds (glucosinolates and phenolics) than mature plants (10–100 times) (Hanlon & Barnes, 2011; Moreno, Pérez-Balibrea, & García-Viguera, 2006). Even though cruciferous foods are recognized for their high glucosinolates content, Brassicaceae foods are also rich in phenolic compounds (flavonoids and anthocyanins), carotenoids, vitamins and minerals (Manchali, Chidambara Murthy, & Patil, 2012). Within the bioactive compounds classes, anthocyanins are water-soluble flavonoids that usually exist in plants in the form of glycosides and acylated form. Their non-carbohydrate moieties (aglycones) are called anthocyanidins. There are many types of anthocyanins, which are distinguished according to the number and position of the hydroxyl and methoxyl groups as substituent on the B ring, type and number of conjugated sugars, and the presence or absence of an acyl group. The six most important types are pelargonidin (Pg), cyanidin (Cy), delphinidin (Dp), peonidin (Pn), petunidin (Pt) and malvidin (Mv) (Jaakola, 2013). Cy and its derivatives, which possess two hydroxyl groups on the B-ring, are the most widely distributed, followed by Dp and its derivatives (De Pascual-Teresa & Sanchez-Ballesta, 2008). They are not only responsible for the red, blue and purple colors of many fruits, vegetables, flowers and seeds, but also protect plants against various biotic and abiotic stresses (Harborne & Williams, 2000). In recent years, human intervention studies have focused on the preventive and suppressive effects of these compounds against obesity and diabetes, reducing inflammation associated with cancer pathogenesis, cardiovascular diseases, improvement of visual function and the positive effects of intake of anthocyanin-rich fruits on memory and on cognitive decline by delaying the deterioration of neural function in aged individuals by inhibition of neuroinflammation (Pojer, Mattivi, Johnson, & Stockley, 2013).

The differences in the total anthocyanin content (TAC) among red radish sprouts varieties are qualitative and quantitative, presenting mainly cyanidin derivatives, glycosylated at C-3, with the presence of one or two cinnamoyl groups (sinapoyl, feruloyl, p-coumaroyl and caffeoyl), and at C-5 position, with the presence of malonyl (Matera et al., 2015; Park et al., 2013; Wu & Prior, 2006).

Exogenous application of elicitors has been considered as a suitable strategy for the activation of secondary metabolites pathways. methyl jasmonate (MeJA), jasmonic acid (JA), salicylic acid (SA), sucrose and glucose have been selected as successful treatments for the accumulation of anthocyanins (Baenas, García-Viguera, & Moreno, 2014a). Previous studies showed that jasmonates could induce defense responses in the plant though encoding PR proteins and genes involved in biosynthesis of flavonoids (phenylalanine ammonia lyase [PAL], chalcone synthase [CHS] and chalcone isomerase [CHI]). The F-box protein coronative insensitive 1 (COI1) functions as a jasmonate receptor in *Arabidopsis*, modulating the up-regulation of PAP1 proanthocyanidin transcription factor, and dihydroflavonol reductase (DFR), anthocyanidin synthase (LDOX/ANS) and UDP-glucose:flavonoid 3-O-glucosyltransferase (UF3GT) specific anthocyanin biosynthetic genes (Shan, Zhang, Peng, Wang, & Xie, 2009). An induction of PAL and CHS activity has been also proved after SA treatment (Ghasemzadeh, Jaafar, & Karimi, 2012; Obinata et al., 2003). The induction of anthocyanin synthesis genes has been studied as sugar specific (Guo, Yuan, & Wang, 2011a; Hara, Oki, Hoshino, &

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Kuboi, 2004; Solfanelli, Poggi, Loreti, Alpi, & Perata, 2006; Teng, Keurentjes, Bentsink, Koornneef, & Smeekens, 2005); interestingly, Solfanelli et al. showed that at least one gene up-regulated by sucrose was detected in each step of the biosynthetic pathway (i.e. flavonoid biosynthetic genes such as PAL, CHI, CHS, and those specific for anthocyanin biosynthesis PAP1, DRF, LDOX/ANS, UF3GT). It is speculated that hypocotyls in radish sprouts take up sucrose rapidly and metabolize it into glucose (Hara, Oki, Hoshino, & Kuboi, 2003). In this work, two varieties of *Raphanus sativus* ready-to-eat sprouts (cv. China rose and Rambo), different in color and visual appearance (white and rose hypocotyls and green cotyledons; and purple and deep red in hypocotyls and cotyledons, respectively), were selected in order to study their anthocyanin pigments, discussing their differences and investigating the potential for enrichment by elicitation of the anthocyanin concentration, as natural healthy foods likely to be consumed daily by the general population.

2. Material and methods

2.1. Plant material and germination conditions

China rose radish (*R. sativus* var. *sativus*) and Rambo radish (*R. sativus* cv. Rambo) seeds were provided by Intersemillas S.A. (Valencia, Spain). Radish sprouts were grown according to Baenas, García-Viguera, and Moreno (2014b) with some modifications; sprouts were covered with perforated aluminum foil for increasing stem elongation in the environment chamber from days 0 to 3. Three replicates per treatment of radish sprouts were collected at day 8 after germination for analysis. All samples were frozen in liquid nitrogen and stored at −80 °C prior to analyses.

2.2. Treatments with elicitors

The phytohormones jasmonic acid (JA) (150 μM), methyl jasmonate (MeJA) (25 μM), salicylic acid (SA) (100 μM) and the oligosaccharides glucose (277 mM) and sucrose (176 mM) were selected as elicitors according to literature review (Baenas et al., 2014a). JA (Sigma-Aldrich, Co., 3050 Spruce Street, St. Louis, MO 63103, USA), MeJA (SAFC, 3050 Spruce Street, St. Louis, MO 63103, USA) and SA (Panreac, S.A., Barcelona, Spain) were dissolved in 0.2 % ethanol in Milli-Q water. Sucrose and glucose (Sigma Chemical Co., 14508, St. Louis, MO 63178, USA) were also dissolved in Milli-Q water. Elicitors were applied as exogenous treatment (spraying) on the cotyledons with 30 mL of test solution per sample (10 mL per tray) from day 3 to day 7 of sprouting (5 days of treatment), using Milli-Q water as control.

2.3. Extraction and determination of anthocyanins

2.3.1. Sample extraction

Freeze-dried samples (100 mg) were extracted with 1.5 mL of methanol/water/formic acid (25:24:1, v/v/v), according to Moreno, Pérez-Balibrea, Ferreres, Gil-Izquierdo, and García-Viguera (2010) with slight modifications. Briefly, samples were vortexed and extracted in an ultrasonic bath for 60 min at room temperature. The samples were kept at 4 °C overnight and sonicated again for 60 min. A centrifugation (model EBA 21, Hettich Zentrifugen) step (9500 ×*g*, 5 min) was used to separate the supernatant from the solid residue. This supernatant was filtered through a 0.22 μm (HPLC-DAD-ESI/MS^n) or 0.45 μm (HPLC-DAD) PVDF filter (Millex HV13, Millipore, Bedford, MA, USA) and stored at 4 °C before the analyses were performed.

2.3.2. Identification of anthocyanins by HPLC-DAD-ESI-MS^n and quantification by HPLC-DAD

Chromatographic analyses with HPLC-DAD-ESI/MS^n for qualitative analysis were conducted as described by Moreno et al. (2010). An HPLC-DAD system (Waters Cromatografía SA, Barcelona, Spain) was employed for the quantification, consisting of a W600E multisolvent delivery system, an in-line degasser, a W717Plus autosampler and a W2996 photodiode array detector set at 520 nm. Anthocyanins were quantified using cyanidin 3-*O*-glucoside-β-glucopyranoside (Polyphenols, Norway) as external standard. Chromatograms were recorded at 520 nm.

The retention time (Rt) of Tables 1 and 2 have different values than those of Table 3 because the study of MS (Tables 1 and 2) has been carried out in a different HPLC equipment than the quantification UV study (Table 3).

2.3.3. Statistical methods

All assays were conducted by triplicate. The data were processed using the SPSS 15.0 software package (LEAD Technologies, Inc., Chicago, USA). We carried out a multifactorial analysis of variance (ANOVA) and the Duncan's multiple range test to determine significant differences at P values < 0.05.

3. Results and discussion

3.1. Qualitative and quantitative analysis of anthocyanins

The identification of anthocyanins was achieved by HPLC-DAD-ESI-MS^n analysis of the lyophilized radish sprouts extracts, according to our results, the most abundant anthocyanins were cyanidin derivatives, diglycosylated at C-3 and glycosylated at C-5 position, mainly with the presence of one or two cinnamoyl groups on the glycosylated fraction at 3 position (sinapoyl, feruloyl, *p*-coumaroyl and caffeoyl) and malonyl at hexose in 5 position, according to the anthocyanins commonly described in Brassicaceae: cyanidin-3-*O*-sophoroside-5-*O*-glucoside derivatives (Andersen & Jordheim, 2006), with quantitative differences among species and crops (Cartea, Francisco, Soengas, & Velasco, 2011; Giusti, Rodriguez-Saona, Griffin, & Wrolstad, 1999; Park et al., 2014; Wu & Prior, 2005). Interpretation of mass spectra was based on previous observations that fragmentation of anthocyanins occurs almost exclusively at the glycosidic bonds, attached to hydroxyls, at the 3 and/or 5 position, in addition to the possible loss of the carbonyl group (−44) or the malonyl radical (−86) (Giusti et al., 1999; Matera et al., 2012). Acylated groups were determined by calculating possible combinations of aliphatic and aromatic acids found in acylated anthocyanins (Wu & Prior, 2005).

Molecular ions of anthocyanins ($[M]^+$, m/z) and MS fragmentation are presented in Tables 1 and 2 (tables have been prepared gathering compounds with similar structure and increasing Rt; the numbers assigned to compounds in Tables 1–2 are not comparable between them, being independent by variety).

The MS screening allowed the detection of 24 anthocyanins in China rose radish (Table 1) and 47 anthocyanins in Rambo red radish (Table 2) sprouts. A mass spectroscopic analysis is absolutely required for anthocyanin characterization because compounds with similar UV spectral characteristics can have similar retention time (Giusti et al., 1999). These pigments showed similar fragmentation patterns and their relative ion intensities according to their abundance are presented in Tables 1–2.

The anthocyanin composition of the varieties China rose and Rambo red radish sprouts are reported here for the first time. Some anthocyanins have been tentatively identified for the first time while others have been reported before in Sango red radish sprouts (Matera et al., 2012; Matera et al., 2015).

Radish cv. China rose showed only acylated anthocyanins: cinnamoyl, malonyl and cinnamoyl malonyl derivatives (Table 1). The glycosylation loss from C-5 was 162 [glycosyl]$^+$ (**5**, **6**, **11**) or 248 (162 + 86) [glycosyl-malonyl]$^+$ (**1–4**, **7–10**, **12–24**) to give rise to the anthocyanidin ion bond to the glycosidic fraction at the 3-position. Moreover, a diglucosyl loss (324) (**1**) with their corresponding cinnamoyl acid ([diglucosyl-acyl]$^+$) (**2–4**, **8**, **9**, **10**, **12–15** and **20**) or [diglucosyl-acyl1-acyl2]$^+$ (**7**, **10**, **15–19** and **21–24**) was observed,

Table 1
Anthocyanins in China rose radish sprouts.[1]

Peak	Rt (min)	$[M]^+$ m/z	MS2 $[M]^+$, m/z (%)				MS3 $[M-162]^+$, m/z (%)	Compound
			−162	−(324 + acyl)	Agly			
5	26.8	979	817 (100)	449 (7)	287 (17)	287 (100)		Cy[2]-3-O-[Si]soph-5-O-glu
6	26.6	948	787 (100)	449 (20)	287 (21)	287 (100)		Cy-3-O-[FE]soph-5-O-glu
11	26.4	**963**[3]	801 (100)	433 (7)	271 (8)	271 (100)		**Pg-3-O-[Si]soph-5-O-glu**[3]
Anthocyanin 3-O-sophoroside-5-O-(malonyl)glucoside derivatives								
							MS3 $[M-324]^+$, m/z (%)	
			−44	−248	−324			
1	17.2	858	–	611 (15)	535 (100)	449 (18)	287 (40)	449 (16), 400 (13), 287 (70)	Cy-3-O-soph-5-O-(MA)glu
Anthocyanin 3-O-(cinnamoyl)sophoroside-5-O-(malonyl)glucoside/sophoroside derivatives								MS3(535/519)$[]^+$, m/z (%)	
					−(324 + acyl)	−(324 + acyl + 44)			
2	24.0	1021	977 (20)	773 (68)	535 (100)	491 (15)	287 (28)	287 (100)	Cy-3-O-soph-5-O-(MA)soph
3	24.9	1035	991 (48)	787 (67)	535 (100)	491 (46)	287 (7)	287 (100)	Cy-3-O-[FE]soph-5-O-(MA)glu
4	26.0	1021	977 (20)	773 (93)	535 (100)	491(31)	287 (17)	517 (29), 449 (22), 287 (100)	Cy-3-O-[CA]soph-5-O-(MA)glu
5	27.6	1035	991 (8)	787 (76)	535 (100)	491 (20)	287 (46)	449 (55), 287 (100)	Cy-3-O-[FE]soph-5-O-(MA)glu
9	27.6	**1065**	1021 (5)	817 (73)	535 (100)	491 (17)	-	287 (100)	**Cy-3-O-[Si]soph-5-O-(MA)glu**
12	28.2	1065	991 (15)	757 (52)	535 (100)	491 (24)	287 (36)	287 (100)	Cy-3-O-[pCoA]soph-5-O-(MA)glu
13	28.4	1035	991 (7)	787 (43)	535 (100)	491 (16)	287 (11)	287 (100)	Cy-3-O-[FE]soph-5-O-(MA)glu
14	29.3	**1065**	1021 (8)	817 (71)	535 (100)	491 (1)	-	287 (100)	**Cy-3-O-[Si]soph-5-O-(MA)glu**
20	31.2	1019	975 (13)	771(68)	535(100)	475(15)	-	433 (100), 271(32)	Pg-3-O-[FE]soph-5-O-(MA)glu
					-(324 + acyl1)	-(324 + acyl1 + acyl2)	[535–44]	MS3 $[M-248]^+$, m/z (%)	
7	27.6	**1227**	1183 (2)	979 (100)	–	535 (38)	–	787 (15), 703 (14), 287 (100)	**Cy-3-O-[CA-Si]soph-5-O-(MA)glu**
10	28.3	1197	1153 (38)	949 (100)	887 (48)	535 (43)	491 (14)	287 (100)	Cy-3-O-[CA-FE]soph-5-O-(MA)glu
15	30.0	**1227**	1183 (12)	979 (100)	679 (8)	535 (50)	491 (8)	602 (6), 287 (100)	**Cy-3-O-[CA-Si]soph-5-O-(MA)glu**
17	30.3	1197	1153 (37)	949 (100)	–	535 (53)	491 (16)	287 (100)	Cy-3-O-[CA-FE]soph-5-O-(MA)glu
22	31.8	**1227**	1183 (23)	979 (100)	–	535 (90)	491 (27)	491 (100), 287 (81)	**Cy-3-O-[CA-Si]soph-5-O-(MA)glu**
23	31.8	1197	1153 (14)	949 (74)	–	535 (100)	-	287 (100)	Cy-3-O-[CA-FE]soph-5-O-(MA)glu
	MS3 (535)$[]^+$, m/z (%)								
16	30.3	**1271**	1227 (8)	1023 (88)	–	535 (100)	491 (11)	287 (100)	**Cy-3-O-[di-Si]soph-5-O-(MA)glu**
18	30.7	1241	1197 (11)	993 (66)	–	535 (100)	-	287 (100)	Cy-3-O-[Si-FE]soph-5-O-(MA)glu
19	31.1	1241	1197 (12)	993 (63)	–	535 (100)	-	287 (100)	Cy-3-O-[Si-FE]soph-5-O-(MA)glu
21	31.5	1211	–	–	963 (100)	535 (41)	491 (14)	287 (100)	Cy-3-O-[pCoA-Si]soph-5-O-(MA)glu or Cy-3-O-(di-FE)soph-5-O-(MA)glu
24	32.5	1211	1167 (11)	–	963 (37)	535 (100)	491 (9)	287 (100)	Cy-3-O-[pCoA-Si]soph-5-O-(MA)glu or Cy-3-O-(di-FE)soph-5-O-(MA)glu

[1] Main observed fragments. Other ions were found but they have not been included.
[2] Cy = cyanidin, glu = glucoside, soph = sophoroside, pCoA = p-coumaroyl, CA = caffeoyl, SI = sinapoyl, FE = feruloyl, MA = malonyl.
[3] The anthocyanins tentatively identified for the first time are in boldface.

giving rise to the anthocyanidin ion bonded to the glycosidic fraction at the 5-position (*m/z* 535/519 in the malonyl derivatives, and 449/433 in the nonmalonated derivatives) (Table 1). Some cyanidin derivatives found were similar and coincident with previously published data on anthocyanins in *Brassicaceae* species (Matera et al., 2012; Matera et al., 2015; Park et al., 2014); nonetheless, we found and tentatively identified some new anthocyanins displayed $[M]^+$ at *m/z* 963 (pelargonidin 3-O-(sinapoyl)sophoroside-5-O-glucoside) (**11**), 1065 (cyanidin 3-O-(sinapoyl)sophoroside-5-O-(malonyl)glucoside) (**9** and **14**), 1227 (cyanidin 3-O-(caffeoyl, sinapoyl)sophoroside-5-O-(malonyl)glucoside) (**7**, **15** and **22**) and 1271 (cyanidin-3-O-(disinapoyl)sophoroside-5-O-(malonyl)glucoside) (**16**).

Red radish cv. Rambo sprouts exhibited a wide range of anthocyanins, with cyanidin being the predominant aglycone, along with smaller amounts of peonidin and delphinidin in this cultivar. Unusually, some anthocyanins have been detected whose glycosylation at position 5 is dihexoside instead of monoglucoside, tentatively identified as sophoroside (**3**, **11**, **12**, **18**, **19**, **23**, **25**, **26**, **28**, **29**–**33**, **38**, **40** and **44**), the fragmentation is similar to that described above. We observed in the malonyl-sophorosides (**11**, **12**, **18**, **19**, **23**, **25**, **28**, **31**, **32**, **38**, **40**, **44**, except for **3**) the loss of *m/z* 410 (324 + 86) due to fragmentation of the glycosydic fraction in 5-position ([diglucosyl-malonyl]$^+$), instead of the *m/z* 248 (162 + 86) ([glucosyl-malonyl]$^+$) found in the malonyl-glucoside derivatives. For the first time, we identified the following anthocyanins in red radish: the $[M]^+$ at *m/z* 757 (pelargonidin-3-O-sophoroside-5-O-glucoside) (**2**), 859 (cyanidin 3-O-sophoroside-5-O-(malonyl)glucuside) (**6**), 873 (peonidin-3-O-sophoroside-5-O-(malonyl)glucoside) (**8**), 1065 (cyanidin 3-O-(sinapoyl)sophoroside-5-O-(malonyl)glucuside) (**13**, **20** and **37**), 1181 (cyanidin 3-O-(*p*-coumaryl, feruoyl)sophoroside-5-O-(malonyl) glucuside) (**46**), 1255 (peonidin 3-O-(feruoyl,sinapoyl)sophoroside-5-O-(malonyl)glucoside) (**47**), 1227 (cyanidin 3-O-(sinapoyl)sophoroside-5-O-(malonyl)sophoroside) (**11** and **18**), 1183 (cyanidin 3-O-(caffeoyl) sophoroside-5-O-(malonyl)sophoroside) (**23**), 1167 (cyanidin 3-O-(*p*-coumaric)sophoroside-5-O-(malonyl)sophoroside) (**31**), 1389 (cyanidin 3-O-(caffeoyl, sinapoyl)sophoroside-5-O-(malonyl)sophoroside) (**25**) and 1359 (cyanidin 3-O-(caffeoyl, feruoyl)sophoroside-5-O-(malonyl)sophoroside) (**28**) presented in Table 2.

Few published works showed that the characterization of anthocyanins in radish was dependent on the studied variety (Giusti & Wrolstad, 2003; Hanlon & Barnes, 2011). Hanlon and Barnes (2011) showed a quantification of anthocyanins by classes (pelargonidin, cyanidin and

Table 2
Anthocyanins in Rambo red radish sprouts.[a,b]

Peak	R_t (min)	$[M]^+$ (m/z)	-162	-324	Agli	MS3 $[M-162]^+$ (m/z)			Compound
Non-acylated anthocyanins									
MS2 $[M]^+$ (m/z)									
1	12.1	773	611 (36)	449 (100)	287 (69)	287 (100)			Cy3-3-O-soph-5-O-glu
2	13.5	**757**[c]	595 (100)	433 (33)	271 (39)	271(100)			**Pg-3-O-soph-5-O-glu**[c]
7	18.9	773	611 (100)	-	303 (11)	465 (100), 303 (11)			Dp-3-rut-5-glu
17	22.5	611	449 (100)	-	303 (31)	368 (5), 303 (100)			Dp-3-rut
Anthocyanin 3-O-sophoroside-5-O-[malonyl]glucoside/sophoroside derivatives									
3	16.2	1021	-	-	287 (6)	287 (100)			Cy-3-O-soph-5-O-(MA)soph
6	17.0	**859**	-	-	287 (21)	287 (100)			**Cy-3-O-[CA]soph-5-O-[MA]glu**
8	19.2	**873**	-	-	-	301 (100)			**Pn-3-O-soph-5-O-(MA)glu**
Anthocyanin 3-O-[cinnamoyl]glucoside derivatives									
10	19.8	611	465 (100)	-	303 (35)	303 (100)			Dp-3-O-[pCoA]glu
Anthocyanin 3-O-[cinnamoyl]sophoroside-5-O-glucoside/sophoroside derivatives				-(324 + acyl)					
4	16.6	979	817 (100)	449 (70)	287 (39)	287 (100)			Cy-3-O-[S]soph-5-O-glu
5	17.0	949	787 (100)	449 (58)	287 (20)	287 (100)			Cy-3-O-[FE]soph-5-O-glu
9	19.5	993	831 (100)	463 (93)	301 (45)	301 (100)			Pn-3-O-[S]soph-5-O-glu
14	20.6	979	817 (100)	449 (46)	287 (34)	287 (100)			Cy-3-O-[S]soph-5-O-glu
15	20.7	949	787 (100)	449 (71)	287 (37)	287 (100)			Cy-3-O-[FE]soph-5-O-glu
				-(324 + acyl1 + acyl2)					
22	25.2	1141	979 (100)	449 (70)	-	773 (100), 449 (16), 287 (38)			Cy-3-O-[CA-S]soph-5-O-glu
27	27.5	1155	993 (100)	449 (4)	-	678 (25), 287 (100)			Cy-3-O-[FE-S]soph-5-O-glu
36	28.9	1155	993 (100)	449 (5)	-	678 (31), 287 (100)			Cy-3-O-[FE-S]soph-5-O-glu
			-324			MS3 $[M-324]^+$, m/z (%)			
26	27.0	1317	993 (100)	611 (2)	-	287 (100)			Cy-3-O-[FE-S]soph-5-O-soph
29	27.5	1287	963 (100)	-	-	287 (100)			Cy-3-O-[diFE]soph-5-O-soph
									or Cy-3-O-[pCoA-FE]soph-5-O-soph
30	27.7	1287	963 (100)	611 (27)	-	287 (100)			Cy-3-O-[diFE]soph-5-O-soph
									or Cy-3-O-[pCoA-FE]soph-5-O-soph
33	28.5	1317	993 (100)	611 (7)	-	287 (100)			Cy-3-O-[FE-S]soph-5-O-soph
Anthocyanin 3-O-[cinnamoyl]sophoroside-5-O-[malonyl]glucoside/sophoroside derivatives									
			-44	-248	-(324 + acyl1)	MS2 [535–44]$^+$	Agli		MS3 $[M-(324+acyl1)+m/z$ (%)
13	20.4	**1065**	-	817 (7)	535 (100)	-	-		287 (100) **Cy-3-O-[S]soph-5-O-(MA)glu**

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16	21.0	1035	–	787 (26)	535 (100)	491 (20)	–	491 (10), 287 (100)	Cy-3-O-(FE)soph-5-O-(MA)glu
20	24.0	**1065**	–	817 (53)	535 (100)	491 (9)	–	491 (7),287 (100)	**Cy-3-O-(SI)soph-5-O-(MA)glu**
21	24.0	1035	–	787 (84)	535 (100)	491 (22)	287 (12)	491 (10), 287 (100)	Cy-3-O-(FE)soph-5-O-(MA)glu
24	25.8	1021	977 (20)	773 (60)	535 (100)	–	–	491 (31), 287 (100)	Cy-3-O-(CA)soph-5-O-(MA)glu
34	28.6	1005	961 (44)	757 (100)	535 (16)	491 (7)	–	287 (100)	Cy-3-O-(pCoA)soph-5-O-(MA)glu
35	28.9	1005	961 (44)	757 (100)	535 (100)	491 (7)	–	287 (100)	Cy-3-O-(pCoA)soph-5-O-(MA)glu
37	29.2	**1065**	1021 (5)	817 (59)	535 (100)	–	–	287 (100)	**Cy-3-O-(SI)soph-5-O-(MA)glu**
38	29.5	1035	–	787 (100)	535 (79)	–	–	287 (100)	Cy-3-O-(FE)soph-5-O-(MA)glu
41	30.6	1241	1197 (44)	993 (28)	535 (100)	491 (10)	–	287 (100)	Cy-3-O-(FE-SI)soph-5-O-(MA)glu
42	31.0	1019	975 (6)	771 (72)	519 (100)	475 (21)	–	475 (12), 271 (100)	Pg-3-O-(FE)soph-5-O-(MA)glu
43	31.3	1211	1167 (16)	963 (100)	535 (74)	–	–	287 (100)	Cy-3-O-(pCoA-SI)soph-5-O-(MA)glu
45	31.5	1241	1197 (10)	993 (70)	535 (100)	491 (9)	–	491 (97), 287 (100)	Cy-3-O-(FE-SI)soph-5-O-(MA)glu
46	32.5	**1181**	1137 (33)	933 (98)	535 (100)	–	–	287 (100)	**Cy-3-O-(pCoA-FE)soph-5-O-(MA)glu**
47	34.3	**1255**	1211 (13)	1007 (74)	549 (100)	–	–	409 (18), 301 (45), 201 (100)	**Pn-3-O-(FE-SI)soph-5-O-(MA)glu**
							MS3 [M-(324 + acyl1)]+, m/z (%)		
			−44	−248	−410	-(324 + acyl1)			
11	19.8	**1227**	1183 (4)	979 (9)	817 (22)	697 (100)	610 (13), 287 (100)		**Cy-3-O-(SI)soph-5-O-(MA)soph**
12	20.3	1197	1153 (10)	–	787 (19)	697 (100)	553 (7), 473 (100), 287 (70)		Cy-3-O-(FE)soph-5-O-(MA)soph
18	22.7	**1227**	1183 (1)	–	817 (29)	697 (100)	287 (100)		**Cy-3-O-(SI)soph-5-O-(MA)soph**
19	23.5	1197	1153 (24)	–	787 (53)	697 (100)	653 (28), 455 (13), 287 (100)		Cy-3-O-(FE)soph-5-O-(MA)soph
23	25.2	**1183**	1139 (15)	–	773 (49)	697 (100)	653 (20), 455 (8), 287 (100)		**Cy-3-O-(CA)soph-5-O-(MA)soph**
31	27.8	**1167**	1123 (13)	–	757 (43)	697 (100)	653 (26), 619 (10), 515 (8),287 (100)		**Cy-3-O-(pCoA)soph-5-O-(MA)soph**
32	28.1	1197	1153 (8)	949 (23)	787 (97)	697 (100)	611 (52), 287 (100)		Cy-3-O-(FE)soph-5-O-(MA)soph
						MS3 [M-410] + m/z (%)			
			−44	−410	-(324 + acyl1)				
25	26.8	**1398**	1345 (8)	979 (100)	697 (69)	773 (100), 287 (46)			**Cy-3-O-(CA-SI)soph-5-O-(MA)soph**
28	27.5	**1359**	1315 (8)	949 (100)	697 (98)	773 (100), 287 (35)			**Cy-3-O-(CA-FE)soph-5-O-(MA)soph**
39	29.6	1373	1329 (3)	963 (100)	697 (61)	595 (100), 287 (46)			Cy-3-O-(pCoA-SI)soph-5-O-(MA)soph or Cy-3-O-(diFE)soph-5-O-(MA)soph
40	30.2	1373	1329 (8)	963 (94	697 (100)	287 (100)			Cy-3-O-(pCoA-SI)soph-5-O-(MA)soph or Cy-3-O-(diFE)soph-5-O-(MA)soph
44	31.3	1373	1329 (2)	963 (63)	697 (100)	654 (17), 455 (73), 287 (100)			Cy-3-O-(pCoA-SI)soph-5-O-(MA)soph or Cy-3-O-(diFE)soph-5-O-(MA)soph

[1] Main observed fragments.
[2] Other ions were observed in MS2 (−248) of compound **8** (611 (9)) and compound **8** (625 (24)).
[3] Cy = cyanidin, Pg = pelargonidin, Pn = peonidin, glu = glucoside, soph = sophoroside, rut = rutinoside, pCoA = p-coumaroyl, CA = caffeoyl, SI = sinapoyl, FE = feruloyl, MA = malonyl.
[4] The anthocyanins tentatively identified for the first time are in bold face.

Table 3
List of individual anthocyanins (mg · 100 g^{-1} F.W.) tentatively identified and quantified in China rose radish and Rambo red radish sprouts by HPLC-DAD.

Peak LC-MS/HPLC	[M]$^+$ m/z DAD	Rt HPLC-DAD	Compound(s)	(3a) China rose radish						
				Control	MeJA	JA	SA	Glucose	Sucrose	$LSD_{0.05}$
1	859	15.5	Cy^a-3-O-soph-5-O-(MA)glu	0.03f	0.03a,b	0.03f	0.03b	0.04b	0.03b	0.004 n.d.
2	1021	20.2	Cy-3-O-soph-5-O-(MA)soph	0.16 e,f	0.25a	0.17 e,f	0.21b	0.21a,b	0.20b,c	0.12**
4	1021	29.0	Cy-3-O-(CA)soph-5-O-(MA)glu	0.95d,e	1.16 c,d	0.60f	1.10c	1.29b	1.52a	0.04**
10	1157	30.8	Cy-3-O-(CA-FE)soph-5-O-(MA)glu	0.93 e,f	1.10d	0.88d,e	1.24b	1.52a	1.20c	0.04***
12 + 13 + 14	1006 + 1006 + 1063	32.26	Cy-3-O-(pCoA)soph-5-O-(MA)glu + Cy-3-O-(FE)soph-5-O-(MA)glu + Cy-3-O-(S)soph-5-O-(MA)glu	7.43c	10.7a	5.88d	8.48b	10.32a	9.72a	0.33***
16 + 17	1221 + 1197	33.8	Cy-3-O-(diS)soph-5-O-(MA)glu + Cy-3-O-(CA-FE)soph-5-O-(MA)glu	1.65c	1.84c	2.29b	2.48b	3.35a	1.74c	0.13***
19 + 20	1241 + 1019	34.0	Cy-3-O-(FE-S)soph-5-O-(MA)glu + Pg-3-O-(FE)soph-5-O-(MA)glu	2.49c	2.71b	2.58c	3.13c	3.77a	2.99c	0.19***
22 + 23	1227 + 1197	34.5	Cy-3-O-(CA-S)soph-5-O-(MA)glu + Cy-3-O-(CA-FE)soph-5-O-(MA)glu	0.79e	0.9 c,d	1.11b	0.94c	1.22a	0.83d,e	0.03***
24	1211	35.2	Cy-3-O-(pCoA-S)soph-5-O-(MA)glu or Cy-3-O-(diFE)soph-5-O-(MA)glu	0.33 e,f	0.38b,c	0.42a,b	0.45a	0.44a	0.37c	0.02**
			Unidentified anthocyanins	0.99d,e	0.56e	1.64b	1.04c	2.63a	0.76d,e	0.12***
				(3b) Rambo red radish						
				Control	MeJA	JA	SA	Glucose	Sucrose	$LSD_{0.05}$
1	773	16.0	Cy-3-O-soph-5-O-glu	0.62b	0.52b,c	0.62b,c	0.78a	0.81a	0.56b,c	0.04***
4	979	19.5	Cy-3-O-(S)soph-5-O-glu	1.1b,c	1.01c	0.88c	1.40a,b	1.57a	1.14b,c	0.09**
11	1227	30.0	Cy-3-O-(S)soph-5-O-(MA)soph	0.75d	0.79 c,d	0.95b,c	1.37a	1.48a	0.99b	0.06***
14	979	30.9	Cy-3-O-(S)soph-5-O-glu	5.38c,d,e	6.26b	5.11d,e	5.65b,c,d	8.35a	5.91b,c	0.21***
27	1155	29.5	Cy-3-O-(FE-S)dglu-5-O-glu	5.3 c,d	5.53b,c	4.75d,e	5.07b	7.61a	5.68b,c	0.18***
32	1197	30.1	Cy-3-O-(FE)soph-5-O-(MA)soph	11.27b,c	11.26b,c	10.77 c,d	12.5b	15.84a	11.61b,c	0.38***
33 + 34	1317 + 1005	30.7	Cy-3-O-(FE-S)soph-5-O-soph + Cy-3-O-(pCoA)soph-5-O-(MA)glu	2.98c	3.88b	3.42d	3.88b	3.84a	3.15b	0.09***
35 + 36	1005 + 1155	32.0	Cy-3-O-(pCoA)soph-5-O-(MA)glu + Cy-3-O-(FE-S)dglu-5-O-glu	23.5b	34.04b	23.35b	23.3bc	35.5a	24.82b	1.37***
37 + 38	1065 + 1035	32.5	Cy-3-O-(S)soph-5-O-(MA)glu + Cy-3-O-(FE)soph-5-O-(MA)glu	50.37c	65.17b	53.49de	56.67 c,d	68.37a	57.53c	1.65***
40	1303	33.6	Cy-3-O-(pCoA-S)soph-5-O-(MA)soph or Cy-3-O-(diS)soph-5-O-(MA)soph	27.2de	28.82b,c	22.83d	29.17b,c	35.62a	30.50b	0.59***
41	1241	33.8	Cy-3-O-(FE-S)soph-5-O-(MA)glu	20.27c	24.69b	15.20e	25.19c	31.26a	26.4c	0.65***
43 + 44 + 45	1211 + 1317 + 1241	34.2	Cy-3-O-(pCoA-S)soph-5-O-(MA)glu + Cy-3-O-(pCoA-S)soph-5-O-(MP)soph or Cy-3-O-pCoA-S)soph-5-O-(MA)soph + Cy-3-O-(FE-S)soph-5-O-(MA)glu	15.19b,c	15.47ab	13.22d	14.66b,c,d	17.34a	16.79a	0.47***
46	1181	35.0	Cy-3-O-(pCoA-S)soph-5-O-(MA)glu	3.41b,c	3.35b,c	2.76d	3.76b	4.31a	3.68b	0.18***
			Unidentified anthocyanins	8.1c	6.78d	12.64a	7.96c	8.78b	8.10c	0.34***

[a] Mean values (n = 3), a–d: Different lowercase letters mean statistically significant differences between treatments.
[b] Least significant difference (LSD) for separating means in their respective columns. The LSD was computed only after analysis of variance indicated a significant ($p < 0.05$) entry effect. Asterisks indicate p value, *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; n.s., $p > 0.05$.
[c] Cy = cyanidin, Pg = pelargonidin, glu = glucoside, soph = sophoroside, rut = rutinoside, pCoA = p-coumaroyl, CA = caffeoyl, S = sinapoyl, FE = feruloyl, MA = malonyl.

delphinidin) in 8 different varieties of *R. sativus* sprouts, finding large differences between them. Several research groups (Giusti & Wrolstad, 2003; Park et al., 2013; Wu & Prior, 2005) also found that the major anthocyanins in radish sprouts are acylated pelargonidins, such as Daikon cultivar (De Nicola et al., 2013), while others reported the isolation of cyanidin-based pigments in red radish (*R. sativus* L. var. Benifuuki) (Tatsuzawa et al., 2010). Purple Bordeaux radish (Lin, Sun, Chen, & Harnly, 2011) and radish cv. Sango sprouts (Matera et al., 2012).

The anthocyanins tentatively identified were then quantified in HPLC-DAD by comparing their retention times and spectra to those of compounds found in the mass spectra experiments, using peak spectral characteristic and the absorption at 520 nm.

The total anthocyanin content (TAC) on China rose radish sprouts was 15.8 mg · 100 g^{-1} fresh weight (F.W.) and in the red radish sprouts was >10 fold more (180 mg · 100 g^{-1} F.W.) (Fig. 1). China rose radish showed its most abundant anthocyanin at minute 32.6, comprising the elution of three compounds with $[M]^+$ at m/z 1005 (**12**, cyanidin 3-O-(*p*-coumaroyl)sophoroside-5-O-(malonyl)glucoside), 1035 (**13**, cyanidin 3-O-(feruloyl)sophoroside-5-O-(malonyl)glucoside), 1065 (**14**, cyanidin 3-O-(sinapoyl)sophoroside-5-O-(malonyl)glucoside) (Table 3a), and representing 7.4 mg · 100 g^{-1} F.W. from the total (15.8 mg · 100 g^{-1} F.W.); (Fig. 1). These anthocyanins presented three different aromatic groups (*p*-coumaroyl, feruloyl and sinapoyl) in C-3 diglycosidic substituent while one aliphatic group (malonic acid) in sugar of C5, as previously described in red cabbage (Park et al., 2014) and Sango radish sprouts (Matera et al., 2012, 2015). The relevant anthocyanins in Rambo red radish sprouts showed $[M]^+$ at m/z 1065 (**37**, cyanidin 3-O-(sinapoyl)sophoroside-5-O-(malonyl)glucoside) and 1035 (**38**, cyanidin 3-O-(feruoyl)sophoroside-5-O-(malonyl)glucoside) (Rt 32.5) (Table 3b), representing almost 30% of the total anthocyanins (181.5 mg · 100 g^{-1} F.W.) (Fig. 1). Also, compounds with $[M]^+$ at m/z 1005 (**35**, cyanidin 3-O-(*p*-coumaryl)sophoroside-5-O-(malonyl)glucoside) and 1155 (**36**, cyanidin 3-O-(feruloyl, sinapoyl)sophoroside-5-O-glucoside) (co-eluting at Rt 32.0), 1373 (**40**, cyanidin 3-O-(*p*-coumaric, sinapoyl)sophoroside-5-O-(malonyl)sophoroside) and 1241 (**41**, cyanidin 3-O-(feruoyl, sinapoyl)sophoroside-5-O-(malonyl)glucoside) were abundant in this sample, each one representing 14% from the total amount of anthocyanins.

Compared to other plants studied for their TAC, China rose radish sprouts might be comparable to the values found in strawberry (19–55 mg · 100 g^{-1} F.W.), plum (10–25 mg · 100 g^{-1} F.W.) and gooseberry (2–40 mg · 100 g^{-1} F.W.), while Rambo red radish was found comparable to red cabbage (50–300 mg · 100 g^{-1} F.W.) (Zabaras, Roohani, Krishnamurthy, Cochet, & Delahunty, 2013), black currant (130–476 mg · 100 g^{-1} F.W.) and blackberry (83–326 mg · 100 g^{-1} F.W.) (De Pascual-Teresa & Sanchez-Ballesta, 2008). Anthocyanin compounds have interesting biological activities connected to cancer prevention,

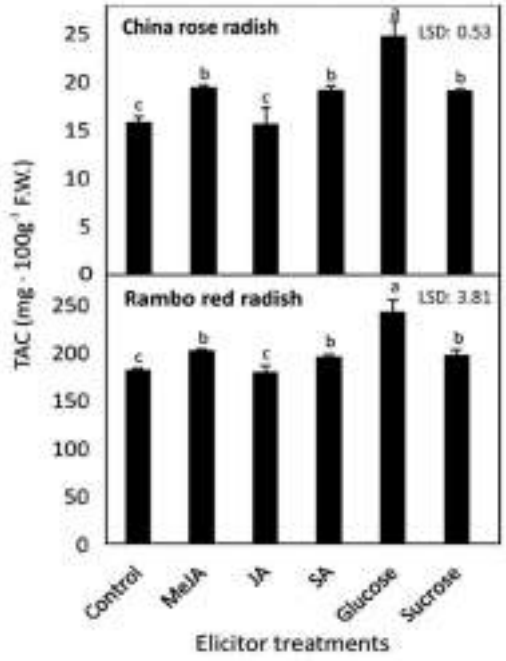

Fig. 1. Total anthocyanin contents (TAC) (mg · 100 g^{-1} F.W.) in radish sprouts after elicitor treatments.

oxidative damage and cardiovascular protection (Pojer et al., 2013). The results obtained in this work showed that radish sprouts are rich sources of anthocyanins, especially in the red radish Rambo variety.

3.2. Elicitors enhance anthocyanin content in radish sprouts

The roles of spray treatments of elicitors as appropriate tools for the enhanced production of anthocyanins in radish sprouts was studied in this work. The effects were determined in 8-day-old sprouts after exposure to elicitors for 5 days.

The signalling molecules salicylic acid (SA), methyl jasmonate (MeJA) and jasmonic acid (JA) play an important role in plant defense signal transduction pathways through the expression of defense-related genes, leading to the biosynthesis of secondary metabolites from the stimulation of the flavonoid biosynthetic genes, such as PAL, CHI and CHS (Ghasemzadeh et al., 2012; Ohinata et al., 2003; Shan et al., 2009; Tovar, Romero, Girona, & Motilva, 2002). MeJA elicitor (25 μM) showed higher effects in radish sprouts, increasing the TAC by 23% and 11% in China rose (19.45 mg · 100 g^{-1} F.W.) and Rambo radish sprouts (201.4 mg · 100 g^{-1} F.W.), respectively. By contrast, TAC in radish sprouts were not affected by JA elicitor (Fig. 1); however, SA treatment increased the TAC by 21% and 7% in China rose and Rambo radish, respectively. The activity of MeJA as up-regulator of PAL was determined by Kim, Park, and Lim (2011), who showed that MeJA-treated buckwheat sprouts had about twice as high as activity that of the control, with an increase of total phenolic compounds. Few results have been found about phytohormone treatments over Brassicaceae species, such as the study done by Park et al. (2013), where the mRNA transcript levels of genes involved in anthocyanin biosynthesis (RsMYB) were higher in MeJA-treated radish sprouts than in the untreated control.

Glucose (277 mM) and sucrose (176 mM) effectively enhanced TAC in China rose radish sprouts by 57 and 20% and, in red radish sprouts, by 33 and 83%, respectively (Fig. 1). In previous studies, sugar-regulated plant secondary metabolite production was observed in broccoli sprouts treated with 88 and 176 mM of sucrose, which increased total anthocyanins by 26 and 44%, respectively (Guo et al., 2011a); the transcription level of PAL treated by sucrose is much higher than controls (Guo, Yuan, & Wang, 2011b). Hara et al. (2004) found an induction of expression of the CHS, DFR and ANS genes after sucrose treatment, increasing as the TAC (7-fold) in red radish sprouts (cv. Comet) after 6 days of sucrose optimized treatment (175 mM). Wie, Miao, and Wang (2011) observed an increase in TAC by 101%, 120% and 83% in radish, Chinese kale and pak choi sprouts, respectively, after a 5% glucose solution treatment. Sugars are an important source of energy and carbon for plant development (Lereti et al., 2008). In addition, the mechanism of sucrose and glucose-specific induction of anthocyanin biosynthesis gene expression were demonstrated in *Arabidopsis* seedlings (Solfanelli et al., 2006).

4. Conclusions

The results supported the hypothesis that anthocyanin synthesis may allow the plant to develop resistance to a number of elicitor treatments by stimulation of the PAL pathway. All individual anthocyanins identified were increased by elicitor treatments, leading to the observed increase of TAC. Sugars were considered the most effective elicitors. The selection of ready-to-eat cruciferous sprouts rich in anthocyanins, as well as the appropriate elicitor treatment, is a candidate strategy to develop novel plant foods with beneficial nutritional and health properties.

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Optimizing elicitation and seed priming to enrich broccoli and radish sprouts in glucosinolates

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ABSTRACT

Elicitation is a cheaper and socially acceptable tool for improving plant food functionality. Our objective was to optimize the treatment doses of the elicitors: methyl jasmonate (MeJA), jasmonic acid (JA) and DL-methionine (MET), in order to find a successful and feasible treatment to produce broccoli and radish sprouts with enhanced levels of health-promoting glucosinolates. Also a priming of seeds as a novel strategy to trigger the glucosinolates content was carried out with water (control), MeJA (250 μM), JA (250 μM) and MET (10 mM) before the elicitor exogenous treatment. The results showed that almost all treatments could enhance effectively the total glucosinolates content in the sprouts, achieving the most significant increases from 34% to 100% of increase in broccoli and from 45% to 118% of increase in radish sprouts after MeJA priming and treatments. Consequently, our work demonstrates the feasibility of using elicitors, such as plant stress hormones, by priming and exogenously, as a way of increase the phytochemical profile of these sprouts to enhance their consumption in the diet.

1. Introduction

Consuming cruciferous vegetables is associated with many health benefits due to their composition in antioxidant compounds (mainly phenolic compounds and vitamin C) and glucosinolates (GLS – sulfur and nitrogen compounds with a glucose and a variable side chain derived from amino acids) (Dinkova-Kostova & Kostov, 2012; Jahangir, Abdel-Farid, Kim, Choi, & Verpoorte, 2009). Particularly, *Brassicaceae* sprouts content higher amount of GLS (20 times more), compared to the mature plants because their young physiological state (Fahey, Zhang, & Talalay, 1997). These bioactive phytochemicals have been widely investigated because their hydrolysis compounds, the isothiocyanates (ITC) and indoles. In plants, GLS are accompanied, but physically separated, by myrosinases (EC 3.2.1.147). These enzymes are responsible of their hydrolysis when there is a tissue disruption, mastication of fresh plants, and also upon ingestion by humans, because β-D-thioglucosidase activity of the gut microflora is largely responsible for converting ingested GLS to their cognate ITC and indoles, biologically active molecules which may impact in diseases prevention (Dinkova-Kostova & Kostov, 2012). The ITC sulforaphane, produced by hydrolysis of the predominant GLS of broccoli glucoraphanin, has demonstrated to have neuroprotective effects (Tarozzi, Angeloni, Malaguti, Morroni, Hrelia, & Hrelia, 2013) and anti-inflammatory and chemoprotective activity (Surh and Na, 2008). Other broccoli ITC, such as iberin and erucin, have shown similar antiproliferative activity in cancer cell lines, even though these compounds have not been widely studied (Wang, Wang, Howie, Beckett, Mithen, & Bao, 2005). The hydrolysis compounds of the GLS glucoraphenin and dehydroerucin, from radish sprouts, also showed inhibition of phase I or induction of phase II xenobiotic metabolizing enzymes (Barillari et al., 2007). Indole GLS, such as glucobrassicin, are hydrolyzed to indole-3-carbinol and its derived compound 3,3′-diindolylmethane, which have potentially biological effects, including activity on carcinogen metabolizing enzyme system (Aggarwal & Ichikawa, 2005).

The glucosinolates content of broccoli and radish sprouts can be manipulated through treatments with elicitors, such as plant hormones (methyl jasmonate (MeJA), jasmonic acid (JA), salicylic acid (SA), ethylene (ET) or abscisic acid (ABA), among others) (Roberto & Solano, 2005), sucrose (Guo, Yuan, & Wang, 2011), sodium chloride (Yuan, Wang, Guo, & Wang, 2010), or the amino acid DL-methionine (MET) (Scheuner, Schmidt, Krumbein, Schonhof, & Schreiner, 2005), which act as stressors in the plants, activating an array of mechanisms similar to the defense responses to pathogen infections or environmental stimuli, affecting the plant metabolism and enhancing the synthesis of phytochemicals. Elicitors are usually applied daily by spraying over the cotyledons, not as irrigation procedure. In this work, using elicitors as a priming

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treatment is a novel tool to increase bioactive compounds, as this method has been widely used only to reduce the time from sowing to radicle emergence. Therefore, this work reports the effect of combination of priming and elicitation with MeJA, JA and MET, in order to maximize the total GLS contents in broccoli and radish sprouts, to include the naturally healthy and functional food in future human clinical trials and to enhance the bioactive compounds intake through dietary interventions, in view of increased interests in healthy foods from natural origin.

2. Material and methods

2.1. Plant material

Seeds for sprouts production were provided by Intersemillas S.A (Valencia, Spain). Two varieties were used: broccoli (*Brassica oleracea* L. var *italica*) and red radish (*Raphanus sativus cv.* Rambo). Seeds were equally hydrated by immersion in 5 g L^{-1} sodium hypochlorite under aeration during 2 h, then, were immersed with aeration in distilled water (control samples), and MeJA, JA and MET (treated samples), involving the priming treatment, during 24 h until radicle protrusion, in order to reduce the time from sowing to emergence. After pouring off the soaking water, the seeds were weighed (day 0) and spreaded on trays (5 g per tray) lined with cellulose (CN Seeds, UK) and irrigated everyday with Milli-Q water. Three replicates (trays) per sample were transferred to an environment controlled chamber with a cycle of 16 h light with 60% relative humidity and air temperature of 25 °C and 8 h dark with 80% relative humidity and 20 °C. Photosynthetically active radiation (PAR) of 400 μmol m^{-2} s^{-1} was supported by a combination of fluorescent tubes (Philips TLD 36 W/83, Hamburg, Germany; Sylvania F36 W/GRO, Danvers, Massachusetts, USA) and metal halide lamps (Osram HQLT 400 W, Munich, Germany). During the first 3 days all trays were kept in controlled dark for increasing the stem elongation of sprouts. Then, three replicates per treatment of broccoli and radish sprouts were rapidly collected at day 8 after germination, in the middle of the light period, for analysis. All samples were weighed (fresh mass), flash frozen in liquid nitrogen and stored at −80 °C prior to analyses.

2.2. Treatments with elicitors: priming and exogenous spraying

The phytohormones jasmonic acid (JA) and methyl jasmonate (MeJA) (25–250 μM), and the amino acid DL-methionine (MET) (1–10 mM) were selected as elicitors according to literature review. JA (SIGMA–ALDRICH, Co., 3050 Spruce Street, St. Louis, MO, 63103, USA) and MeJA (SAFC, 3050 Spruce Street, St. Louis, MO, 63103, USA) were dissolved in 0.2% ethanol in Milli-Q water. DL-methionine (Alfa Aesar GmbH & Co KG, Karlsruhe, Germany) was dissolved in 0.04% ethanol in Milli-Q water.

Priming was performed with 100% imbibition and aeration of the seeds for 24 h, with three different treatments: MeJA and JA in a concentration of 250 μM and MET in 10 mM. Elicitors during germination of sprouts were applied as exogenous spraying on the cotyledons (not as soaking or irrigation solution) with 30 mL of test solution per sample (10 mL per tray) from day 4 to day 7 of sprouting (4 days of treatment), using Milli-Q water as control.

2.3. Extraction and determination of glucosinolates

2.3.1. Sample extraction

Freeze-dried samples powder (50 mg) were extracted with 1 mL of methanol 70% V/V, then heated at 70 °C for 30 min in a heating bath, with shaking every 5 min and centrifuged (17,500 ×g, 5 min). The supernatants were collected and methanol was removed using a rotary evaporator. The dry material obtained was re-dissolved in Milli-Q water and filtered (0.45 μm Millex-HV13 filter, Millipore, Billerica, MA, USA).

2.3.2. HPLC-PAD-ESI-MS^n analysis of glucosinolates

The qualitative and quantitative analysis of glucosinolates was performed according to Baenas, García-Viguera, and Moreno (2014) protocol. Briefly, the intact GLS were identified following their MS^2 $[M-H]^-$ fragmentations patterns in an HPLC-PAD-ESI-MSn (Agilent Technologies HPLC 1200, Waldbronn, Germany; coupled to a mass detector Bruker in series, model UltraHCT, Bremen, Germany). Chromatograms were recorded at 227 nm. Mass spectrometry data were acquired in the negative ionization mode for glucosinolates. Then, the extracted samples were analyzed and quantified in a Waters HPLC-DAD system (Waters Cromatografía S.A., Barcelona, Spain) as described by Pérez-Balibrea, Moreno, and García-Viguera (2011). The intact GLS were identified following their UV spectra and order of elution already described for similar acquisition conditions. Glucosinolates were quantified using sinigrin and glucobrassicin as standard of aliphatic and indole GLS, respectively (Phytoplan, Germany).

2.4. Statistical methods

The data were processed using the SPSS 15.0 software package (LEAD Technologies, Inc., Chicago, USA). The assays were conducted by triplicate. We carried out an ANOVA and the Tukey's Multiple Range Test to conclude significant differences at P values < 0.05.

3. Results and discussion

3.1. Glucosinolates profiles of broccoli and radish sprouts

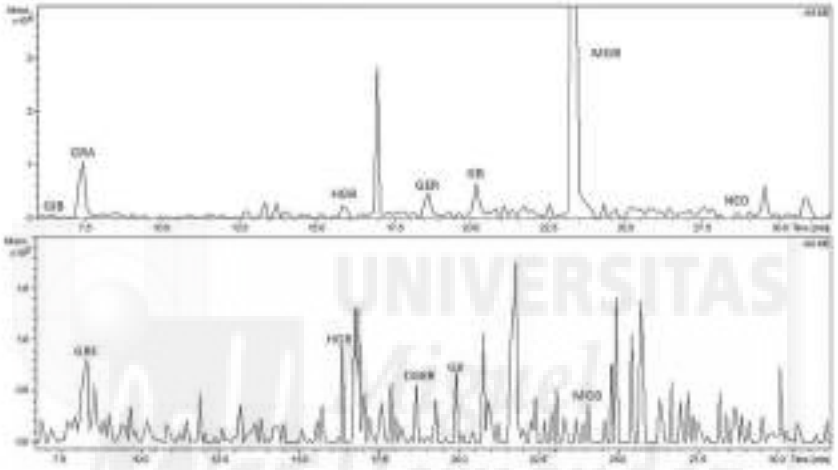
The glucosinolates content in *Brassicaceae* vegetables varies with genotype, and environmental and growth conditions (Cartea & Velasco, 2008). Broccoli (*B. oleracea* var *italica*) and radish (*R. sativus* cv. Rambo) 8-day-old sprouts show different glucosinolates profiles (Fig. 1). These species are interesting due to their high content in total GLS, being 302.84 and 379.71 mg g^{-1} F.W., in broccoli and radish sprouts, respectively (Tables 1 and 2), if compared with other 7 and 8-days-old sprouts (100–250 mg g^{-1} F.W.; Pereira, Rosa, Fahey, Stephenson, Carvalho, & Aires, 2002; Zhou, Zhu, & Luo, 2013), and adult plants (30–100 mg g^{-1} F.W.; Verkerk et al., 2009). These results are fairly consistent with previous studies from our group, using controlled growth conditions to reduce the influence of external factors to the minimum (Baenas et al., 2014). The predominant individual GLS have been widely studied because of their hydrolysis products, the ITC and indoles (derived from tryptophan), which might play a role in diseases prevention through the anti-inflammatory and chemopreventive pathways (Wagner, Terschluesen, & Rimbach, 2013). In broccoli sprouts, the predominant glucosinolate is glucoraphanin (4-methylsulphinylbutyl), accounting for almost the 50% of the total, 144 mg g^{-1} F.W. (Table 1), which belongs to the aliphatic group (mainly derived from methionine, but also from alanine, leucine, isoleucine, and valine) and is hydrolyzed to the ITC sulforaphane. Also glucoiberin (3-methylsulphinylpropyl), precursor to the ITC iberin, and glucoerucin (4-methylthiobutyl), precursor to the ITC erucin, are aliphatic GLS which account for the 15% of the total GLS in broccoli sprouts (48.06 and 45.21 mg g^{-1} F.W, respectively). The GLS glucoraphenin (4-methylsulphinyl-3-butenyl) and dehydroerucin (4-methylthio-3-butenyl, also known as glucoraphasatin), are the predominant in radish sprouts (162.20 and 195.22 mg g^{-1} F.W, respectively). Both, broccoli and radish sprouts, contain indole

A.

Code	Glucosinolate (GLS)	Semisystematic name	R_t (min)	[M-H]$^-$ (m/z)	MS2 and MS3	Broccoli	Radish
GIB	Glucoiberin	3-methylsulfinylpropyl-gls	6.5	422		+	
GRE	Glucoraphenin	4-methylsulfinyl-3-butenyl-gls	7.1	434			+
GRA	Glucoraphanin	4-methylsulfinylbutyl-gls	7.4	436		+	
4-HGB	4-Hydroxyglucobrassicin	4-hydrony-3-indolylmethyl-gls	16.9	463		+	+
GER	Glucoerucin	4-methylthiobutyl-gls	18.6	420	259 and 97	+	
DER	Dehydroerucin	4-methylio-3-butenyl-gls	19.9	418			+
GB	Glucobrassicin	3-indolylmethyl-gls	20.1	447		+	+
MGB	4-Methoxyglucobrassicin	4-methoxy-3-indolylmethyl-gls	23.5	477		+	+
NGB	Neoglucobrassicin	N-methoxy-3-indolylmethyl-gls	28.4	477		+	

R_t, retention time; +, compound presence

B.



C.

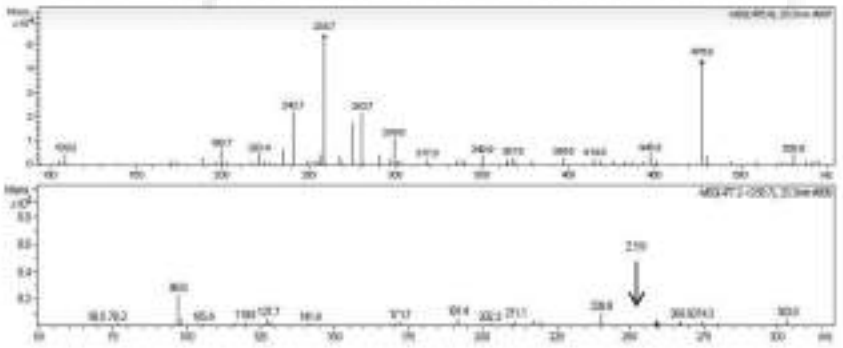


Fig. 1. (A) Identification of individual GLS present in broccoli and radish sprouts. (B) Full MS data of broccoli and radish sprouts. (C) MS2 and MS3 spectra of the fragmentation of MGB glucosinolate, consisting of the aglycone m/z 259 and the MS3 of this ion, a fragment of m/z 97, corresponding to the sulfate molecule.

GLS, accounting for the 20% and 5% of the total GLSs, respectively. Glucobrassicin (3-indolylmethyl) (GB), 4-hydroxiglucobrassicin (4-hydroxy-3-indolylmethyl) (HGB) and 4-methoxyglucobrassicin (4-methoxy-3-indolylmethyl) (MGB) are common to both species, but broccoli sprouts also present the GLS neoglucobrassicin (*N*-methoxy-3-indolylmethyl) (NGB).

3.2. Effect of the application of elicitors: priming and exogenous spray

In this work, MeJA (250 μM), JA (250 μM) and MET (10 mM) were applied as priming treatment during 24 h, finding only slight enhanced GLS content; but when priming was applied in combination with exogenous spray from day 4 to 7 of germination, the

Table 1
Individual and total glucosinolates (mg 100 g $^{-1}$ F.W.) in broccoli sprouts under priming and elicitation treatments.

Treatment	Glucosinolates														Total	
	GIB		GRA		4-HGB		GER		GB		MGB		NGB			
Control	48.06	j	144.36	i	19.07	b–e	45.21	a–c	17.27	i	13.93	g	16.95	g	302.84	i
Methyl jasmonate																
25 μM	59.75	h–g	185.48	c–f	18.37	b–e	48.48	a–c	31.08	h–j	15.86	e–g	53.81	gh	420.45	e–i
50 μM	61.99	e–j	173.36	e–h	19.11	b–e	44.00	a–c	31.17	h–j	14.88	fg	67.11	g	415.95	f–j
125 μM	58.98	e–j	165.09	f–i	15.75	d–f	36.78	b–c	32.81	g–i	14.12	fg	78.09	hi	405.87	f–j
250 μM	**78.42**	**a–d**	**187.71**	**c–f**	21.15	a–e	40.59	a–c	40.79	d–g	20.30	c–f	**135.59**	**de**	**537.80**	**bc**
Prim[b] (250 μM)	67.02	d–h	177.27	d–g	23.64	a–c	41.96	a–c	**53.09**	**ab**	16.98	e–g	48.86	j–l	427.59	e–i
Prim + 25 μM	71.75	a–f	176.30	e–g	17.75	b–f	36.59	bc	45.05	b–e	17.48	d–g	115.74	fg	480.04	c–e
Prim + 50 μM	**82.85**	**a–c**	**198.77**	**b–e**	23.54	b–f	40.77	a–c	**49.49**	**bc**	21.24	c–f	**133.44**	**d–f**	**564.45**	**ab**
Prim + 125 μM	68.43	c–g	151.37	g–j	15.89	d–f	33.10	c	37.31	e–h	16.13	e–g	107.26	g	437.55	d–h
Prim + 250 μM	**83.34**	**ab**	**205.49**	**b–d**	17.82	b–f	34.40	c	**42.54**	**c–f**	**22.80**	**b–e**	**189.21**	**a**	**626.46**	**a**
Jasmonic acid																
25 μM	55.80	g–j	144.96	i	17.16	c–f	38.12	a–c	36.33	f–h	17.42	d–g	54.86		360.93	i–k
50 μM	64.82	d–i	174.74	e–g	18.12	b–e	37.53	a–c	44.62	b–f	18.50	d–g	80.91	k	450.27	d–f
125 μM	49.64	j	151.70	g–i	14.64	ef	30.12	a–c	41.33	c–g	18.56	d–g	151.86	cd	467.83	d–f
250 μM	55.68	g–j	135.38	j	10.77	f	40.62	a–c	29.85	h–j	16.28	e–g	117.83	e–g	385.59	g–j
Prim (250 μM)	57.25	e–j	162.17	f–i	21.67	a–e	43.97	a–c	35.20	i–l	16.40	e–g	25.67	mn	366.07	j–l
Prim + 25 μM	59.88	e–j	166.49	f–i	18.72	b–e	36.92	a–c	41.86	c–f	18.27	d–g	96.36	gh	486.70	f–j
Prim + 50 μM	59.55	e–j	168.10	f–i	16.83	c–f	36.06	bc	43.31	c–f	18.84	d–g	77.55	hi	420.82	e–i
Prim + 125 μM	69.24	h–g	183.64	f–i	18.50	b–e	50.18	a–c	**49.31**	**b–d**	**24.72**	**a–d**	**165.54**	**bc**	**544.38**	**bc**
Prim + 250 μM	51.51	h–j	145.38	hi	16.60	d–f	36.35	a–c	44.12	c–f	**24.93**	**a–d**	**178.50**	**ab**	**495.84**	**cd**
DL-Methionine																
1 mM	58.20	e–j	186.69	c–f	24.69	ab	35.37	c	43.59	c–f	16.32	e–g	31.38	l–n	385.94	g–j
2.5 mM	66.15	d–g	178.34	d–g	17.88	b–f	42.35	a–c	23.30	j–l	15.09	fg	21.72	n	363.41	i–l
5 mM	60.93	e–j	180.42	d–f	21.17	a–e	54.75	ab	27.30	i–k	14.78	fg	22.58	n	379.40	h–j
10 mM	51.27	ij	144.12	i	12.00	c–f	40.43	a–c	20.65	kl	12.65	g	16.55	n	307.68	kl
Prim (10 mM)	51.68	ij	145.26	hi	16.17	d–f	41.61	a–c	19.07	kl	20.88	c–f	16.10	k–m	280.55	l
Prim + 1 mM	72.48	a–e	**221.60**	**b**	**26.78**	**a**	53.76	a–c	**60.26**	**a**	**32.27**	**a**	43.49	n	**496.76**	**cd**
Prim + 2.5 mM	61.40	e–j	170.81	f–i	19.70	a–e	43.78	a–c	37.37	e–h	27.42	a–c	25.97	mn	379.76	h–j
Prim + 5 mM	**85.77**	**a**	**264.45**	**a**	21.17	a–e	54.75	a–c	27.30	i–k	14.78	fg	22.58	n	**555.98**	**bc**
Prim + 10 mM	77.81	a–d	**209.25**	**bc**	22.68	a–d	53.11	a–c	42.95	c–f	38.3	ab	20.60	n	450.14	d–g
$LSD_{0.05}$[c]	3.77		7.29		3.81		4.91		2.175		1.95		4.83		10.88	

The most effective treatments are indicated in bold, as well as, the results of intact and total GLS.
[a] Mean values (n = 3); a–d, Different lowercase-letters mean statistically significant differences between treatments (for each glucosinolate).
[c] Least Significant Difference (LSD) for separating means in the respective row. The LSD was computed only after analysis of variance indicated a significant ($p < 0.05$) entry effect. ANOVA p value, $p < 0.001$.
[b] Prim (Priming).

effect was improved in terms of total GLS. Conrath, Beckers, Langenbach, and Jaskiewicz (2015) defined defense priming as an induced physiological state in which cells respond to very low levels of a stimulus in a more rapid and robust manner than unprimed cells. The higher increase of total GLS content after combination of priming seeds and exogenous application of elicitors, may be related to an activation of the seeds resistance, which produce enhance molecular mechanism of defense in sprouts. This idea could be substantiated by the fact that plants treated with elicitors such as UV radiation, salt or temperature, caused an increased resistance for subsequent generations, and also treatment of seeds with JA and MeJA primes plants enhance herbivore resistance weeks later and in next generations (Rasmann et al., 2012). These elicitors applied exogenously activate a large set of cellular defense responses in the plant, including the expression of enzymes involving in the synthesis of bioactive compounds, as well as occurs after the attack by pathogens, insects or in response to abiotic stresses, widely studied because of their effect in crop protection and health promotion (Bradler, Tas, & Palva, 2001).

There exist no single established and standardized protocol for elicitor application as a sustainable and safe method to enrich crucifers or other plant species in bioactive compounds, therefore, this study provide useful information about the appropriate dose of application of these natural elicitors in sprouts. Under MeJA treatment, there was a statistically significant increase in the total GLS in both species, not only after exogenous application of the elicitor (50–250 μM) but also after priming treatment (250 μM) together with spray treatment on the cotyledons. According to Ku, Jeffery, and Juvika (2014), the increase of total GLS is not correlated with the concentration of the elicitor applied, may be because of a saturation of the elicitor molecule in the plant tissues after a certain dose. Therefore, selecting a cost-effective concentration of the elicitor for each species under treatment could be highly effective. In 7 selected both for broccoli and radish sprouts: Prim + 50 μM MeJA, Prim + 125 μM JA and Prim + 5 mM MET (Tables 1 and 2). Even though several authors showed a certain degree of specificity between specie and elicitor, such as after application of MeJA in broccoli florets and kale leafs (Ku & Juvik, 2013); or after application of DL-methionine in broccoli heads or radish hypocotyls (Scheuner et al., 2005), in the present study, the results obtained showed that sprouts have a similar metabolism in response of elicitors. This could be probably because of their young physiological state and metabolism, unlike happens when elicitation is carried out in different tissues in the adult plant, such as after the application of MeJA in roots, leaves or broccoli heads (Ku et al., 2014), where the increase of total GLS was different according to the organ treated.

The elicitors treatments in these sprouts were effective, as well as other results found in bibliography which show an increase in GLS after applying exogenous MeJA 250 μM on broccoli florets (60% increase) (Kim & Juvik, 2011) or MeJA 10 μM on broccoli sprouts (22% of increase) (Pérez-Balibrea et al., 2011), the variations in the

Table 2
Individual and total glucosinolates (mg 100 g^{-1} F.W.) in radish sprouts under priming and elicitation treatments.

Treatment	Glucosinolates									Total	
	GRE		4-HGB		DER		GB		MGB		
Control	162.20^{f}	k	9.48	k	195.22	g	3.42	l	7.05	g	379.71	l
Methyl jasmonate											
25 µM	215.46	g–k	14.58	e–k	240.84	b–g	18.10	g–k	11.74	d–g	507.23	e–i
50 µM	300.24	b–f	18.05	d–h	265.91	a–f	20.23	f–j	16.39	b–f	660.75	b–e
125 µM	253.50	d–i	17.01	d–i	230.05	c–g	22.01	e–i	12.81	d–g	557.00	d–h
250 µM	**304.82**	**b–e**	20.20	b–f	280.31	a–g	35.11	c–e	16.81	a–e	**678.33**	**a–d**
Prim^{b} (250 µM)	**312.49**	**a–d**	**24.73**	**a–c**	**356.74**	**a**	51.87	d–f	7.56	g	**721.20**	**a–c**
Prim + 25 µM	236.80	f–i	20.77	b–e	247.83	b–g	33.45	c–e	12.43	d–g	563.81	e–h
Prim + 50 µM	**374.23**	**a**	**28.75**	**a**	**339.07**	**ab**	**68.78**	**ab**	17.88	a–d	**829.88**	**a**
Prim + 125 µM	**331.14**	**a–c**	**25.09**	**ab**	308.88	a–e	43.34	b–d	12.87	d–g	**788.86**	**ab**
Prim + 250 µM	294.39	b–f	21.79	b–d	212.73	e–g	**45.63**	**a–c**	12.98	c–g	610.63	b–g
Jasmonic acid											
25 µM	198.94	i–k	10.95	i–k	199.06	fg	14.20	h–l	8.51	e–g	419.14	hi
50 µM	204.47	h–k	12.10	h–k	194.68	g	20.10	f–j	8.38	g	437.59	hi
125 µM	261.65	d–i	14.03	f–k	193.48	g	28.42	e–g	12.50	d–g	501.96	f–i
250 µM	**345.16**	**ab**	19.74	b–g	219.35	d–g	**53.98**	**ab**	**23.99**	**a**	659.34	b–f
Prim (250 µM)	228.30	g–j	13.07	h–k	239.68	b–g	8.98	i–l	13.03	c–g	512.10	e–i
Prim + 25 µM	223.69	g–k	13.47	g–k	215.63	e–g	22.33	e–h	12.38	d–g	483.65	g–i
Prim + 50 µM	246.06	e–i	10.10	jk	216.41	e–g	28.52	e–g	12.85	d–g	508.00	e–i
Prim + 125 µM	**312.96**	**a–d**	20.31	b–f	267.48	a–g	**45.34**	**a–c**	**21.39**	**ab**	**660.44**	**b–f**
Prim + 250 µM	**328.62**	**a–c**	17.28	d–i	216.92	e–g	**58.20**	**a**	**20.38**	**a–c**	**638.91**	**b–g**
DL-Methionine											
1 mM	188.17	i–k	13.05	f–k	261.84	a–g	7.87	j–l	13.53	c–g	485.27	g–i
2.5 mM	214.79	g–k	13.22	g–k	271.58	a–g	5.31	kl	13.38	c–g	517.52	e–i
5 mM	276.81	d–h	17.15	d–i	309.36	a–e	7.49	j–l	14.63	b–g	624.92	b–g
10 mM	172.39	jk	9.97	jk	226.81	c–g	3.74	l	9.35	fg	422.30	hi
Prim (10 mM)	206.58	h–k	10.88	i–k	196.75	fg	4.52	l	12.26	d–g	431.01	hi
Prim + 1 mM	191.57	i–k	13.84	d–k	260.42	a–g	7.20	j–l	14.18	b–g	488.69	g–i
Prim + 2.5 mM	248.54	d–i	16.26	d–j	318.13	a–d	6.42	kl	13.94	c–g	604.16	b–g
Prim + 5 mM	**277.26**	**c–g**	18.37	c–h	**326.64**	**a–c**	8.66	kl	12.70	d–g	**641.53**	**b–g**
Prim + 10 mM	190.53	i–k	13.21	g–k	278.57	a–g	12.86	h–l	10.71	d–g	505.40	e–i
$LSD_{0.05}$[c]												

The most effective treatments are indicated in bold, as well as, the results of intact and total GLS.
[a] Mean values ($n = 3$). a–l. Different lowercase letters mean statistically significant differences between treatments (for each glucosinolate).
[b] Least Significant Difference (LSD) for separating means in the respective row. The LSD was computed only after analysis of variance indicated a significant ($p < 0.05$) entry effect. ANOVA p value, $p < 0.001$.
[c] Prim (Priming).

% of increases of GLS could be produced by different environmental factors, physiological states of the plant or doses of treatment.

The physiological and molecular mechanisms by which jasmonates regulate the expression of certain transcription factors have not yet been determined, since the metabolic pathway that is affected is not linear, but through an extensive network of cellular responses, including induction of pathogenesis-related proteins and enzymes of oxidative stress protection, the activation of defense-related genes, changes in the potential of plasma membrane cell and enhanced ion fluxes, rapid changes in protein phosphorylation, lipid oxidation, structural defensive barriers and the activation and the *de novo* biosynthesis of transcription factors, which directly regulate the expression of genes involved in secondary metabolites production, such as glucosinolates (García-Brugger et al., 2006). Regarding MET as elicitor; an increase of this compound in the cells could enhance the biosynthesis of GLS, as the side-chain elongation of this amino acid involves the formation of the GLS core structure, representing a key component in the regulation of aliphatic derived GLS biosynthesis, as well as has been studied when sulfur availability is increased by fertilization, a mineral which also takes part in the GLS formation (Kaur, Gupta, Sukhija, & Munshi, 1990).

In broccoli sprouts (Table 1) not all compounds were increased equally: the aliphatic GLS glucoiberin was enhanced to a maximum around 75% after some treatments, such as priming + 50 and 250 µM MeJA, and priming + 5 mM MET. The aliphatic GLS glucoraphanin (recently accepted as safe, GRAS), increased by 40% and 70% after some MeJA (250 µM exogenous spray with or without priming) and MET (priming + 1–10 mM exogenous application) treatments, respectively, achieving significant differences compared to the control (Table 1). However, JA treatments had a very limited effect on this GLS. The aliphatic GLS of radish sprouts, such as GRE and DER also were enhanced after MeJA, JA and MET treatments (Table 2). GRE achieved the maximum concentration of 374 mg g^{-1} F.W. after priming + 125 µM MeJA, 345 mg g^{-1} F. W. after 250 µM JA and 277 mg g^{-1} F.W. after priming + 5 mM MET. Regarding DER, the elicitor JA did not show any effect, however, this compound increased to a maximum of 80% in case of MeJA priming with or without exogenous treatment, and a maximum of 67% after MET priming + exogenous application of this elicitor (Table 2). According to these results, using the amino acid DL-methionine as elicitor in sprouts, could favored the biosynthesis of aliphatic GLS, as this elicitor works as precursor of this metabolic pathway (Scheuner et al., 2005).

Studying indole GLSs, GB from broccoli sprouts was enhanced almost by all treatments under study, achieving a 2-fold increase with MeJA priming (53 mg g^{-1} F.W.) and MET priming + 1 mM of exogenous spray (60 mg g^{-1} F.W.) (Table 1). In case of radish sprouts, GB was increased 13-fold and 16-fold by priming + the higher concentrations of MeJA and JA exogenous treatments, respectively. The GLS 4-HGB only was increased in radish sprouts after MeJA and MET treatments (to a maximum of 2-fold in MeJA

priming + 50 µM) and in broccoli sprouts after MET treatments. We also found an increase of 2-fold and 3-fold in MGB in both sprouts species, and 10-fold increase of NGB concentration in broccoli sprouts, after application of the higher concentrations of MeJA and JA priming + exogenous treatments. We could highlight that phytohormones were more effective in increasing indole GLS than MET, which is only implicated in the biosynthesis of aliphatic GLS. The first step in biosynthesis of the core structure of GLS is the conversion of amino acids to the corresponding aldoximes. This step is catalyzed by cytochromes P450 from the CYP79 gene family. Mikkelsen et al. (2003) showed an increase of CYP79B2 and CYP79B3 expression levels by MeJA, according to an increase of indoles GLS. However, not dramatically different gene expression levels (CYP83A) involved in aliphatic GLS biosynthesis after application of elicitors have been studied (Ku et al., 2013). Further studies with elicitors are needed to understand their molecular mechanism of action in defense-related genes.

Sprouts could be consumed uncooked, allowing the activity of the enzyme myrosinase and, therefore, the ITC and indoles production and absorption is more extensive than when crucifers are subjected to cooking (Cramer & Jeffery, 2011). Priming and exogenous elicitation with plant hormones (generally accepted as safe, GRAS) are a sustainable tool to improve the content of health-promoting compounds (achieving in this work upon 2-fold increase of GLS concentration after MeJA priming + 250 µM in broccoli sprouts), enhancing the concentrations of potentially anti-inflammatory, anticarcinogenic and antioxidant bioactives in the consumers daily diet.

4. Conclusions

Combining priming of seeds and spray treatments with natural elicitors, mainly with very low dosages of phytohormones (MeJA 50–250 µM), offer effective and environmentally friendly strategies to trigger the synthesis of target natural products in cruciferous foods without using transgenic technology. Understanding the metabolism pathways where elicitors act in the plant should be a target in this field in order to make effective their use. These enriched ready-to-eat sprouts can be used in preclinical and clinical trials with potential for protective effects in cells against oxidative and inflammatory processes, and therefore to study and research on the prevention of the development of neurodegenerative, cardiovascular diseases and certain types of cancer, through dietary interventions with naturally healthy foods.

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3. EVALUATION OF *IN VITRO* AND *IN VIVO* BIOLOGICAL ACTIVITIES OF BROCCOLI AND RADISH SPROUTS

(Publications 5, 6 and 7)

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Metabolism and antiproliferative effects of sulforaphane and broccoli sprouts in human intestinal (Caco-2) and hepatic (HepG2) cells

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Abstract The purpose of this work was to study the absorption and metabolism of sulforaphane (SFN) from broccoli sprouts, their major glucosinolate glucoraphanin and its hydrolysis product SFN. This was done by monitoring SFN's main metabolites, i.e. SFN, SFN-glutathione and SFN-cysteine, in two different cell models of absorption and metabolism (Caco-2 colorectal carcinoma cells and HepG2 hepatocellular carcinoma cells), during 3, 6, and 24 h of treatment, using a selective UHPLC-QqQ-MS/MS procedure. Concentrations ranging 0.5–90 nmol/l were found within the cells and released in the medium, depending on the type of analyte under study. Cells were capable of conjugative metabolism, since the SFN mercapturic derivatives could be identified in the cell medium. The antiproliferative activity of broccoli sprouts, glucoraphanin and sulforaphane was compared in Caco-2 and HT-29 human colorectal carcinoma cells, and HepG2 hepatocellular carcinoma cells, establishing the minimal concentration of a given compound to achieve half inhibition of the maximal cell growth (IC_{50}) for broccoli sprouts extract and sulforaphane. However, glucoraphanin did not show an antiproliferative effect in the cells under study.

Keywords *Brassica oleraceae* · Metabolism · Cytotoxicity · UHPLC-QqQ-MS/MS

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Introduction

There is consistent epidemiological evidence that a diet rich in vegetables, particularly cruciferous, is inversely related to the risk of suffering certain types of cancer and more specifically colon cancer (Tse and Eslick 2014). The chemopreventive benefit of these vegetables is attributed in part to the glucosinolates (GLS) in them. However, the biological activity attributed to cruciferous is mainly caused by glucosinolate hydrolysis metabolites isothiocyanates (ITC), which are known to stimulate phase II carcinogen detoxifying enzymes, such as glutathione *S*-transferase (GST) and quinone reductase (Wallig et al. 1998). The induction of cellular enzymes of phase II are largely mediated by the antioxidant responsive element (ARE) which is regulated by the transcription factor Nrf2 (Keum 2012). Recent studies suggest that Nrf2-mediated signaling, which controls the expression of several genes responsible for detoxification of carcinogens and

protection against oxidative stress, is regulated by ITC such as sulforaphane (SFN) (Yeh and Yen 2009).

The most potent stimulator of phase II enzymes found in broccoli sprouts is SFN [1-isothiocyanato-4-(methylsulfinyl)-butane], which is a hydrolysis product of the inactive precursor glucoraphanin (GRA) by the thiohydrolase myrosinase, found endogenously in broccoli, or by the microflora present in the colon (Angelino and Jeffery 2014). Also the chemopreventive activity of SFN by blocking cancer initiation via inhibition of the metabolic activation of carcinogens by cytochrome P450 (CYS) (Phase I enzymes) has been studied (Clarke et al. 2008). Once cancer is initiated, SFN can act via several mechanisms that modulate cell growth and cell death signals to suppress cancer progression (Clarke et al. 2008).

The mechanisms by which ITC might exert their anticarcinogenic effects remain unclear and the evaluation of anticarcinogenic and antiproliferation effect of SFN is very limited. Some recent results suggest that the chemopreventive activities of ITC may involve other mechanisms in addition to the activation of detoxifying enzymes, as specific mechanisms that could also act at the DNA level or affect signal transduction pathways leading to growth arrest or cell death (Gamet-Payrastre et al. 2000).

Caco-2 and HT-29 cells feature many characteristics of intestinal epithelial cells, representing a widely accepted in vitro model for human intestinal absorption and metabolism. The hepatoma cell line, HepG2, not only resembles morphologically hepatocytes, but has also been shown to retain many of the enzymes involved in xenobiotic metabolism (Lenaerts et al. 2007).

SFN and other ITC are known to be metabolized in the enterocytes and the liver through the mercapturic acid pathway (Angelino and Jeffery 2014). An initial reaction between the –N=C=S group of ITC and the cysteine sulfhydryl group of glutathione (GSH) can take place spontaneously and is enhanced by glutathione *S*-transferase (GST). SF-GSH metabolites have been found in plasma and urine. The liver is able to carry out enzymatic modifications of the GSH moiety, forming cysteinylglycine-(cys-gly), cysteine-(cys); and the final *N*-acetyl-cysteine-(NAC-) conjugate is formed in the kidney (Angelino and Jeffery 2014).

The aim of this work was to examine the absorption of SFN metabolites of broccoli sprouts extract, pure SFN and GRA, in order to identify the possible metabolites in human colon Caco-2 and liver HepG2 cells. The effect of lyophilized broccoli sprouts, as a food matrix, the glucosinolate GRA, and its metabolite SFN on the proliferation of intestinal and hepatic human cancer cell lines, Caco-2 and HT-29 colon cells; and HepG2 liver cells using the colorimetric assay method MTT was also studied. Different concentrations of broccoli sprouts, GRA and SFN were tested in order to find their IC_{50}.

Materials and methods

Standards

Glucoraphanin (GRA) and sulforaphane (SFN) were purchased from CRA-CIN (Rome, Italy) and Sigma (St. Louis, MO, USA), respectively. The standards of SFN-glutathione and SFN-cysteine (SFN-GSH, and SFN-Cys, respectively) were from Santa Cruz Biotech (Santa Cruz, CA, USA). All LC–MS grade solvents were obtained from J. T. Baker (Phillipsburg, NJ, USA). Sinigrin monohydrate was obtained from Phytoplan (Heidelberg, Germany).

Plant material

Broccoli seeds were provided by Intersemillas, S.A. (Valencia, Spain). Broccoli sprouts were germinated for 8 days according to Baenas et al. (2014) with a slight modification; sprouts were covered with perforated aluminum foil for increasing stem elongation in the environment chamber from day 0 to 3. Then, 8-day-old sprouts were collected, rapidly frozen in liquid nitrogen and lyophilized prior to analyses.

For the development of the metabolism and cytotoxicity assay, 1 g of broccoli sprouts dry powder was hydrolyzed following Cramer and Jeffery (2011) protocol. Then, the supernatant was lyophilized, dissolved again in the culture cells medium according to the concentration of SFN understudy, and filtered by 0.22 μmol/l before adding to the cells.

Determination of bioactive compounds

Dry samples were hydrolyzed following Cramer and Jeffery (2011) method in order to quantify the sulforaphane content by the UHPLC-QqQ-MS/MS method described by Dominguez-Perles et al. (2014).

The identification and quantification of individual glucosinolates and total GLS was carried out in a HPLC–DAD-ESI-MSn (Agilent Technologies HPLC 1200, Waldbronn, Germany; coupled to a Bruker mass detector in series, model UltraHCT, Bremen, Germany) and HPLC–DAD system (Waters Cromatografia S.A., Barcelona, Spain), respectively, as previously described by Baenas et al. (2014). Glucosinolates were quantified using sinigrin as external standard, because of the similar structure to the glucosinolates in the sample.

Cell cultures

Caco-2 and HT29 (human colorectal adenocarcinoma) and HepG2 (human hepatocellular carcinoma) cell lines were plated in 75 cm^2 tissue culture flasks. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10 % heat-inactivated fetal bovine serum (FBS), 1 % (v/v) non-essential amino acids, and 1 % penicillin/streptomycin (5000 U/ml) (Lonza, Barcelona, Spain) in the presence of 5 % CO_2 at 37 °C and humidified atmosphere until confluence. The culture medium was changed every 2–3 days. After a confluent monolayer appeared, subculturing was carried out using Trypsin/EDTA solution (Lonza, Barcelona, Spain).

Absorption and Metabolism assay using UHPLC-QqQ-MS/MS

Caco-2 and HepG2 cells were seeded (1.5×10^5 cells/well) in 6-well plates (Sarstedt, Nümbrecht, Germany) until monolayer was formed. After that, cells were incubated with broccoli sprouts extract (containing 1 µmol/l SF), GRA (50 µmol/l) or SFN (1 µmol/l) dissolved in serum-free DMEM. After 3, 6 and 24 h of treatment the media was collected, and the cells were washed twice and collected in PBS, all samples were frozen until metabolite analysis. As for cell lysate preparation, samples were defrosted and incubated on ice for 15 min, shaking each 5 min in a vortex. Then, were mixed in an ultrasound for 5 min and transferred into an ultra-speed centrifuge (15 min, 9500×*g*). The analysis of SFN and its derivatives (SFN-GSH and SFN-Cys) in the different cell supernatants and culture mediums was carried out in a UHPLC-QQQ-MS/MS method described by Domínguez-Perles et al. (2014). Multiple reactions monitoring (MRM) was performed in positive electrospray ionization (ESI). MRM transitions were exclusive to a compound, so, GRA (438.1 → 196), SFN-GSH (484.9 → 178), SFN-Cys (299 → 178), SFN (178 → 114). The most abundant fragment ion produced was considered as the product ion for MRM. Data acquisition was performed using MassHunter software version B.04.00 (Agilent Technologies, Waldbron, Germany). The limit of quantification (LOQ) was 10 nmol/l for SFN-GSH and for GRA, 8 nmol/l for SFN and 1 nmol/l for SFN-CYS. The limit of detection (LOD) was 4 nmol/l for all compounds, except for SFN-CYS which was 0.5 nmol/l. A higher dose of GRA compared to SFN was selected in order to see whether there has been absorption of this compound within the cells or hydrolysis of this compound to SFN in the cell supernatant before absorption.

Cytotoxicity assay

The growth inhibitory effect of SFN, GRA, and broccoli sprouts extracts against Caco2 and HT29 cells was evaluated by using a MTT assay. Briefly, cells were plated in 96-well plates (1×10^4 cells/well) (Sarstedt) and cultured for 24 h at 37 °C in 5 % CO_2. Cells were treated with different concentrations of SFN (100, 85, 70, 55, 40, 25 and 10 µmol/l in 0.1 % DMSO), GRA (100, 85, 70, 55, 40, 25 and 10 µmol/l in 0.1 % DMSO) and broccoli sprouts extracts (containing 20, 10, 5, 1, 0.5, 0.1 and 0.05 µmol/l of SFN in 0.1 % DMSO) dissolved in serum-free DMEM. After 24 h of incubation 20 µl of a MTT solution (5 mg/ml in PBS) was added to each well and incubated for 4 h at 37 °C in 5 % CO_2. Formazan crystals formed in the wells were solubilized in 200 µl of DMSO (Panreac, Barcelona, Spain). Absorbance was measured at 570 nm employing a microplate reader PowerWave™ XS (BioTek Instruments, Inc., Winooski, VT, USA). The assay was repeated with three independent experiment replications. The viability was calculated considering controls containing a solvent control (0.1 % DMSO) as 100 % viable. DMSO at experimental concentrations was nontoxic to cells.

Statistical analysis

All analyses were conducted by triplicate, processed by Graphpad Prism to determine IC_{50} and SPSS 15.0 (IBM Corp., Armonk, NY, USA) to carry out a

multifactorial analysis of variance (ANOVA) and Tukey's Test to determine significant differences at p values <0.05.

Results and discussion

Analysis of broccoli sprouts

The contents of total glucosinolates as well as the individual glucosinolates in 8-day-old broccoli sprouts were tentatively identified following their MS^2 $[M-H]^-$ fragmentations by HPLC–DAD–ESI-MSn and quantified by HPLC–DAD system, according to previous reports (Baenas et al. 2014). The content of glucosinolates in broccoli sprouts decrease over the germination time. 8-day-old sprouts were selected as optimum for consumption according to the length and bioactive compounds content (Baenas et al. 2012). The concentration of GRA in broccoli sprouts was analyzed after the inactivation of the myrosinase activity to prevent the hydrolysis of GRA to SFN (Mellon et al. 2002). Total GLS amount was 377.64 mg 100 g fresh weight (F.W.), being GRA the most predominant GLS, accounting 160 mg·100 g F.W. of the total (Fig. 1), in accordance with several authors (Ávila et al. 2014; Guo et al. 2011).

In the hydrolyzed extracts of broccoli sprouts, only SFN was quantified by UHPLC-QQQ-MS/MS and the concentration was 13 mg 100 g F.W. These data are in line with previous reports (Dominguez-Perles et al. 2014; Guo et al. 2014). According to these results, the broccoli sprout extracts for cell culture tests were prepared. Thus, the bioactivity of a similar concentration of SFN as a pure compound (1 µmol/l) as a component of a food matrix (broccoli sprouts), and a higher concentration of GRA (50 µmol/l) were compared in cell culture experiments.

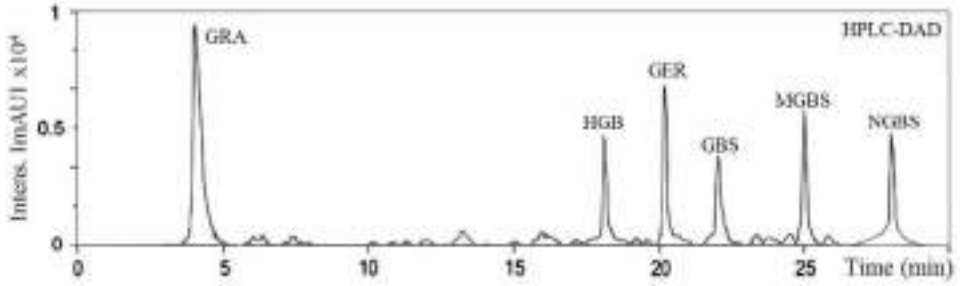
Cell metabolism

The levels of sulforaphane (SFN) and its derivatives (SFN-GSH and SFN-CYS) were measured in HepG2 and Caco-2 cells. After treating the cells with broccoli sprouts extract containing 1 µmol/l SFN, glucoraphanin (50 µmol/l) and pure SFN (1 µmol/l) for 3, 6 and 24 h. Metabolites were analyzed both, in whole-cell lysates and in the culture medium (Figs. 2, 3, 4). Metabolites of sulforaphane were absent in both control groups and present in all treated groups at nanomolar concentrations (0.5–90 nmol/l). Results after 24 h of exposing the HepG2 cells to the broccoli sprouts extract could not be measured because of the high cytotoxicity of this product in this cell model.

SFN in cells, both intracellular and in the medium was principally accumulated as glutathione (GSH) conjugates (SFN-GSH) (Figs. 2, 3) in accordance with different authors (Callaway et al. 2004; Zhang 2001).

Glucosinolates (mg · 100g F.W.)*

GRA	HGB	GER	GBS	4-MGBS	NGBS	Total GLS
161.01 ± 8.52	36.79 ± 1.03	65.09 ± 1.73	36.11 ± 0.50	40.86 ± 0,50	37.78 ± 2,85	377.64 ± 9.37



*Mean values (n = 3 ± SD). GRA: Glucoraphanin, HGB:4-Hydroxyglucobrassicin, GER: Glucoerucin, GBS: Glucobrassicin, 4-MGBS: 4-Methoxyglucobrassicin, NGBS: Neoglucobrassicin.

Fig. 1 Individual and total glucosinolates present in 8-day-old broccoli sprouts (mg 100 g F.W.)

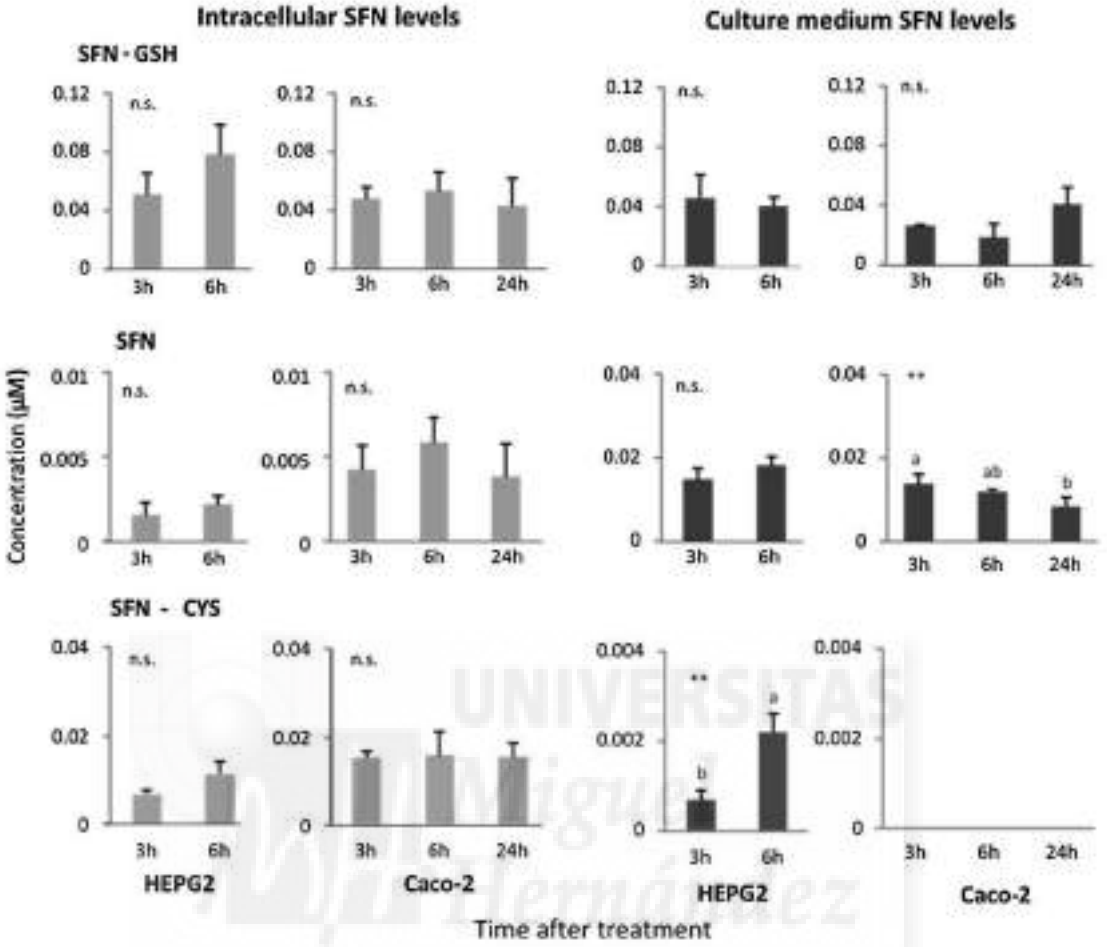


Fig. 2 Concentration (µmol/l) of sulforaphane (SFN) and its metabolites sulforaphane-glutathion (SFN-GSH) and sulforaphane-cysteine (SFN-CYS) inside of HepG2 and Caco-2 cells, and their release in the culture medium after the application of broccoli sprouts extract containing 1 µmol/l of sulforaphane. Lower case letters show statistically significant differences at $^{**}p < 0.01$ and *n.s.* not significant $p > 0.05$

After 6 h of broccoli sprouts extract treatment, higher amounts of SFN-GSH were found in cell lysates than in the culture medium in both HepG2 and Caco-2 cells (Fig. 2), then, similar levels were found in Caco-2 at 24 h of incubation, probably due to the release of the molecules into the medium. The highest concentration of SFN-GSH was found in cell culture medium after 24 h of incubation with 1 µmol/l SFN in Caco-2 cells (89 ± 8 nmol/l) (Fig. 3). It should be noted that the formation of SFN-GSH, not only requires the action of the phase II enzyme glutathione *S*-transferase (GST), involved in first pass metabolism in enterocytes (Petri et al. 2003), but also depletes the cell of GSH, resulting in a rapid increase in GSH production within the cell, therefore assisting carcinogen metabolism on two fronts. Kim et al. (2003) showed that when cells were treated with a GSH-depleting agent, the SFN could not be accumulated in the cells, and the subsequent induction of phase II enzymes was blocked. Phase II enzyme induction is considered the most likely contributing factor to the anticarcinogenicity of SFN. Petri et al. (2003), showed that a significant proportion of SFN absorbed into enterocytes was effluxed back into the lumen as SFN-GSH conjugate, based on

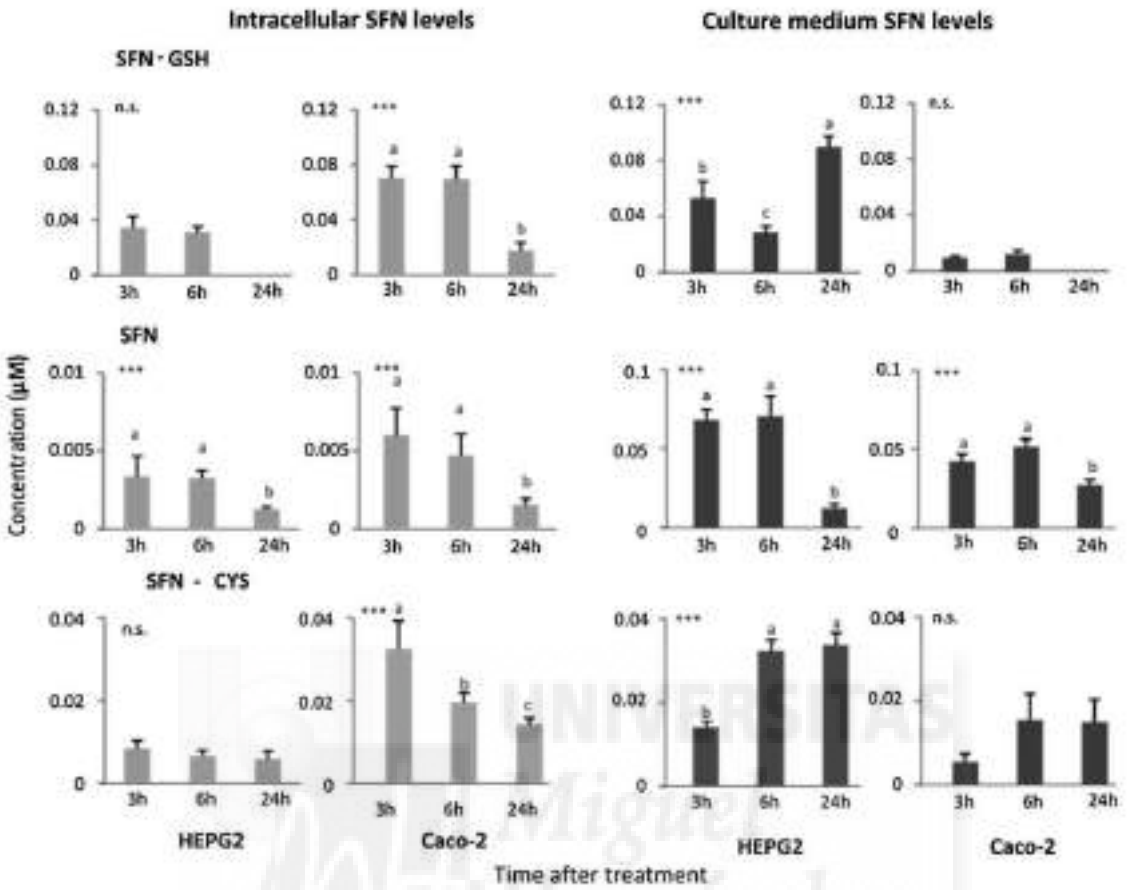


Fig. 3 Concentration (µmol/l) of sulforaphane (SFN) and its metabolites sulforaphane-glutathion (SFN-GSH) and sulforaphane-cysteine (SFN-CYS) in the interior of HEPG2 and Caco-2 cells and their release in the culture medium after the application of 1 µmol/l of sulforaphane. Lower case letters show statistically significant differences at $^{**}p < 0.01$ and *n.s.* not significant $p > 0.05$

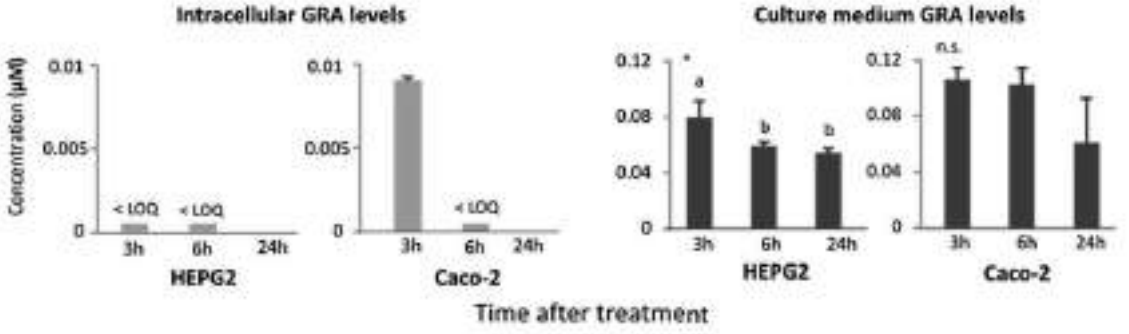


Fig. 4 Concentration (µmol/l) of glucoraphanin (GRA) and sulforaphane (SFN) in the interior of HEPG2 and Caco-2 cells and their release in the culture medium after the application of 50 µmol/l of glucoraphanin. *Lower case letters* show statistically significant differences at $^{**}p < 0.01$ and *n.s.* not significant $p > 0.05$

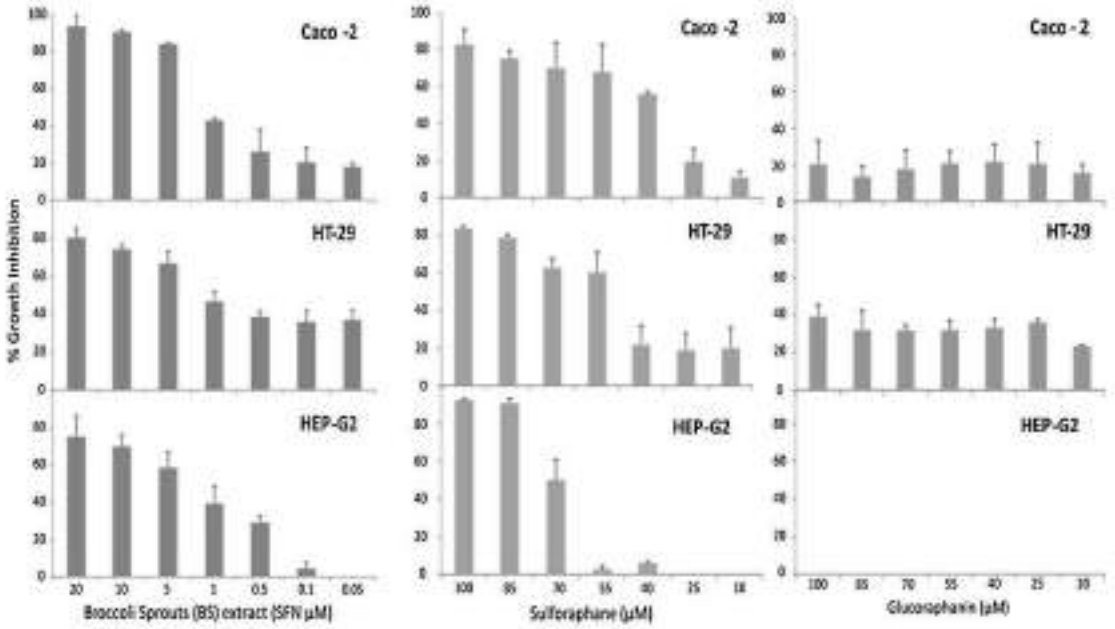


Fig. 5 Cell growth Inhibition (%) after application of broccoli sprouts extract, sulforaphane and glucoraphanin solutions. IC_{50} shows the half-maximal inhibitory concentration

previous reports in cultured human cells, P-glycoprotein is likely to be responsible for the efflux of the SFN conjugate (Zhang 2001).

Our results also showed the presence of SFN and SFN-CYS, after the application of lyophilized broccoli sprouts extract and pure SFN to the HepG2 and Caco-2 cells, in both media and cells (Figs. 2, 3). Higher levels of pure SFN were found in the culture medium than inside the cells, where this compound rapidly undergoes conjugation with GSH (Zhang and Callaway 2002). Intracellular SFN and its metabolites remained unchanged at the different times of incubation (3, 6 and 24 h) in both cell types after broccoli sprouts extract application (Fig. 2). Not significant differences were found in the accumulation of SFN metabolites after 3 and 6 h of broccoli sprouts and pure SFN treatments. However, a clear decrease in this metabolite after 24 h was found inside and in the culture medium of cell lines treated by pure SFN (Fig. 3). After the application of pure SFN, it seems that the release of metabolites of SFN outside the cell is faster in HepG2 than in Caco-2 cells, where we found higher values of SFN-GSH and SFN-CYS in cell lysates (Fig. 3).

The concentration of SFN-CYS was similar in both cell lines after the application of broccoli sprouts extract (Fig. 2). Nonetheless, higher levels of this metabolite were found at 3 h of application of pure SFN inside the cells and in the culture medium (Fig. 3).

GRA was found in the medium in those samples treated with broccoli sprouts extract, showing concentrations <LOQ (data not shown), therefore, this GRA, found in mmol/l concentrations in the extract, could be rapidly hydrolyzed to SFN. In those samples where GRA was added directly to the cell medium, concentrations around 0.11 µmol/l after 3, 6 and 24 h in Caco-2 and HepG2 cells were found (Fig. 4). We also found that the standard GRA (50 µmol/l) added to the cells was hydrolyzed to sulforaphane in the cell medium, since little concentrations of SFN (<LOQ) were found (data not shown). Also concentrations of GRA (Fig. 4) and SFN under LOQ were found inside these cells. As far as we are concerned none works using GRA as bioactive compound in cell cultures are found in the literature.

In spite of strong epidemiological data which support the potential role of dietary factors in cancer risk, such as increased consumption of cruciferous vegetables, particularly broccoli (Dinkova-Kostova and Kostov 2012; Michaud et al. 2000), there are

limited in vitro and in vivo studies examining the absorption of the cruciferous bioactive compounds. The results here presented showed that cells are capable of conjugative metabolism, since, as we have shown in our experiments, SFN mercapturic derivatives could be identified in the incubation medium, as well as in the cell lysate. SFN effect as modulator of absorption and metabolism in enzymatic systems has been proved before (Lubelska et al. 2012). Hence, these cell lines are a good model for the examination of metabolism regulation; even there are significant differences with human enterocytes (Petri et al. 2003). It is important to understand absorption and metabolism of these compounds in order to properly translate this work into future human clinical trials.

Cell proliferation

To study the antiproliferative effect of broccoli sprouts (containing 20–0.05 μmol/l SFN) and its metabolites SFN (100–10 μmol/l) and GRA (100–10 μmol/l) on different cell lines, we examined their cytotoxicity on Caco-2, HT-29 and HepG2 cells after 24 h treatment. The inhibition of cell growth by broccoli sprouts extract and its metabolites is shown in Fig. 5. Data obtained was dose and time dependent for broccoli sprouts and SFN, but not for GRA, which did not achieve the half-maximal inhibitory concentration (IC_{50}). Moreover they showed to have no effect in HepG2 cells (Fig. 5), consistent with observations made in other experiments where SFN induced a dose dependent decrease in HT-29 cells using the MTT assay (Gamet-Payrastre et al. 2000) and WST-1 assay (Frydoonfar et al. 2004). Both GRA and a broccoli sprout extract, in which the glucosinolate was not hydrolyzed to isothiocyanates due to heat-induced myrosinase inactivation, have been studied to demonstrate their little antiproliferative activity compared to extracts with isothiocyanates content (Tang et al. 2006).

The lowest IC_{50} was observed after broccoli sprouts application; the IC_{50} was 1.6 and 3.2 μmol/l, in both Caco-2 and HT-29 cells lines, and in HepG2, respectively. The broccoli sprouts extract showed the highest antiproliferative activity in all cells, even containing lower concentration of SFN, suggesting that other compounds in the food matrix may act biologically similar to SFN, and may not interfere with the antiproliferative activity (Tang et al. 2006). Lower concentrations of the SFN molecule (10–0.05 μmol/l) were also tested, nonetheless, the viability of cells were not reduced (data not shown). The bioactivities of different samples varied with the different cell lines. An IC_{50} of 37.5, 50.9 and 69.9 was observed in Caco-2, HT-29 and HepG2 cells, respectively, after SFN application, according to previous studies, such as Bonnesen et al. (2001), who obtained a IC_{50} of 55 μmol/l in Caco-2 cells after 24 h; Lubelska et al. (2012), who obtained a IC_{50} of 33.4 μmol/l in Caco-2 cells after 48 h; and Jakubikova et al. (2005), who reported an IC_{50} of 23 μmol/l after 72 h of treatment. Another study has shown SFN (15 μmol/l) induced cell cycle arrest and apoptosis in colon adenocarcinoma HT-29 cells (24 h after treatment) (Parnaud et al. 2004).

The inhibitory effect on cell proliferation have been confirmed also in in vitro experiments with other cancer cell lines, including prostate cancer cells (PC3), colon cancer cells (HCT116) (Singh et al. 2004) and Barrett esophageal adenocarcinoma cells (BEAC), where SFN has been shown to inhibit cell cycle progression, induce apoptotic cell death, and inhibit angiogenesis (Qazi et al. 2010). This cycle arrest was correlated with the loss of viability and decrease of G0/G1, S, and G2/M phase cells by SFN (Chaudhary et al. 2014; Parnaud et al. 2004; Singh et al. 2004; Tang et al. 2006). Apoptosis was associated with activation of MAPK pathways (ERK,JNK, p38) (Jakubikova et al. 2005), down-regulation of nuclear factor κB (Xu et al. 2005), mitochondrial damage, relocation of cytochrome c, cleavage/activation of caspase-9 and caspase-3, as well as cleavage of poly(ADP-ribose)-polymerase (Tang et al. 2006). Other protective mechanisms of SFN related to cell cytotoxicity have been studied, including the induction of phase II detoxification enzymes (such as NADPH:quinone reductase, UDP-glucuronyl transferase or glutathione-*S*- transferase (GST)) and inhibition of phase I carcinogen-activating enzymes (Jakubikova et al. 2005). Harris and Jeffery related the decrease in cell viability by SFN treatment (20–40 μmol/l) with an increase of MRP1 and MRP2 mRNA levels and protein levels in a dose-dependent manner in HepG2, through the activation of the transcription factor Nrf2. Activated Nrf2 enters to the nucleus and interacts with the antioxidant response element (ARE) in the promoter region of target genes, altering the expression. The up-regulation of enzymes involved in glutathione biosynthesis (GST and α-

GCS) in Nrf-2 overexpressing cells has been also studied (Shih et al. 2003).

Conclusions

The results of this study indicate that broccoli sprouts and sulforaphane inhibit proliferation of cancer cell lines providing support to the role of Brassica foods in reducing the risk of certain cancers, nonetheless, it is necessary to study the potential synergy of SFN combined with other food components, as in this work, broccoli sprouts extract, with lower sulforaphane concentration, had a greater antiproliferative effect than SFN its self. On the other hand, concentrations of SFN-metabolites found inside Caco-2 and HepG2 cells showed absorption of SFN after the application of broccoli sprouts extract to the cells, and showed its conjugation as mercapturic derivatives, assisting carcinogen metabolism in the cell. These results should encourage further in vivo assays to understand glucosinolates bioavailability, and preventive efficacy as therapeutic agents within the confines of animal studies or human trials for any form of cancer. Broccoli sprouts could be an excellent choice for developing a food product for future human studies. Their consumption or use as ingredient in food industry would enrich the composition in health-promoting bioactives of new foods. The development of human preventive studies focusing on the components of cruciferous vegetables would be advisable if an inhibitory effect was detected in vitro.

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Metabolic Activity of Radish Sprouts Derived Isothiocyanates in *Drosophila melanogaster*

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Abstract: We used *Drosophila melanogaster* as a model system to study the absorption, metabolism and potential health benefits of plant bioactives derived from radish sprouts (*Raphanus sativus* cv. Rambo), a *Brassicaceae* species rich in glucosinolates and other phytochemicals. Flies were subjected to a diet supplemented with lyophilized radish sprouts (10.6 g/L) for 10 days, containing high amounts of glucoraphenin and glucoraphasatin, which can be hydrolyzed by myrosinase to the isothiocyanates sulforaphene and raphasatin, respectively. We demonstrate that *Drosophila melanogaster* takes up and metabolizes isothiocyanates from radish sprouts through the detection of the metabolite sulforaphane-cysteine in fly homogenates. Moreover, we report a decrease in the glucose content of flies, an upregulation of *spargel* expression, the *Drosophila* homolog of the mammalian PPARγ-coactivator 1 α, as well as the inhibition of α-amylase and α-glucosidase *in vitro*. Overall, we show that the consumption of radish sprouts affects energy metabolism in *Drosophila melanogaster* which is reflected by lower glucose levels and an increased expression of *spargel*, a central player in mitochondrial biogenesis. These processes are often affected in chronic diseases associated with aging, including type II diabetes mellitus.

Keywords: *Brassicaceae*; sulforaphene; radish; *spargel*; energy metabolism

1. Introduction

Obesity and related diseases, such as diabetes and cardiovascular diseases, are a growing and serious health problem in both industrialized and developing countries [1]. The consumption of cruciferous plants (*Brassicaceae* family) has been associated with beneficial metabolic effects, although the underlying cellular and molecular mechanisms have not yet been fully elucidated [2–4]. *Brassicaceae* contain high amounts of glucosinolates (GLS), bioactive compounds that are enzymatically hydrolyzed to several breakdown products, including isothiocyanates (ITC, Figure 1). Treatment with indole-3-carbinol (I3C) and 3,3′-diindolylmethane (DIM), hydrolysis products of the GLS glucobrassicin, has been shown to significantly decrease blood glucose levels in C57BL/6 mice receiving a high fat diet [5]. Furthermore, patients suffering from type II diabetes exhibited significantly improved fasting glucose and lower insulin levels as well as an augmented homeostasis model assessment of insulin resistance (HOMA-IR) index following the consumption of 10 g/day broccoli sprout powder for four weeks [6]. Radish sprouts have been widely studied because of their high content of potentially health-promoting GLS [7,8]. The main compounds glucoraphenin and glucoraphasatin, belonging to the aliphatic group

of GLS, are hydrolyzed by the plant endogenous enzyme myrosinase (thioglucoside glucohydrolase, EC 3.2.1.147), following plant tissue disruption to sulforaphene (SFE; 4-methylsulfinyl-3-butenyl ITC) and raphasatin (RPS; 4-methylsulfanyl-3-butenyl ITC), respectively [9]. Also, indole GLS, such as glucobrassicin, 4-hydroxyglucobrassicin and 4-methoxyglucobrassicin, are present in radish sprouts and have been studied because of their breakdown product I3C, which has been associated with improved glucose tolerance and modulated expression of adipokines and lipogenic-associated gene products, including acetyl-CoA carboxylase and peroxisome proliferator-activated receptor-γ [10].

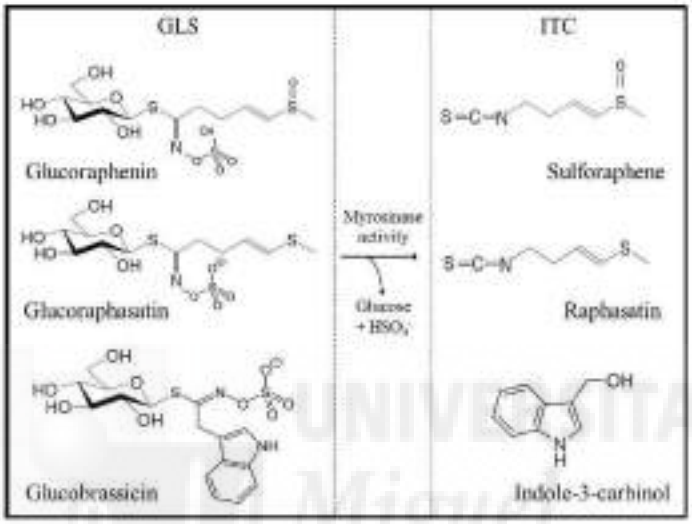

Figure 1. Glucosinolates in radish sprouts and their corresponding hydrolysis to isothiocyanates.

In mammals ITC are known to be metabolized in the enterocytes and the liver through the mercapturic acid pathway. Initially, a reaction between the –N=C–S group of the ITC and the cysteine sulfhydryl group of glutathione (GSH) catalyzed by glutathione-S-transferase (GST) takes place. Next, hepatic enzyme modifications of the GSH moiety to cysteinylglycine (–cys–gly), cysteine (cys), and *N*-acetyl-cysteine (NAC) conjugates are formed in the kidney of mammalian species [11]. Little is known about the absorption, metabolism and metabolic effects of ITC in model organisms. In this study, the fruit fly *Drosophila melanogaster* is used as a model system for studying the absorption and metabolism of ITC in lyophilized radish sprouts. In addition, the bioactivity of these compounds in terms of glucose and energy metabolism is evaluated.

2. Results and Discussion

2.1. Glucosinolate and Isothiocyanate Content in Radish Sprouts

The GLS profile of *Brassicaceae* species varies by genotype [12]. Radish sprouts (*Raphanus sativus* cv. Rambo) display a characteristic GLS profile, which has been extensively studied because of its hydrolysis products, ITC and indoles. ITC and indoles have been suggested to have protective effects on disease development through anti-inflammatory, chemopreventive and epigenetic pathways [13]. The concentrations of GLS are the highest in seeds and decline exponentially with sprout development. Aliphatic GLS are the predominant compounds in eight-day-old radish sprouts. Glucoraphenin and glucoraphasatin account for approximately 40% and 50% of the total GLS, respectively, as summarized in Table 1.

Following endogenous myrosinase hydrolysis of glucoraphenin and glucoraphasatin, these compounds released SFE and RPS, respectively. RPS is the oxidized counerpart of SFE and was not detected with our UHPLC method because a commercial standard was not available. However, this

compound has been reported to be highly unstable, with a half-life of less than 30 min [9]. Besides its instability RPS has been reported to significantly induce the expression of detoxifying enzmyes [14]. In addition to SFE, we also detected sulforaphane (SFN) in the hydrolyzed samples. The detection of SFN is interesting because glucoraphanin, the precursor of SFN, was not detected in our radish sprouts. However, these molecules only differ chemically by one double bond, suggesting that SFN exists as a natural product or that the presence of SFN is an artifact of the analytical technique, as we did not find any reports of this conversion taking place. We also detected indole GLS, which accounts for 15% of the total GLS in our radish sprouts. In particular, 4-hydroxyglucobrassicin and 4-methoxyglucobrassicin were present at higher amounts than glucobrassicin and neoglucobrassicin (Table 1). Their main hydrolysis compound, I3C (1 mg/100 g F.W. (Fresh Weight)), has been shown to lead to decreases in body weight via its effect on fat accumulation and blood glucose levels in mice [5,10].

Table 1. Quantification of glucosinolates and isothiocyanates in radish sprouts.

Glucosinolate Content in Radish Sprouts (mg/100 g F.W.)	
Glucoraphenin	202 ± 18.3
4-Hydroxyglucobrassicin	19.9 ± 1.32
Glucoerucin	8.74 ± 1.85
Glucoraphasatin	250 ± 23.5
Glucobrassicin	6.48 ± 0.36
4-Methioxyglucobrassicin	19.5 ± 0.72
Neoglucobrassicin	6.51 ± 0.31
Aliphatic GLS	461 ± 42.3
Indole GLS	52.4 ± 1.97
Total	514 ± 44.0
Isothiocyanate Content in Radish Sprouts (mg/100 g F.W.)	
Sulforaphene	9.93 ± 0.01
Sulforaphane	0.97 ± 0.02
Indole-3-carbinol	1.00 ± 0.09
Total	11.9 ± 0.11

Mean values ($n = 3$) ± SD. F.W. (Fresh Weight).

2.2. Evaluation of Food Intake and Fitness in *Drosophila melanogaster*

Food intake did not differ between control flies and radish sprout-treated flies (Figure 2a). Climbing ability, as a marker of overall fitness of the flies, did not show statistically significant differences between groups (Figure 2b), indicating that supplementation of the SY medium with lyophilized radish sprouts did not affect the movement of the flies.

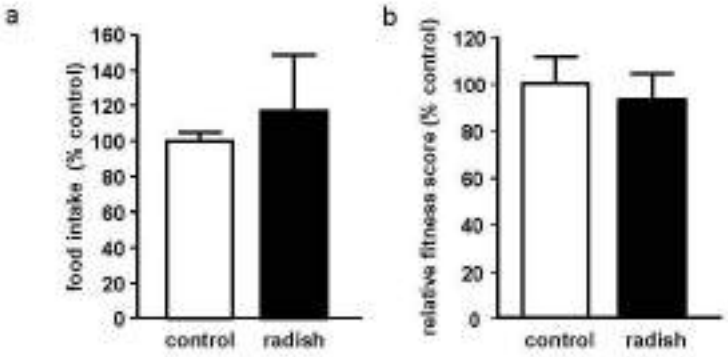

Figure 2. Effect of 10-day radish sprout supplementation on male *Drosophila melanogaster*. (**a**) relative food intake analyzed by the gustatory assay ($n = 3$ + SEM; extraction from 3 × 15 flies); (**b**) relative fitness score detected by the RING assay ($n = 3$ + SEM).

2.3. Sulforaphene, Sulforaphane, Indole-3-Carbinole, and Sulforaphane-Cysteine Concentrations in Fly Homgenates

The isothiocyanates SFE, SFN and I3C were present in our flies in concentrations of nanomol per gram on a fresh weight basis. Thus, under the conditions investigated, the natural conversion of glucosinolates to isothiocyanates, by the plant-derived enzyme myrosinase but also by gut microflora-derived myrosinase, may have occurred [3].

Flies principally accumulate the ITC SFE (1.11 nmol/g F.W. in flies, Figure 3). SFN was also found to be present in radish sprout-fed flies, but we cannot confirm whether SFN derives from the radish sprout extract, whether it is formed in the organism or whether SFN is formed due to a spontaneous conversion between SFE and SFN. Support for a metabolic origin of SFN can be obtained by finding an additional SFN conjugate—SFN–cysteine (SFN–CYS)—which was also detectable in our flies (0.7 nmol/g F.W.). This finding suggests that GLS and ITC were metabolized in the flies and that the initial reaction between ITC and GSH may be performed as a first step in SFN–CYS conjugation [11]. It has been shown that SFN treatment elevated cellular GSH levels [15,16] which prevents the accumulation of free radicals inside the cells and thereby reduces oxidative stress, which is generally associated with the development and progression of diabetes and its complications [17]. Interestingly, the distribution of GLS and ITC present in radish sprouts is partly reflected in ITC levels of our flies. The presence of the SFN–CYS metabolite in radish sprouts fed flies suggests that the metabolization of brassica-derived bioactive compounds in *Drosophila melanogaster* is similar to mammalian species.

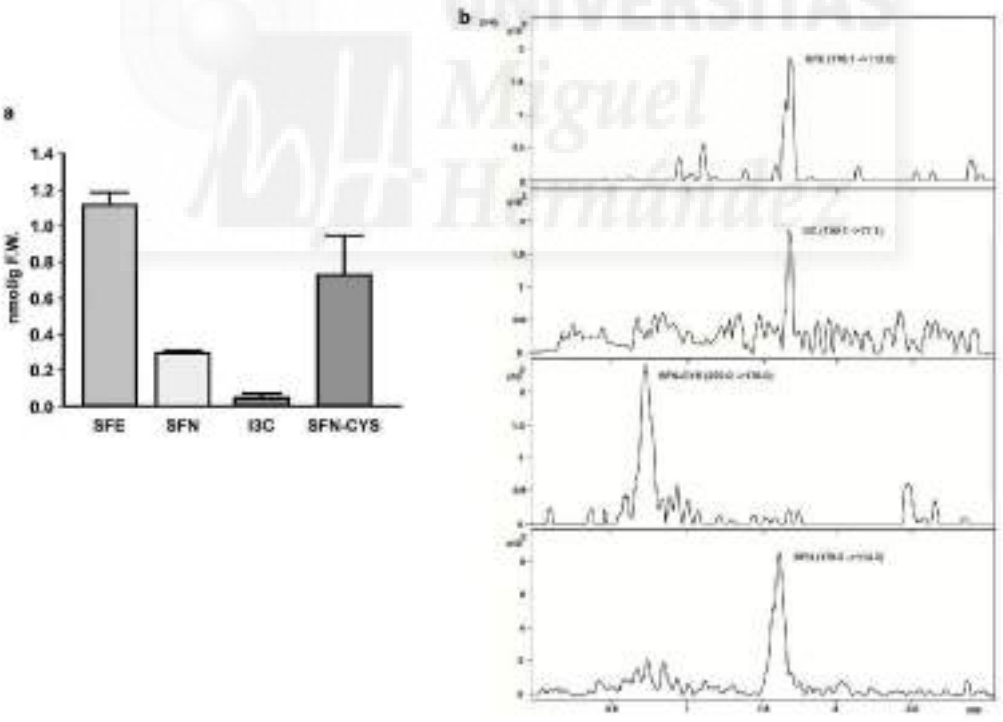

Figure 3. (**a**) Metabolites present in *Drosophila melanogaster* following the consumption of radish sprouts for 10 days; (**b**) Representative chromatogram of metabolites found in *Drosophila melanogaster* following the consumption of radish sprouts for 10 days. F.W. = fresh weight, SFE = sulforaphene, SFN = sulforaphane, I3C = indole-3-carbinole, SFN–CYS = sulforaphane–cysteine; $n = 3 + SD$.

2.4. Inhibition of α-Amylase and α-Glucosidase in Vitro by Radish Sprouts

An aqueous extract of lyophilized radish sprouts had an inhibitory effect on α-amylase and α-glucosidase activity *in vitro* (Table 2). The calculated concentration of the extract required to achieve

half of the maximal inhibitory concentration (IC_{50}) was higher in the α-glucosidase inhibition assay (60.7 ± 1.2 mg/mL) than in the α-amylase inhibition assay (33.8 ± 4.0 mg/mL). The concentration of 10.6 mg/mL radish sprouts which we have used in our *Drosophila melanogaster* experiments resulted in a 23% inhibition of the α-amylase activity *in vitro*, while the *in vitro* α-glucosidase activity was not affected (data not shown).

Table 2. *In vitro* α-glucosidase and α-amylase inhibitory activity of an aqueous extract of radish sprouts.

Inhibitory Activity of Radish Sprouts	
α-Glucosidase IC_{50}	60.8 ± 1.16 (mg/mL)
α-Amylase IC_{50}	33.8 ± 4.00 (mg/mL)

Mean values ($n = 3$) ± SD; IC_{50} (concentration which shows 50% of the inhibitor's response).

Inhibition of the enzyme α-amylase in the intestines delays the degradation of starch and oligosaccharides to monosaccharides before they can be absorbed. The enzyme α-glucosidase catalyzes the final step in the digestion and breakdown of carbohydrates; thus, its inhibition can be effective for the regulation of Type II diabetes through the control of glucose absorption [18]. The inhibition of α-glucosidase may retard the digestion and absorption of carbohydrates. In addition, it may suppress post-prandial hyperglycemia, decrease calorie uptake, and result in lower levels of glucose in *Drosophila melanogaster*. Thus, radish sprouts may improve glucose homeostasis and provide a dietary strategy to control hyperglycemia in diabetic and obese patients. However, additional evaluation of the *in vivo* potential of anti-diabetic activity of radish sprouts bioactives is necessary to verify these beneficial effects.

2.5. Energy Metabolism in *Drosophila melanogaster*

The glucose levels in our radish sprout-treated flies were significantly lower than those in control flies (Figure 4a). This finding is consistent with results obtained by Okulicz and co-workers [19], who observed that I3C, a breakdown product also present in radish sprouts, affects glucose uptake in adipocytes under basal as well as under insulin-stimulated conditions.

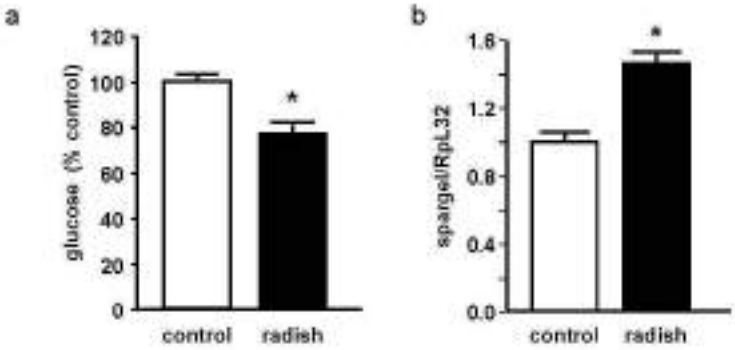

Figure 4. Effect of 10-day radish sprout supplementation on male *Drosophila melanogaster*. (**a**) relative glucose levels ($n = 9$ + SEM; extraction from 9 × 5 flies); (**b**) relative mRNA levels of *sargel* related to the housekeeping gene *RpL32* ($n = 3$ + SEM; extraction from 3 × 5 flies). * indicates significant differences between control and radish sprout-fed flies ($p < 0.05$, Student's *t*-test).

Because we have detected inhibition of α-amylase *in vitro* by radish sprouts and decreased glucose levels in our flies, we suggest that intestinal glucose absorption decreased as a consequence of radish sprout treatment. Glucose has been described to be a pro-aging factor and to interact with several age-associated processes in the organism [20,21]. High glucose availability has been shown to shorten life span whereas a glucose restriction increased life span in the model organism

Caenorhabditis elegans [22]. Interestingly, PGC-1α has been suggested to be involved in the regulation of glucose homeostasis in mammals [23,24]. Along with decreased glucose levels, we also showed that there was an upregulation of the PGC-1α homologous gene *srl* in *Drosophila* (Figure 4b). PGC-1α/*srl* plays an important role in the stimulation of mitochondrial biogenesis, in the reduction of ROS levels in enterocytes and stem cells, in the induction of several ROS-detoxifying enzymes and in the maintenance of optimal intestinal homeostasis [25]. Furthermore, an overexpression of PGC-1α/*srl* in the intestine of *Drosophila melanogaster* has been reported to be associated with life span extension [25].

Thus, we could not detect an improved resistance of our radish sprout-treated flies against both hydrogen peroxide and paraquat-induced stress (data not shown), which may be attributed to the relatively short intervention period of 10 days. However, there appears to be a connection between insulin resistance and mitochondrial dysfunction because patients suffering from type II diabetes have been reported to exhibit lower levels of mitochondria-related OXPHOS genes and PGC-1α in their skeletal muscles [26,27]. In addition, insulin has been shown to induce PGC-1α expression in skeletal muscle [28]. Therefore, insulin resistance may lead to a decrease in PGC-1α expression and mitochondrial dysfunction, which, in turn, increases insulin resistance further [26]. An increase in PGC-1α expression may provide a means to disrupt this vicious circle so that glucose homeostasis in diabetic and obese patients can be improved. Furthermore, Fernandes and co-workers have demonstrated that there is an approximately 40% increase in cellular PGC-1α levels as a result of SFN administration in cultured rat cardiac myoblasts [29]. Thus, we suggest that radish sprout-derived bioactives may affect glucose homeostasis—at least partially—through the modulation of PGC-1α-expression.

3. Materials and Methods

3.1. Radish Sprout Production

Red radish sprouts (*Raphanus sativus* cv. Rambo) were germinated for 8 days, according to the protocol of Baenas *et al.* [7]. Briefly, sprouts were collected, flash frozen in liquid nitrogen, and stored at −80 °C prior to analyses. Samples were then lyophilized and ground into a fine powder before being extracted for analyses and used as fly food supplement.

3.2. Analyses of GLS and ITC in Radish Sprouts and Drosophila Melanogaster by HPLC-DAD-ESI-MSn and UHPLC-QqQ-MS/MS

GLS in radish sprouts were extracted and quantified by HPLC-DAD-ESI-MSn, according to the protocol of Baenas [7]. Briefly, GLS were first identified following their MS2 [M–H]-fragmentations and were then quantified following their UV spectra and order of elution as previously described for the acquisition conditions. Sinigrin and glucobrassicin were used as external standards for aliphatic and indole GLS, respectively. ITC in radish sprouts were extracted according to the protocol of Cramer and Jeffery [30] and quantified by UHPLC-QqQ-MS/MS, according to the protocol of Dominguez-Perles *et al.* [31]. Also, this method was used to analyze metabolites in *Drosophila melanogaster*. First, 200 mg of flies were extracted with 5 mL MeOH:H_2O (70:30), mashed with a mortar until a homogenous liquid was obtained, filtered by 0.22 µm PVDF and analyzed with UHPLC-QqQ-MS/MS. The standards SFE, SFN, SFN–CYS and I3C were identified and quantified using MRM transitions and positive or negative ESI mode for quantification and confirmation of the target analytes. Analyses of GLS and ITC of radish sprouts were conducted in triplicate.

3.3. In Vitro α-Amylase and α-Glucosidase Assay of Radish Sprouts

The α-amylase and α-glucosidase inhibition assay was performed using a protocol modified from Phan *et al.* [32]. Samples of lyophilized radish sprouts ($n = 3$) were extracted with distilled water. The extracts were tested for α-glucosidase and α-amylase inhibition as previously described [33].

Acarbose was used as a positive control and was equally dissolved in distilled water. IC_{50} values were calculated using the program GraphPad prism (La Jolla, CA, USA).

3.4. *Drosophila melanogaster* Stocks and Treatment

In the present study, W^{1118} *Drosophila melanogaster* was used for the experiments. Flies were maintained under conventional conditions on sugar yeast medium (SY) containing 10% sucrose (Carl Roth, Karlsruhe, Germany), 10% inactive dry yeast, 2% agar, 0.3% nipagin (all Dominique Dutscher SAS, Brumath, France), and 0.3% propionic acid (Carl Roth) in a climate chamber (HPP 1018, Memmert, Schwabach, Germany) under the following constant conditions: a temperature of 25 °C, relative humidity of 60% and 12-h day/night cycle. For all of the experiments, age-matched flies from synchronized eggs were used [34]. The SY medium was supplemented with radish sprouts at a concentration of 10.6 g/L, containing 50 µmol/L of the ITC SFE.

3.5. Gustatory Assay

This method was performed to exclude differences in food intake between control flies and radish sprouts treated flies. The gustatory assay was performed as described earlier [34]. In brief, 15 flies were kept on SY medium or SY+radish sprouts. Both were supplemented with 0.2% *w*/*v* sulforhodamine B sodium salt (Sigma-Aldrich, Steinheim, Germany) and kept under standard conditions for 500 min. Next, the flies were homogenized in PBS (Life Technologies by Thermo Fisher Scientific, Darmstadt, Germany) plus 1% Triton™ X-100 (Sigma-Aldrich) using a Qiagen TissueLyser II (Hilden, Germany). The flies were then centrifuged, and the absorbance was measured in an Infinite 200 spectrophotometer (Tecan, Crailsheim, Germany) at 535/25 nm excitation and 590/20 nm emission wavelength.

3.6. Negative Geotaxis Assay: Climbing Activity

Climbing ability was considered to be an indicator of overall fitness of the flies, which were maintained under control conditions (SY) and under SY+radish sprouts for 10 days. On day 10, flies were transferred into empty vials to perform the rapid iterative negative geotaxis (RING)-assay as previously described [33].

3.7. Glucose Analysis

Flies were maintained either on SY ($n = 20$) or SY+radish sprouts under standard conditions for 10 days. Five flies per sample were homogenized in PBS/1% Triton™ X-100 using a Qiagen TissueLyser II. The supernatant was removed and analyzed for glucose content with Fluitest®GLU (Analyticon Biotechnologies, Lichtenfels, Germany) according to the manufacturer's protocol. Sample concentrations were calculated via the standard curve and related to the corresponding fly weights. The weighing of flies was performed using previously described methods [35].

3.8. Real-Time PCR

Total RNA was isolated from 5 flies per sample using peqGOLD TriFast™ (Peqlab, Erlangen, Germany) according to the manufacturer's protocol. RNA concentration was determined using a NanoDrop® spectrophotometer (Thermo Scientific, Schwerte, Germany). Primer sequences for *Drosophila melanogaster spargel* (*srl*) are described elsewhere [36]. Primers for the housekeeping gene *Drosophila melanogaster Ribosomal protein L32* (*RpL32*) (forward 5′-GGCAAGCTTCAAGATGACCA-3′; reverse 5′-GTTCGATCCGTAACCGATGT-3′) were designed by Primer3 software (Whitehead Institute for Biomedical Research, Cambridge, MA, USA). All primers were purchased from MWG Biotech (Ebersberg, Germany). Real-time PCR was performed using the SensiFastTM SYBR® No-ROX One-Step kit (Bioline, Luckenwalde, Germany) on a Rotor-Gene 6000 cycler (Corbett Life Science, Sydney, Australia). The expression of *srl* was related to the expression of the housekeeping gene *RpL32*.

3.9. Statistics

The results are presented as the mean ± SEM unless otherwise indicated. All data were analyzed using SPSS software (Statistical Package for the Social Sciences, IBM, Armonk, NY, USA). The significance of the differences between the control and radish sprout-treated flies was evaluated using a Student's *t*-test. All data were tested for normality of distribution (Shapiro–Wilk) and the homogenicity of variances (Levene). Significance was accepted at p-values <0.05.

4. Conclusions

In the present study, we showed, for the first time, that plant bioactives present in radish sprouts are absorbed and hydrolyzed by *Drosophila melanogaster* either due to the presence of plant-derived or *Drosophila*-derived microbial myrosinase in the gut. The presence of SFN–CYS in fly homogenates suggests that ITC are metabolized by fruit flies. The intake of radish sprouts decreased the glucose content in our flies and increased *srl* expression levels, thereby modulating energy metabolism. Understanding the factors that determine the absorption and effects of ITC in *in vivo* models is critical for identifying effective dosages that can be used in nutritional studies. As we have only analyzed flies on a radish sprout-supplemented diet for 10 days, analyses of flies receiving radish sprout-supplemented food for a longer period are sorely needed to assess the robustness of our results. To confirm the involvement of *srl* in the potential beneficial metabolic effects of radish sprouts, life span studies are important. Additionally, studies in mammalian species are needed to confirm our results that were obtained in the model organism *Drosophila melanogaster*.

Author Contributions: Nieves Baenas, Stefanie Piegholdt, Diego A. Moreno, Cristina García-Viguera, Gerald Rimbach and Anika E. Wagner conceived and designed the experiments; Nieves Baenas, Stefanie Piegholdt and Anke Schloesser performed the experiments; Nieves Baenas, Stefanie Piegholdt and Anke Schloesser analyzed the data; Nieves Baenas, Stefanie Piegholdt, Gerald Rimbach and Anika E. Wagner wrote the paper.

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

GLS	glucosinolates
ITC	isothiocyanates
PGC1α	PPARγ co-activator 1 α
PPARγ	peroxisome proliferator activated receptor γ
GSH	glutathione
GST	glutathione-S-transferase
NAC	*N*-acetyl-cysteine
cys	cysteine
cys–gly	cysteinylglycine
SFE	sulforaphene
SFN	sulforaphane
RPS	raphasatin
SFN–CYS	sulforaphane-cysteine
RpL32	*Drosophila melanogaster* ribosomal protein L32
Srl	spargel

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Antinociceptive effects of *Brassica oleracea* var. *italica* sprouts involves opioid neurotransmission in experimental models in rodents*

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4. SHELF-LIFE QUALITY AND SAFETY OF ECO-GROWN BROCCOLI AND RADISH SPROUTS

(Publication 8)

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Chapter 5. General Results and Discussion

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The interest of consuming sprouts is increasing because these plant foods are a healthy dietary alternative with higher nutritive value compared to the adult plants (Cevallos-Casals and Cisneros-Zevallos, 2010; Fahey *et al.*, 2001; O'Hare *et al.*, 2007). According to the general objective of this Doctoral Thesis, a characterization of the bioactive compounds (GLS and phenolic compounds) and antioxidant capacity, as well as the study of the biomass development of the sprouts, was performed for 4, 8 and 12 days of germination (**Publication 1**), being 8-day-old sprouts the best option for consumption, along with the studied *Brassicaceae* species broccoli (*Brassica oleracea* var. *italica*), turnip (*B. rapa* var. *rapa*), rutabaga (*B. napus* var. *napobrassica*), and radish (*Raphanus sativus)* sprouts, which were selected among others (red cabbage, *B. oleracea* var. *capitata*; kohlrabi, *B. oleracea* var. *gongylodes*; and turnip greens, *B. oleracea* subsp. *rapa*), as the best in terms of their profile in bioactive compounds and size for manipulation.

Then, different treatments with elicitors (phytohormones, sugars or methionine) were investigated in order to enhance the phytochemical contents in these sprouts, according to the general and second partial objective. Generally, exogenous spray of elicitors in different micromolar or millimolar concentrations over the cotyledons, for four or five days, was effective to increase the content of GLS in almost all tests allowing to choose a cost-effective dosage (**Publications 2 and 4**).

Anthocyanins, compounds belonging to phenolic compounds, were also studied in radish sprouts cv. China Rose and Rambo in order to characterize these novel varieties and elicit their red coloured flavonoids (**Publication 3**).

According to the obtained results, the phytohormone MeJA was selected as the most effective treatment to increase health-promoting GLS in *Brassica* and *Raphanus* sprouts. On the other hand, priming seeds with elicitors in combination with exogenous spray was highly effective as a novel strategy to trigger the GLS contents (**Publication 4**).

Once GLS were identified and quantified in sprouts, broccoli and radish cv. Rambo sprouts were selected as species rich in health-promoters (GRA and GRE, respectively), in order to investigate the absorption and metabolism of their bioactive hydrolysis compounds, isothiocyanates and indoles, as well as to validate their functionality to comply with the general objective of this Ph. D. Thesis (**Publications 5, 6 and 7**).

Regarding to this, broccoli sprouts as a food matrix, and the pure compound SFN, showed antiproliferative effect in human intestinal (Caco-2 and HT-29) and hepatic (HepG2) cancer cells. Furthermore, results showed that SFN and GRA were metabolized in the cells through the detoxification mercapturic acid pathway, contributing to the induction of Phase II enzymes involved in anticarcinogenesis mechanisms, since metabolites of SFN were measured in cell lysates and the culture medium (**Publication 5**).

Also a beneficial effect in the energy metabolism, reducing the glucose levels and increasing expression of *spargel* gen, central player in mitochondrial biogenesis was demonstrated by the *in vivo* (*Drosophila melanogaster*) study of the absorption and metabolism of isothiocyanates and indoles from radish cv. Rambo sprouts (**Publication 6**).

The antinociceptive (analgesic) effect of broccoli sprouts was evaluated in rodent models of induced pain, and also gastric protection and no-sedative effects were demonstrated. These effects may be originated by the positive modulation of the anti-inflammatory and antioxidant mechanisms in the cells related to the presence of these bioactive compounds (**Publication 7**).

Finally, the shelf-life of broccoli and radish sprouts was studied simulating two storage conditions, showing that sprouts could be maintained in refrigeration for 14 days, not presenting pathogenic microorganisms, and should be consumed as soon as possible from harvest, due to the rapid decline of phytochemicals during storage (**Publication 8**).

The figure 5.1. summarizes the activities and results obtained in this Thesis.

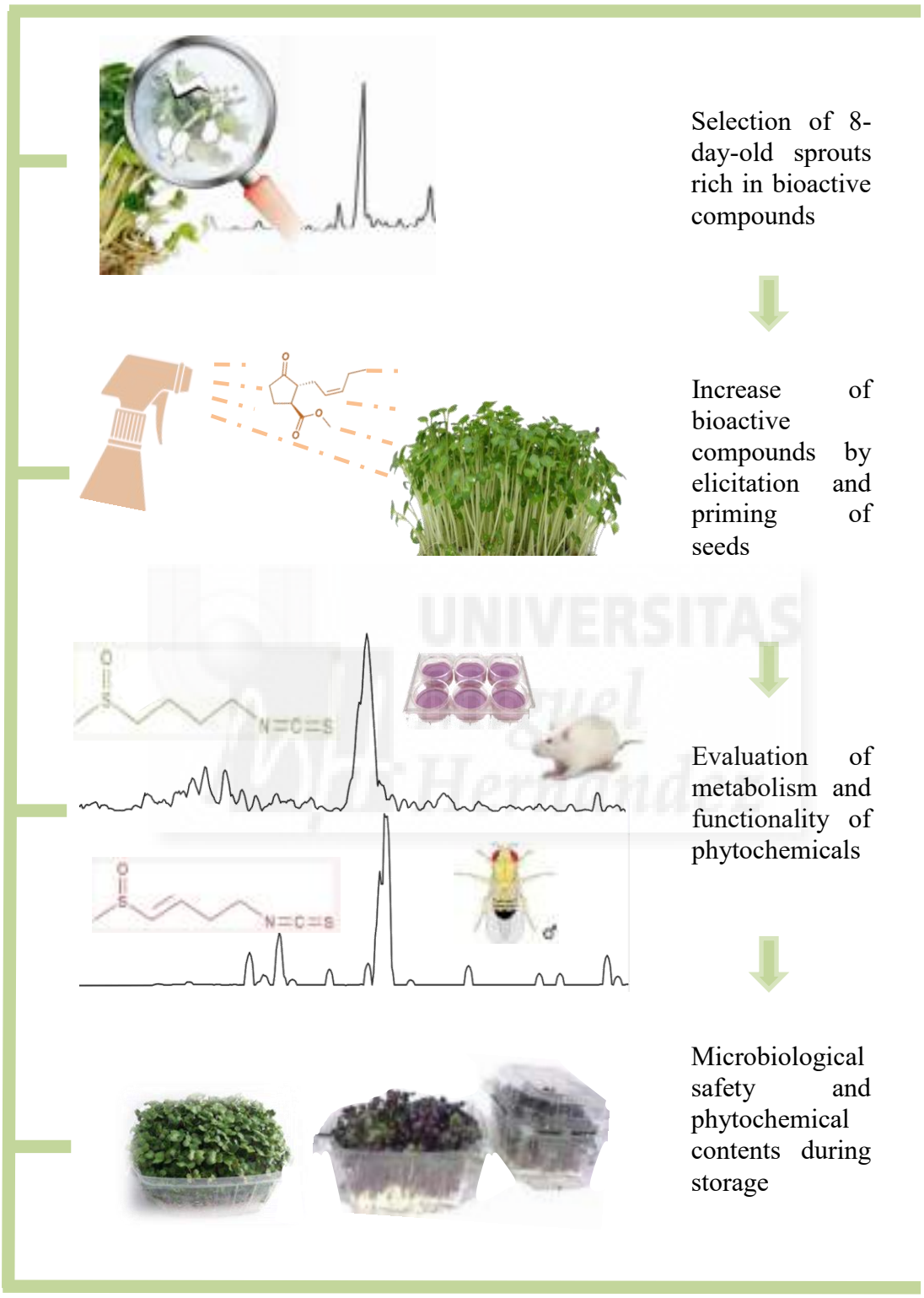

Figure 5.1. *Work-flow of the Doctoral Thesis project.*

The results showed that the general trend for the majority of the GLS and phenolic compounds, presented in the sprouts, was a decrease over germination time. Seeds have the highest amount of bioactive compounds and nutrients, as reserve organ, and the reduction of compounds with germination could be due to a dilution effect of tissue expansion leads to an intermediate phytochemical profile between seeds and adult plants, in accordance with other authors (Ciska *et al.*, 2008; Pérez-Balibrea *et al.*, 2008). Regarding our results, aliphatic GLS represented the 70 – 80 % of the total in broccoli, kohlrabi, red cabbage, rutabaga, turnip greens, turnip and radish sprouts, except for garden cress and mustard, species usually consumed as condiments, which showed aromatic GLS as the predominant class. Aromatic GLS, such as gluconasturtiin, accounted for less than 5 % in the rest of species. As examples of specific GLS profile, broccoli and kohlrabi (*Brassica oleracea*) presented the aliphatic GRA as the predominant GLS (40 % of the total), GRE was the higher GLS in radish (*Raphanus sativus*) (65 % of the total), and gluconapin was the main GLS in turnip varieties (*Brassica rapa*) (50 % of the total). Aliphatic GLS, such as GRA and GRE, have been studied because of their hydrolysis compounds, the isothiocyanates (ITC), which have shown anti-inflammatory and antioxidant activities, reducing the risk for particular cancers, cardiovascular, neurodegenerative and chronic diseases (Dinkova-Kostova, Kostov, 2012; Wagner *et al.*, 2013). As for indole GLS, 4-hydroxyglucobrassicin, glucobrassicin, 4-methoxyglucobrassicin and neoglucobrassicin were generally present in all sprouts, accounting for less than the 30 % of total GLS in all species. Glucobrassicin is hydrolysed by myrosinase to indole-3-carbinol (I3C), which undergoes spontaneous condensation in acidic medium, such as in the stomach, giving the compound 3,3-diindolylmethane (DIM).

Both compounds have shown, as well as other ITC, stimulation of the cellular detoxification pathways, decreasing NF-κB activation and inducing apoptosis in the post-initiation phases of cancer (Watson *et al.*, 2013).

Regarding phenolic compounds, hydroxycinnamic acids (sinapic, chlorogenic and ferulic acid derivatives) were the predominant class present in cruciferous sprouts, representing sinapic and ferulic acid derivatives more than 90 % of the total phenolics in *B. oleracea* (broccoli, kohlrabi, red cabbage, rutabaga) and *Raphaus sativus* sprouts, and the 80 % of the total in B. rapa varieties (turnip). Flavonols (mainly quercetin, kaempferol and isorhamnetin derivatives) achieved around a 17 % of total phenolics in *B. rapa* varieties. Chlorogenic acid derivatives represented between 1 and 4 % of the total phenolics in *Brassicaceae* sprouts. As for the GLS, the concentrations of phenolic compounds found in seeds were higher than in sprouts, except for the aromatic garden cress and white mustard, where the amount of phenolics increased from day 0 to day 4, and slightly decreased up to day 12. Phenolic compounds have shown different biological activities, such as anti-inflammatory and antimicrobial, but the most studied is the antioxidant activity, protecting mammals from the development of various chronic diseases (Cartea *et al.*, 2011; Crozier *et al.*, 2009). The antioxidant capacity of sprouts measured by the DPPH· and FRAP methods was used as a comparison criterion in this study. This parameter also decreases during germination, and values obtained exhibited significant correlation with contents of phenolics and reached a 10-fold increase when compared to adult vegetables (Ou *et al.*, 2002). The sprouts with better biomass yield, broccoli and turnip greens, were consistent with the greater length (4 – 5 cm) and selected for production (Gu *et al.*, 2012). According to our results, the

genotype may be the main factor of variation of the phytochemical profile in cruciferous sprouts; therefore, the selection of species rich in health-promoters compounds (GLS and phenolics) may benefit the consumers even without increasing the overall vegetable consumption.

In order to increase these bioactive compounds some modifications in the cultivation were introduced as it is well known that phytochemicals are affected by physiological changes with the application of elicitors, inducing the defence response systems in the plants (SAR, ISR), which modulate metabolic processes leading to the increase of phytochemicals in the tissues. In our work, the phytohormones MeJA (25 µM), JA (150 µM) and SA (100 µM); the sugars glucose (277 mM) and sucrose (146 mM); and the amino acid DL-methionine (5 mM) were employed, according to literature, as elicitor treatments for broccoli, turnip, rutabaga and radishes cv. China Rose and Rambo, 8-day-old sprouts. Results showed that sucrose and DL-methionine were the most effective treatments for biomass production, may be because sucrose were supply carbon for cell growth (Stewart *et al.*, 2011), and methionine caused an overexpression of aliphatic GLS specific biosynthetic genes related to stronger growth phenotypes (Gigolashvili *et al.*, 2007). Almost all treatments increased significantly the total amount of GLS of 8-day-old sprouts. The elicitor MeJA was highly effective, increasing GLS by 84, 125, 50 and 25 % in broccoli, rutabaga, turnip and radishes varieties, respectively. It is noteworthy that the individual GLS associated with potential health benefits (GRA, GRE and glucobrassicin) were enhanced. The molecular mechanisms by which phytohormones regulate the expression of certain transcription factors involving GLS biosynthesis have not been determined, however, jasmonates activate an extensive network of cellular defence

responses, such as induction of pathogenesis-related proteins and enzymes of oxidative stress protection, which regulates the expression of genes involved in secondary metabolites production (Ferrari, 2010; Garcia-Brugger *et al.*, 2006). GRA and GRE also were increased by sugars treatments, which could up-regulate genes involved in aliphatic GLS pathway in response to carbohydrate availability (Gigolashvili *et al.*, 2007; Guo *et al.*, 2011b). Indole GLS in all species were found to either increase or remain stable after elicitation.

Since China Rose and Rambo radishes varieties have rose and purple colours, respectively, the anthocyanins qualitative and quantitative profiles of both sprouts were studied by HPLC-DAD-ESI-MS^n, showing mainly cyanidin derivatives, diglycosylated at C-3 and glycosylated at C-5 position with the presence of cinnamoyl (sinapoyl, feruoyl, *p*-comaroyl and caffeoyl) and malonyl groups. China Rose radish sprouts but especially Rambo red radish sprouts were a rich source of anthocyanins, containing ~16 and 180 $mg \cdot 100g^{-1}$ F.W., respectively. Also these contents were enhanced by elicitors, being glucose (277 mM) the most effective since, in addition to serve as source of energy, glucose enhance the anthocyanins biosynthetic pathway. Phytohormones also increase the total anthocyanin contents by similar mechanisms, as have been studied to induce the "late" anthocyanin biosynthetic genes (DFR, LDOX, UF3GT) by up-regulation of the ternary complex composed of MYB, bHLH and WD4O transcription factors (Shi and Xie, 2014).

After the evaluation of the bioactive compounds content in cruciferous sprouts, as well as the effectivity of different elicitors treatments on enhancing their phytochemical content, broccoli and radish cv. Rambo sprouts were selected as

interesting varieties rich in bioactive compounds, mainly GLS health-promoters, and acceptable biomass at day 8 of germination (Figure 5.2.).

Figure 5.2. *Broccoli and radish sprouts at day 8 of germination*.

The elicitors are usually applied as exogenous spray over the cotyledons, the organ with higher concentration of phytochemicals in the sprouts, as well as over the organs and leaves in the adult plants. However, the application of elicitors as seed priming was also investigated as strategy to induce the plant defence system in the seeds of broccoli and radish sprouts. The highest increase of total GLS was found after combination of seed priming and exogenous spray, maybe because the activation of seeds resistance enhanced the molecular mechanisms of defence in the sprouts (Rasmann *et al.*, 2012). A 2-fold increase of GLS in broccoli and radish sprouts was obtained after application of MeJA (250µM) as priming treatment, as well as exogenous spray for 4 days. There exist no single established protocol for elicitor application to enrich crucifers in GLS, therefore, different concentrations of elicitors were used in order to provide useful information about appropriate dosage, as the response in terms of GLS biosynthesis after treatments was no correlated with

the dose applied. Seeds priming with MeJA (250 µM), JA (250 µM) and methionine (10 mM) combined with exogenous spray with MeJA (50 µM), JA (125 µM) and methionine (5 mM), respectively, could be selected as sustainable treatments to induce significantly increases of GLS contents. Under these treatments the sprouts did not show any visual differences in aspect or in biomass, whereas some authors reported that MeJA and SA altered the phenotype in *Arabidopsis thaliana* with smaller leaves. On the other hand, both sprouts showed similar response to elicitors, not showing specificity for any elicitor, as seen in adult plants (Ku and Juvik, 2013; Scheuner *et al.*, 2005).

Once the previous results were obtained, the evaluation of the absorption and metabolism of bioactive compounds from broccoli sprouts was approached, by the human cancer Caco-2 and HepG2 cell lines, which feature characteristics of intestinal epithelial cells and hepatocytes, respectively. These cell lines were treated with aqueous broccoli sprouts extracts (containing 1 µM of SFN) as a food matrix, and pure GRA (50 µM) and SFN (1 µM), for 3, 6 and 24 hours. SFN, as well as other ITC as it, is known to be metabolized in the enterocytes and the liver through the mercapturic acid pathway, giving rise to metabolites conjugated with glutathione, cysteine and N-acetylcysteine. In our work, cells were capable of metabolize SFN, accumulated predominantly as glutathione conjugates (SFN-GSH) but also as SFN-cysteine (SFN-CYS), which were determined in cells lysates and in the culture medium in nanomolar concentrations (0.5 – 90 nM). Results after 24 h of exposing the HepG2 cells to broccoli sprouts could not be analysed due to the high cytotoxicity of this plant product in this cell model. The higher amounts of SFN-GSH found in the culture medium involves the release of these molecules outside the

cells, assisting the carcinogen metabolism on two fronts: enhancing the activity of the phase II enzyme glutathione S-transferase (GST) and depleting the cell of GSH inducing ROS generation and apoptosis (Byun *et al.*, 2016; Clarke *et al.*, 2008). In addition, broccoli sprouts and SFN showed dose-time dependent antiproliferative activity in Caco-2, HT-29 and HepG2 cancer cell lines, while GRA did not achieve the half-maximal inhibitory concentration (IC_{50}). A lower IC_{50} was observed after broccoli sprouts treatment (~2 μM) compared to SFN (~50 μM), suggesting that other compounds present in the food matrix may act biologically in synergy with SFN. When lower concentrations of SFN were tested, no reduction of viability of the cells was found. This inhibitory effect of cell proliferation may involve cycle arrest of G0/G1, S, and G2/M phase cells through inactivation of cyclinB/CDK complex, apoptosis associated with activation of MAPK pathways, ROS generation and HDAC inhibition (Clarke *et al.*, 2008), and down-regulation of NFκB factor (Stefanson and Bakovic, 2014).

Moreover, to study the absorption and metabolism of bioactive compounds present in radish sprouts cv. Rambo we used *Drosophila melanogaster*, as a model organism. Flies were subjected to a diet supplemented with radish sprouts (10.6 $g \cdot L^{-1}$) for 10 days, containing 50 μM of sulforaphene (SFE). Flies homogenizes after this treatment showed nanomolar concentrations, per gram on a fresh weight basis, of SFE (1.11 $nmol \cdot g^{-1}$ F.W. in flies), as the predominant ITC, and SFN and I3C (< 0.4 $nmol \cdot g^{-1}$ F.W. in flies), in lower amounts. Therefore, the natural conversion from GLS to ITC by the plant enzyme myrosinase or by *Drosophila*-derived microbial myrosinase-like enzymes in the gut may have occurred. In order to support the conversion of SFE to SFN and the metabolism of SFN through the mercapturic

acid pathway in the flies, the conjugated metabolites were studied, showing homogenized flies the presence of SFN-CYS, suggesting that metabolism of SFN in the *D. melanogaster* is similar to mammalian species. In addition, we suggest that intestinal glucose absorption in the flies could be decreased by radish sprouts treatment, as we found inhibition of 23 % of the enzyme α-amylase with radish sprouts (10.6 $g \cdot L^{-1}$) and lower glucose levels (20 %) in the radish sprouts-treated flies. Interestingly, the homologous gene of PGC-1α in mammals, the *spargel* gen, was up-regulated in flies after radish intake, associated with the regulation of glucose homeostasis in mammals (Yoon *et al.*, 2001), as well as stimulation of mitochondrial biogenesis, reduction of ROS, induction of several detoxifying enzymes and, with increased life span extension in model organisms (Rera *et al.*, 2011).

After studying the absorption and metabolism of bioactive compounds, elicited broccoli sprouts (by exogenous spray of MeJA at 250 µM for 4 days before harvest) were evaluated for the potential to affect the nervous system by the antinociceptive effects and the gastric protection in rodent models of induced pain. Results showed analgesic effects of aqueous broccoli sprouts extracts in a dose-dependent manner in both routes of administration, oral (p.o., 50, 100, 250 and 500 $mg \cdot kg^{-1}$) and intraperitoneal (i.p., 500, 1000 and 2000 $mg \cdot kg^{-1}$), resembling the response of the analgesic tramadol (TRADOL®), by using two models of nociception: the writhing test (an acute pain model) in mice and the formalin paw test (with an early phase of neurogenic nociception and a late phase of inflammatory nociception) in rats. It has been previously described that broccoli sprouts may positively modulate the inflammatory and oxidant processes in the cells through the action of their bioactive compounds, inhibiting inflammatory processes, such as LPS,

ROS, iNOS, COX-2 and TNF-α, via inactivation of NFκB (Heiss *et al.*, 2001; Yun *et al.*, 2008). This anti-inflammatory activity is in agreement to the knowledge that the human intake of broccoli sprouts, *per se*, modulates the inflammatory and vascular prostainoids (Medina *et al.*, 2015). The presence of naloxone (an opioid antagonist) (United States pharmacopeia reference standard) reduced the effect of broccoli sprouts in both phases of the formalin test, suggesting an opioid mechanism of action at both central and peripheral levels of pain modulation. Broccoli sprouts did not cause any gastric damage, and produced gastric protection against ulcerogenic substances, which might be associated with stimulation of Nrf-2 gene-dependent antioxidant, protecting and repairing cells from oxidative damage (Yanaka *et al.*, 2005). Also the possible sedative effect of the extract, often observed in the adverse effect of the opioid analgesia, was evaluated, not showing any sedative effects *per se*, but a synergism with the sedative-hypnotic sodium pentobarbital was observed. Optimizing the bioactive compounds intake in the daily diet may be useful for improving pain management in both central and peripheral nociceptors.

Finally, keeping in mind practical application of the results and commercialization of sprouts, as well as their possible use in preclinical and clinical trials, we studied the microbial safety and bioactive compounds contents (GLS, ITC and phenolic compounds) of broccoli and radish sprouts in shelf-life. Vegetables once harvested are stored in open grocery display cabinets (> 7 °C), even though lower temperatures of storage are recommended (5 °C). Therefore, sprouts safety and phytochemical contents were evaluated under storage at two different temperatures, 5 and 10 °C, for 7 and 14 days. Data of microbiological analysis showed no pathogenic bacteria (*Salmonella* and *Listeria* spp.) development in sprouts, fulfilling the

Regulation (EC) No 2073/2005 for food stuffs. Other microorganisms such *C. perfrigens* and *E. coli* showed < 1 log CFU/g, while *S. aureus* showed < 2 log CFU/g. Similar counts of *Enterobacteriaceae*, aerobic mesophilic and psychrophiolic bacteria, moulds and yeasts (8 – 10 log CFU/g) were found in both sprouts at day 0 (after harvest) and during storage, showing a slight and roughly similar growth at two temperatures during the 14 days of storage. Even this high microbial load in the sprouts could inhibit the growth of pathogenic bacteria through competition during sprouting and storage (EFSA, 2011), the time after harvest should be controlled, as important factor for microbial content. Regarding the content of bioactive compounds in the sprouts, significant decreases of GLS, ITC and phenolic compounds were found depending on temperature and time of storage, being lower temperatures (5 °C) essential to the adequacy of shelf-life conditions, and decreases in compounds were very high when stored at 10 °C.

GLS present in broccoli (470 mg·100g^{-1} F.W.) and radish (720 mg·100g^{-1} F.W.) sprouts at harvest (day 0), decreased by 30 and 20 % during 7 days of storage at 5 °C, respectively, and an additional 20 % on day 14. In spite of that, the first week of storage was more relevant for GLS decreases, since no significant differences were found in broccoli sprouts stored at 5 °C for 7 and 14 days. It is noteworthy that slight losses (~7 %) were found in GRA and GRE contents, the predominant GLS in broccoli and radish sprouts, respectively, after 7 days of storage at 5 °C. Therefore, sprouts after one week of storage continue being a rich source of these compounds compared to commercialized broccoli heads (Rangkadilok *et al.*, 2002) and radish mature taproots (Yi *et al.*, 2016). The contents of ITC and I3C in the sprouts during storage showed a marked reduction, being this decrease more than 80 % in all

compounds studied except for SFN in broccoli sprouts, which concentration was reduced a 50 % after 7 days of storage at 5 °C. This high loss could be due to the decrease of the activity of myrosinase at low temperatures, slowing down the hydrolysis of GLS to ITC and indoles (Lim *et al.*, 2015).

Regarding phenolic compounds, only sinapic and ferulic acid derivatives were found in broccoli and radish sprouts, and contents were also affected by time and temperature of storage. As well as for GLS, the loss of phenolic compounds was higher during the first 7 days of storage, being 40 and 15 % in case of broccoli and radish, respectively, since no significant differences were found up to day 14 of storage. However, similar contents of phenolics (120 $mg \cdot 100g^{-1}$ F.W.) were found in both varieties at day 7 of storage at 5 °C. In spite of the microbial content and losses of phytochemicals reported, sprouts did not show any visible sign of spoilage during storage. However, low temperatures and days of storage are crucial to maintain the quality acceptability of sprouts during shelf-life.

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Chapter 6. Conclusions

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- The selection of cruciferous sprouts according to biomass production and phytochemical profile will maximize their health-promoting properties, without increasing the overall vegetable consumption.

- Exogenous spray application and seed priming with elicitors, particularly using methyl jasmonate or sucrose at very low concentrations, could be established as an effective strategy to enrich cruciferous sprouts in bioactive compounds.

- Sulforaphane, from broccoli sprouts, is bioavailable and bioactive in cells assisting their anticancer mechanisms, by inhibition of the proliferation.

- Sulforaphene, from radish sprouts, is absorbed by the same pathway of sulforaphane and it may be used for the modulation of the glucose metabolism *in vivo*.

- Broccoli sprouts induce *in vivo* central and peripheral antinociceptive activity, suggesting its potential use for treatment of pain without the adverse effect of analgesic drugs.

- Appropriate refrigeration conditions during shelf-life are necessary to provide to consumers with safe broccoli and radish sprouts, rich in bioactive compounds.

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- Las propiedades beneficiosas para la salud derivadas del consumo de brotes de crucíferas, se pueden favorecer mediante la selección de variedades con mayor biomasa y ricas en fitoquímicos bioactivos, sin tener que aumentar la cantidad de brotes consumida.

- Una estrategia efectiva para enriquecer los brotes de crucíferas en compuestos bioactivos es la aplicación foliar y la inducción de las semillas con elicitadores, especialmente con jasmonato de metilo y sacarosa a muy bajas concentraciones.

- El sulforafano, presente en los brotes de broccoli, ha demostrado ser biodisponible y bioactivo en líneas celulares de cáncer, promoviendo mecanismos anticancerígenos a través de la inhibición de su proliferación.

- El sulforafeno, presente en los brotes de rábano, se absorbe por la misma ruta metabólica que el sulforafano y puede ser modulador del metabolismo de la glucosa *in vivo*.

- Los brotes de brócoli poseen actividad antinociceptiva *in vivo*, pudiendo ser útiles para el tratamiento del dolor sin provocar los efectos adversos de algunos analgésicos farmacológicos.

- Es imprescindible una refrigeración adecuada durante el almacenamiento para ofrecer a los consumidores brotes de broccoli y rábano seguros y ricos en compuestos bioactivos.

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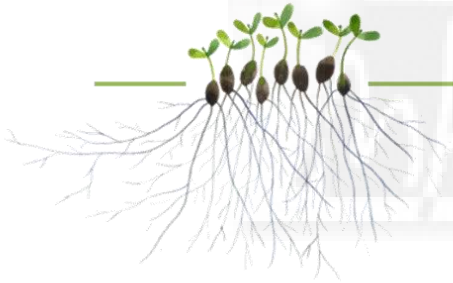

Chapter 7. Annexes

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1. BIOACTIVE COMPOUNDS FROM *BRASSICACEAE* AS HEALTH PROMOTERS

(Annex 1)

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Natural Bioactive Compounds from Fruits and Vegetables, 2016, 27-47 27

CHAPTER 2

Bioactive Compounds from *Brassicaceae* as Health Promoters

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Abstract: This work provides an up to date review of the information available about bioactive compounds present in the *Brassicaceae*family (glucosinolates, phenolics and vitamins) in relation to human health. The *Brassicaceae*plant family includes a large variety of species and cultivars, some of the most known are *Brassica oleracea* (*e.g.* broccoli, cabbage, Brussels sprouts), *Brassica rapa* (*e.g.* turnips), *Brassica napus* (*e.g.* rapeseed), Raphanus sativus (radishes), and Sinapis alba (mustards). In the recent years, these crops are increasingly consumed for possible health benefits as a good source of bioactive compounds. The sulphur containing compounds glucosinolates are almost exclusively found in this family, being their beneficial health effect supposed to be induced by their hydrolysis products, the isothiocyanates. In *in vitro* (human cell lines) and *in vivo* studies (animal models and human intervention assays) isothiocyanates have demonstrated their protective effects in carcinogenesis, chronic inflammation and neurodegeneration. The phenolic compounds mainly studied are flavonols, anthocyanins and hydroxycinnamic acids, which principal bioactivity is their antioxidant capacity. The carotenoids β-carotene, lutein and zeaxanthin, as well as, vitamins C, E and K have also been considered as nutrients with biological activity. The phytochemical wealth of *Brassica* foods is gathering attention from the scientific community for being potentially protective for the cardiovascular system and against certain types of cancer, and neurological disorders, mainly because of their anti-inflammatory and antioxidant properties.

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Luis Rodrigues da Silva and Branca Maria Silva (Eds.)

Even it is not yet possible to recommend a particular "daily dose" for human consumption of cruciferous foods for disease prevention, there is growing evidence regarding the protective effects of *Brassica* bioactive compounds for health via regulation of signaling pathways and cellular metabolism

Keywords: Antiinflammatory, Antioxidant, *Brassicaceae*, Cardiovascular disease, Carotenoids, Chemoprevention, Cruciferous, Glucosinolates, Isothiocyanates, Minerals, Neurodegeneration, Phenolic compounds, Vitamins.

INTRODUCTION

Brassicaceae family, commonly termed the mustard family or Cruciferae, represents a monophyletic group including approximately 350 genera and 3,700 species, which has been the subject of much scientific interest, with many crops of socioeconomical relevance (food and spices, condiments, oils), forage or ornamental. This family includes common species of food staples such as: broccoli, cauliflower, Brussels sprouts, cabbages, belonging to *Brassica* oleracea; turnips and Chinese cabbages of *Brassica* rapa; oilseeds of *Brassica* napus (rapeseed, leaf rape); mustards (*Sinapis alba*); and radishes (*Raphanus sativus*), among others. *Brassicaceae* crops are dated in Europe and northern Asia for at least 600 years and in the earlier part of the 20th century they have grown in North America, with productions in Europe around the 70 million tons/annum [1].

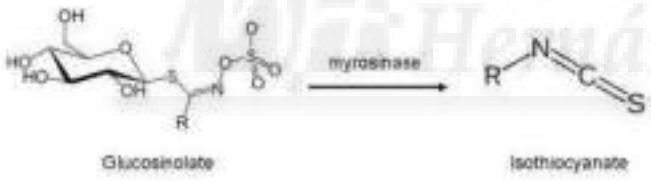
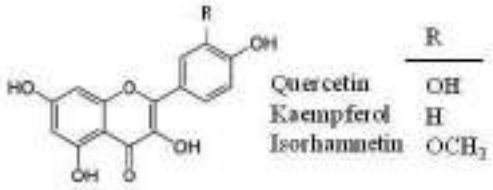
Brassicaceae crops are widely distributed in the World: Southwestern and Central Asia, Mediterranean Europe, and North and South America. *Brassica* production and consumption has increased worldwide in the last years, but only from a few cultivated genera [2]. There are numerous further species with great potential for exploitation in 21st century agricultural and food commodities, particularly as sources of bioactive phytonutrients.

PHYTONUTRIENTS IN CRUCIFEROUS PLANTS AND FOODS

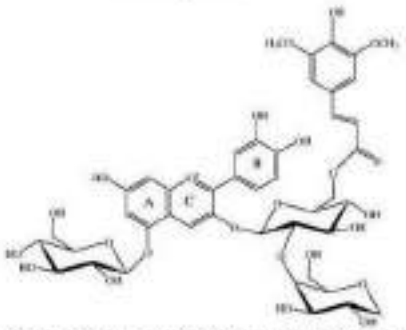
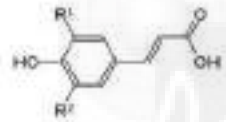
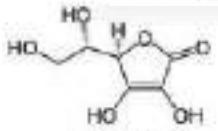
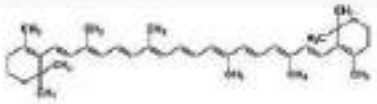
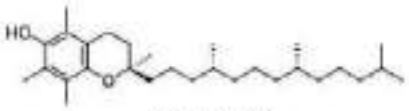
The *Brassicaceae* vegetables have been widely studied for their beneficial effects on human health through epidemiological studies [3], being nutritive foods rich in essential nutrients and phytochemicals that may act synergistically in the food matrix to modulate the cell metabolism and help in the prevention and treatment

of certain types of cancer, cardiovascular health problems, and neurodegenerative conditions of the aging human being (Table **1**) [4]. Although vegetable cruciferous plants are sources of fiber, folate, vitamins (A, E, C, and K) and minerals (Ca, Fe, K, Cu, Zn, P, Mn, and Mg, among others), the major body of evidence in the scientific literature is concentrated in the contents of secondary metabolites, such as flavonoids and carotenoids, and specially glucosinolates (GLSs). These compounds are mainly present in this family and are hydrolyzed to isothiocyanates (ITCs), which may be responsible of the chemoprotective activity and the reduction in the risk of suffering a number of cancers associated with the intake of cruciferous foods. Also the health-promoting effects of crucifers have been attributed at least in part to their bioactive composition rich in natural antioxidants, such as vitamins (C, A, E, K, *etc.*), carotenoids and phenolic compounds [5].

Table 1. Nutrients and phytochemicals presents in cruciferous plants and their physiological functions.

Compounds and chemical structures	Physiological functions	References
GLSs and ITCs  Glucosinolate Isothiocyanate	Induction of detoxification enzymes Apoptosis and arrest of tumor cell growth Decrease adipogenesis and inflammation Reduce oxidative stresses	[4, 6 - 8]
Flavonols  Quercetin R = OH Kaempferol R = H Isorhamnetin R = OCH_3	Prevent the oxidation of LDL Capillary protective effect Reduce serum levels of glucose Tumor inhibitory effect Anti-inflammatory, antimicrobial and anti-allergic	[5, 9, 10]

(Table 1) contd....

Compounds and chemical structures	Physiological functions	References
Anthocyanins  Cyanidin-3-(sinapoyl)diglucoside-5-glucoside	Actioxidant power and antigenotoxic	[11]
Hydroxycinnamic acids 	Cellular defense of peroxynitrite-mediated disorders	[12, 13]
Vitamins and β-carotene  Vitamin C  β-carotene  Vitamin E	Protection against free radicals Cytoprotective functions Preserve protein integrity	[14 - 17]
Minerals Fundamentally the elements or Mainly the elements K, Ca, Na, Mg, Fe, Zn, Se and Mn	Participation in metabolic activities Biochemical and nutritional functions	[3, 18]

Glucosinolates and Bioactive Isothiocyanates

The GLSs are secondary metabolites, sulphur and nitrogen-containing compounds with a common structure which comprises a *β*-D-thioglucose group, a sulphonated oxime moiety, and a variable aglycone side-chain derived from natural amino acids, that determine the final structure, being mainly derived from methionine, tryptophan or phenylalanine. Therefore, GLSs can be classified by their precursor amino acids as aliphatic (derived from alanine, leucine, methionine or valine), aromatic (from phenylalanine or tyrosine) and indolic (from tryptophan). The studies of the profile of GLSs indicate significant differences among species, according to the type and intensity of environmental stress, growth conditions and storage, processing and cooking methods [3, 19 - 21]. The GLSs load in plant tissues is highly variable, being seeds the plant part with the highest contents, followed by the germinating seeds and sprouts –that may present a 10-fold increase compared to commercial inflorescences or heads from adult plants– and generally followed by leaves and roots. This amount of GLSs may range from 1% to 10% (on a dry weight basis) in the seeds of some species [2].

GLSs are hydrolyzed to ITCs, their biologically active hydrolysis metabolites, both by the action of the enzyme myrosinase (thioglucoside glycohydrolase EC EC:3.2.1.147), which comes into contact with GLSs when there is a tissue disrupted by crushing or herbivory/chewing or by the action of the gut microflora upon human ingestion. Intact GLSs have no known biological activity; thus, the bioavailability of bioactive hydrolysis products is dependent not only on ingestion of GLSs, but also on their conversion prior to passage across the gut wall [6]. Inactivation of the plant myrosinase also decreases bioavailability of ITCs because the enzyme is heat sensitive, as occurs when *Brassica* vegetables are ingested cooked, thus boiling or steaming for more than 3-5 min and blanching previous frozen production will lead to loss of its activity [22].

The bioavailability of GLSs is measured by analyzing the mercapturic acid pathway products (mercapturates) which acts as a bioindicator or marker of intake and also are useful to study the bioaccesibility and bioavailability of the GLSs breakdown products, the ITCs, which gives rise to N-acetyl-cysteine conjugates. *In vitro* and *in vivo* studies mainly focused in the use of sulforaphane from

broccoli (SFN), has shown the influence of this bioactive compound on the cellular cytoprotective mechanisms involved in all the stages of development of cancer, through the selective induction of detoxification Phase II enzymes, to detoxify the products (electrophilic metabolites) of the activity of phase I enzymes to avoid the damage on the DNA (glutathione S-transferases, UDP-glucuronosyl transferases, and quinone reductase) [23] and through the limitation of the activity of Phase I enzymes [24]. A diet of 3-5 servings per week is sufficient to cause a 30% or 40% decrease in risk for a number of cancers [25].

Glucoraphanin (GRA) is the GLS precursor of the bioactive ITC SFN, which is being widely studied as a potent protector of carcinogenesis. Histone deacetylases (HDAC), which remove acetyl groups from proteins, has been studied recently and SFN metabolites were reported as inhibitors altering their gene expression and protein function [26]. One major step to determine the absorption of SFN is the hydrolysis of GRA by the action of the enzyme myrosinase. When comparing the intake of supplements rich in GRA with inactivated myrosinase, against the intake of fresh broccoli sprouts with the active enzyme, Clarke *et al.*, observed a much limited SFN absorption in healthy adults (7-fold lower) [27]. Also Vermeulen *et al.*, observed higher excretion of SFN metabolites after consumption of raw *versus* cooked broccoli – by 11% [28]. Other interesting work showed in plasma and urine higher levels of total SFN metabolites (3-5 times) in fresh broccoli sprouts consumers compared to myrosinase-treated broccoli sprouts extract containing SFN but not GRA; therefore, GRA hydrolysis to produce SFN is not the only one factor affecting the absorption of SFN, other compounds present in the broccoli sprout food matrix, such as minerals, vitamins, other nutrients and phytochemicals and fiber may facilitate the transport of SFN across cell membranes [29]. In SFN bioavailability, also the total amount of SFN estimated could derive from the interconversion of erucin, from the GLS glucoerucin, to SFN *in vivo* [8].

Not only the ITC SFN, but also erucin, from the precursor GLS glucoerucin, iberin from glucoiberin, sulforaphene from glucoraphenin (which differs from GRA by a double bond), phenethyl ITC from gluconasturtiin, and indole--carbinol (I3C) from indole GLSs (4-Hydroxi-, 4-Methoxy-, Neo- and glucobrassicin), have been studied because their bioactivity, triggering the

transcription factor Nrf2 into de nucleus, where the antioxidant response element (ARE) promoter region activate multiple genes, including both phase II detoxification enzymes and several antioxidant enzymes, among others, and induce cell cycle arrest and apoptosis [30].

Recent studies have shown that the I3C plays important roles in apoptosis and arrest of cell growth in breast and prostate cancer cells [31], and may potential benefits in preventing obesity and its comorbidities through different mechanisms including the reduction of adipogenesis and inflammation, and the increased thermogenesis [32].

Further *in vitro* and *in vivo* assays to understand GLSs bioavailability, would encourage the use of cruciferous vegetables as preventive and health food within the confines of animal studies or human trial for any form of cancer.

Phenolic Compounds

Phenolic compounds are ubiquitous phytochemicals in plants and plant foods (more than 8,000 described, characterized by having at least one aromatic ring with one or more hydroxyl groups attached). The structure of phenolic compounds may be very simple and with low molecular-weight, with single aromatic-ringed compounds or very complex (*i.e.*, tannins and other (poly)phenolics) [33, 34]. These compounds perform a variety of functions in the plant, generally centered on responses to pathogen attacks, UV protection, colour and sensory characteristics [35]. The phenolic compounds may be classified according to their number and arrangements of their carbon atoms in flavonoids (flavonols, flavones, flavan-3-ols, anthocyanidins, flavanones, isoflavones and others) and non-flavonoids (phenolic acids, hydroxycinnamates, stilbenes and others) [36].

Diets rich in plant-derived foods rich in phenolic compounds, such as those from the cruciferous family, have been reported to exert health-promoting benefits at different levels: anti-inflammatory, enzyme inhibition, antimicrobial, antiallergic, vascular and cytotoxic antitumor activity, and the most widely cited action of phenolics, their antioxidant activity [37, 38]. Phenolic compounds can play important roles in scavenging free radicals and up-regulation of certain metal-chelation reactions. The reactive oxygen species (ROS, singlet oxygen,

peroxynitrite, hydrogen peroxide), must be eliminated from cells to maintain healthy metabolic functions, and such reductions are positively associated with the ion transport systems and may affect the redox signaling in the cells [34]. Despite the beneficial effects of phenolic compounds it must be taken into account that only a small percentage of the dietary phenolics get inside the cells and are absorbed and metabolized. The plasma concentrations after intake of polyphenol-rich foods depend on the food source, and the polyphenol chemistry, and for example, it may vary from 0.3–0.75 μmol/L after consumption of 80–100 mg quercetin equivalents [39]. Moreover, (poly)phenolics are modified during their metabolism in the gastrointestinal tract and these modifications involve conjugation to produce glucuronides or sulphate conjugates by intestinal and/or hepatic detoxification enzymes. However, the major part of these molecules is metabolized by the colonic microflora rendering the so called microbial metabolites. Those microbial metabolites can be analysed in blood (plasma) and urine extractions, after ingestion, but only very small fraction of non-conjugated phenols in their original form can be found. This implies that these microbial metabolites rather than the native phenolics are responsible for the beneficial biological effects in the body [33].

Brassica vegetables are generally rich in polyphenols, although the profile and content of those compounds in the plant may vary depending *e.g.* on climatic conditions and harvest season [14, 40 - 42]. Moreover, differences in phenolic content can be expected between different cultivars as well as within plant organs [43]. Food processing may also affect phenolic content [44 - 47]. Phenolic contents may vary from 15.3 $mg \cdot 100g^{-1}$ in white cabbage (on a fresh weight basis), to 337 $mg \cdot 100g^{-1}$ in broccoli. Cartea and co-workers [48] extensively reviewed the phenolic profiles in many different species of *Brassicaceae* and the most widespread and diverse group of polyphenols in these species are the flavonoids (mainly flavonols, but also anthocyanins) and the phenolic acids.

Flavonoids

Flavonoids are low molecular weight plant-secondary metabolites, consisting of 15 carbon atoms with two aromatic rings (A and B), connected by a three-carbon bridge (C6–C3–C6) configuration that usually in the form of a C-heterocyclic

ring. The flavonoids described in *Brassica* spp. are *O*-glycosides of quercetin, kaempferol and isorhamnetin. Besides, they may be conjugated with different organic acids, most frequently at the 3-position of the C-ring, but substitutions may be also placed in the 5, 7, 4′, 3′ and 5′ positions [33]. To date, more than 20 flavonols have been described in *Brassica* vegetables such as kale, white cabbage, cauliflower, and broccoli as well as in *B. napus* and *B. rapa* leaves. Among them, the main flavonols were identified as kaempferol and quercetin 3-*O*-sophoroside-7-*O*-glucosides and combinations with hydroxycinnamic acids (*i.e.*, kaempferol and quercetin 3-*O*-(caffeoyl/sinapoyl)-sophoroside-7-*O*-glucoside). In the *B. rapa* group, in addition to quercetin and kaempferol derivates, it can be found derivatives of the flavonol isorhamnetin. In cruciferous varieties for fresh-cut and baby leaf supply including *Diplotaxis erucoides* L., *D. tenuifolia* L., *Eruca sativa* L., *Bunias orientalis* L., and *Nasturtium officinale*, quercetin and kaempferol glycosylated derivatives are the major flavonols [38, 48].

The glycosylated flavonols (3-sophoroside-7-glucosides of kaempferol) are receiving more attention in terms of beneficial health effects such as reduction in the risk of suffering certain age-related chronic health problems [38]. Accordingly, quercetin glycosides found at high concentrations in broccoli displayed the ability to prevent the oxidation of LDL by scavenging free radicals and chelating transition metal ions [9]. As a result, quercetin glycosides may help against certain conditions relevant to adult health: cancer, atherosclerosis and chronic inflammation [9]. On the other hand, isorhamnetin glycosides in mustard leaves were named responsible for the hypoglycemic effect using an antioxidant capacity test [49].

Anthocyanins are also present in *Brassica* vegetables conferring red pigmentation in red cabbages, red radishes, purple cauliflowers and broccolis. The major anthocyanins identified in these crops are cyanidin derivatives. In red cabbage and broccoli sprouts the major anthocyanins identified were the cyanidin 3-*O*-(sinapoyl)(feruloyl) diglucoside-5-*O*-glucoside and the cyanidin 3-*O*-(sinapoyl)(sinapoyl) diglucoside-5-*O*-glucoside [50]. The biological activity of the anthocyanins have been related also with the antioxidant capacity and the antigenotoxic properties, also repairing the cytological injuries caused by Cu^{2+} stress on lymphocytes [11].

Phenolic Acids and Derivatives

The compounds present in the phenolic acids class are consisting in two groups, the hydroxybenzoic and the hydroxycinnamic acids. The hydroxybenzoates include gallic, *p*-hydroxybenzoic, protocatechuic, vanillic and syringic acids, which have the C6–C1 structure in common. In the hydroxycinnamic acids, the compounds are aromatic with a three-carbon side chain (C6–C3), being caffeic, ferulic, *p*-coumaric and sinapic acids the most common in *Brassicas*. It is common to find the phenolic acids conjugated also with sugars or with other hydroxycinnamic acids [48]. This phenolic class is abundant in *B. oleracea* crops, such as kale, cabbage, broccoli, and cauliflower. In these crops, hydroxycinnamoyl gentiobiosides (1-*O*-caffeoylgentiobiose and 1,2,6-tri-*O*-sinapoylgentiobiose) and hydroxycinnamoylquinic (5-caffeoyl quinic acid) acids are the major representatives [51].

The antioxidant scavenger properties of *Brassica*extracts rich in phenolic acids have also been proved *in vivo*. As a result, the intervention in human subjects with a diet rich in Brussels sprouts showed a reduction on DNA damage in terms of a decreased excretion 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG) in urine [52]. It has been also reported that even with a short intervention, with broccoli sprouts in rats, a strong protection in the heart against oxidative stress and cell death caused by ischemia-reperfusion or diabetes, can be measured [12]. The sinapic acids, also present in high amounts in cruciferous foods, may also contribute to the cellular defense mechanisms avoiding peroxynitrite-mediated disorders [13].

NUTRIENTS: MINERALS AND VITAMINS

Minerals

The essential minerals (Na, K, Ca, Mg, Cl and P) are required in high amounts in diet (>50 mg/day), while the metals and trace elements (Fe, Zn, Cu, Mn, I, F, Se, Cr, Mo, Co, Ni) are needed in much lower concentrations (<<50 mg/day). The mineral nutrients are involved in different life processes, *e.g.* as electrolytes, as enzyme constituents, building materials in bones and teeth, *etc.* [18]. The microelements content in cruciferous foods are fundamentally K, Ca, Na, Mg, Fe,

Zn, Se and Mn. Ready-to-eat cruciferous sprouts are an excellent source of these compounds, showing higher concentrations of minerals than seeds (12-45% higher according to the compound) [53]. The content of the main minerals could vary among species and crops. Broccoli, cauliflower, turnip, cabbage, red cabbage and rutabaga show values ranging 170-300 $mg \cdot 100g^{-1}$ (on a fresh weight basis of K in raw products, being the predominant mineral in crucifers. The contents of P, Ca, Na and Mg range 10 - 60 $mg \cdot 100g^{-1}$ (on a fresh weight basis), and for Fe and Zn, 0.3 - 0.8 $mg \cdot 100g^{-1}$ F.W. [54]. Seasonal variations could affect the content of minerals, such as Fe, Ca and Zn, being higher in wet season than dry season [55]. Broccoli can be suggested as a 'good source' of Ca and Mg for human nutrition, with comparable bioavailability to that of milk, and therefore, may be considered an important alternative source of Ca in those population groups with limited access or intake of dairy products [56]. Different cooking methods (boiling, steaming, microwaving and frying) not affected significantly to the mineral content of broccoli florets; therefore, on average, an edible portion (200g of raw broccoli) could provide, over 20% of the daily requirements of minerals [21, 57].

Vitamins and Carotenoids

The vitamins present in cruciferous vegetables are: vitamin C, A, E, B and K, thiamin, riboflavin, niacin and folate. The major natural antioxidants in cruciferous foods are the vitamins (C, E, *etc.*), the carotenoids, and the (poly)phenolics [5]. The variation in the contents of these intrinsic antioxidants is caused by many factors: variety, organ and maturity at harvest, soil conditions and agricultural practices, post-harvest management, industrial and domestic processing, inducing all, many differences in the health-promoting properties of these vegetables when reaching the plate [58, 59]. These nutrients and phytochemicals are radical scavengers that inhibit the chain initiation or break the chain propagation (the second defense line) of the oxidative stress reactions. Diverse studies have shown a synergetic effect of both hydrosoluble (vitamin C) and lipid-soluble antioxidants (carotenoids and vitamin E), as in combinations of α-tocopherol or vitamin C plus phenolic compounds [60].

Vitamin C (Ascorbic Acid and Dehydroascorbic Acid), has many biological roles in human physiology, through its protective effects against free radicals,

prevention of DNA mutation in the cells, as well as the protection against lipid peroxidative damage, and repairing amino acid residues to preserve the protein integrity [58, 61]. These mechanisms of actions involve the prevention of certain cancer and cardiovascular diseases [62, 63]. For instance, vitamin C was established as responsible of 10–12% of the total antioxidant capacity of broccoli or cabbage [5]. The vitamin C can not be synthesized in the human body and therefore needs to be taken through diet. *Brassica* vegetables generally contain high amounts of vitamin C, and depending on dietary habits and geographical locations, may provide up to 50% of the daily RDI (recommended dietary intake) for human adults [58]. Among species, the vegetables from the genera *Brassica* (such as broccoli, red cabbage, Brussels sprouts, and kale) exhibit higher content of this vitamin (ranging 50-200 $mg \cdot 100g^{-1}$ F.W) than other species, depending distinct plant organs and physiological stages [54]. Also cooking methods decrease the Vitamin C content, causing steaming the lowest loss comparing to microwave and boiling [58].

On the vitamin E group, α-tocopherol is the most common and biologically active form, and like all essential nutrients, a minimum level of vitamin E is also essential for wellness and health. It reduces the peroxyl radicals produced from polyunsaturated fatty acids (PUFAs) in phospholipidic membranes or lipoproteins. The severe deficiency of vitamin E results in various neurological problems including ataxia (impaired balance and coordination), myopathy (muscle weakness) and damage to the retina. Suboptimal dietary intakes (or plasma levels of vitamin E below normal) are associated with increased risk of cardiovascular disease, some cancers and decreased immune function [15, 64].

Vitamin K, being phylloquinone the major dietary form, is found ranging 15-100 $\mu g \cdot 100g^{-1}$ F.W in common *Brassica* vegetables, such as broccoli, cabbage, red cabbage and cauliflower, and act as cofactor for the enzyme γ-glutamyl carboxylaseis, involved in the blood coagulation cascade and catalysis of the carboxylation of osteocalcin in bone [65].

Thiamine (B1) and riboflavin (B2) have been studied in cruciferous sprouts, radish, rapeseed and white mustard seeds contain vitamin B1 (0.41-0.70 $mg \cdot 100g^{-1}$ D.W.); however, its amount found in the ready-to-eat sprouts were 40% lower.

In contrast, the content of vitamin B2 in ready-to-eat sprouts were 3 -fold higher when compared to the seeds (0.096-0.138 mg·100g^{-1} D.W.).

Brassica crops show high levels of folate (15-60 µg·100g^{-1} F.W.), which is a scarce and important vitamin related to the reduced risk of vascular diseases, cancer and neural tube defects [3].

Carotenoids (carotenes and xanthophylls) are yellow, orange, and red lipid-soluble compounds characteristic of many fruits and vegetables. Leafy *Brassica*s are sources of carotenoids, particularly lutein, zeaxanthin and *β*-carotene [54]. These compounds are also reported for antioxidant functions such as quenching of singlet oxygen and other electronically charged molecules produced in reactions after photo or chemical excitation and they also react with peroxyl or alkoxyl radicals. The carotenoids are precursors of vitamin A (*i.e.β*-carotene, *γ*-carotene, and *β*-cryptoxanthin), and due to their conjugated double bonds they are both radical scavengers and quenchers of singlet oxygen [66, 67]. Brussels sprouts (6.1 mg·100g^{-1} F.W.), broccoli (1.6 mg·100g^{-1} F.W.), red cabbage (0.43 mg·100g^{-1} F.W.), and white cabbage (0.26 mg·100g^{-1} F.W.) are the species with higher content of these compounds. Several studies have demonstrated that carotenoids significantly down-regulated the expression of pro-inflammatory cytokines, possibly due to alterations of the NF-κB pathway and impacted Nrf2, a transcription factor related to the expression of detoxifying enzymes, in addition to directly quenching ROS, all related to the cytoprotective systems that reduce the risk for cardiovascular diseases and different types of cancer [68].

Even *Brassicaceae* vegetables are a good source of antioxidants, the potential health benefits mainly depend on the genotype and subspecies, as after studying different varieties and cultivars of cabbage, Chinese cabbage, cauliflower, broccoli and Brussels sprouts, the major source of Vitamin C (52.9 mg·100g^{-1} F.W.), β-carotene (0.81 mg·100g^{-1} F.W.), lutein (0.68 mg·100g^{-1} F.W.), DL-*α*-tocopherol (vitamin E) (0.47 mg·100g^{-1} F.W.) and phenolics (63.4 mg·100g^{-1} F.W.) was represented by broccoli [69].

OTHER NUTRIENTS

The vegetables of the cruciferous family are good sources of other additional

macronutrients. When comparing *Brassica* vegetables with other plant foods of high water content, the levels of fiber are relatively high. In white cabbage more than 30% of its total carbohydrate content is made from dietary fiber. Similarly, and depending on the crop, they can also represent significant sources of amino acids and proteins. Two hundred calories of steamed broccoli will provide 20g of protein [70]. Raw broccoli, cabbages and cauliflowers also contain folates, a relatively scarce and relevant nutrient that acts as a coenzyme in the synthesis of DNA, RNA and protein components, as well as in many single carbon transfer reactions [3]. Recently, it has been described that germinating seeds or prouts from cruciferous varieties can be also a good source of other antioxidants such us melatonin and serotonin [71]. As a conclusion, regular dietary *Brassica*vegetables it may account for an important promising chemopreventive dietary constituents (GLSs, vitamins, phenolics, minerals, fiber, *etc.*) which may protect the cell systems against free radical damages, LDL oxidation, pathogenesis of cardiovascular problems, and the DNA damages leading to cancer processes.

FUTURE PERSPECTIVES

The increasing awareness among scientists, food manufacturers, more and more health-conscious consumers worldwide, on the beneficial effects of the *Brassicaceae* phytochemical-rich foods, has prompt the production of these vegetables in sustainable practices. From the plant genetics approaches, through traditional breeding programs or through bioengineering of the secondary metabolism, to induce the accumulation of a particular nutrient or phytochemical, not many advatages have been reported. To the best of our knowledge, breeding programs to increase or decrease the content of a particular phenolic compound related to human health with horticultural *Brassica*crops have not been carried out. However, the modification of the synthesis of GLSs is being currently carried out in different crops such as broccoli. Through the introgression of a *Brassica* villosa MYB28 allele, that enhances sulphate assimilation and accumulates methionine-derived GLSs, it was developed commercially broccoli F1 hybrids with increased concentrations of GRA [72]. These investigations on increasing levels of *Brassica* phytochemicals may have a potential for human intervention studies to investigate the effects of a specific compound on human health.

On the other hand, as a result of the increasing available information about the health-promoting properties of cruciferous plants, there is an increasing demand of foods and food products enriched in *Brassica* bioactives. Minimally processed broccoli byproducts can be used as a source of bioactive ingredients, mainly GSLs and phenolic compounds to design novel beverages. A squeezed liquid composition is described by Kumazawa [73] that is rich in GLSs and has a good balance of vitamin C, colour, flavour and similar attributes. Moreover, the use of plant ingredients rich in *Cruciferae* bioactive compounds as functional foods and ingredients provide additional routes for the industrial exploitation of these attractive natural plant products. Only recently, pharmaceutical forms (pills, powders, capsules, vials, *etc.*) containing GLSs as active principles (commonly, broccoli extracts claiming sulforaphane presence in the formulation) have appeared in the markets, even with mixes of varieties of sources to supply SFN and I3C. In a very close future, a better understanding of the bioavailability, metabolism and physiological relevance of these dietary bioactive compounds and the intervention of the gut microbiota in the relationship will all help to elucidate the mechanisms by which these compounds are useful and suitable tools in the dietary interventions to treat and manage diseases as well as the maintenance of a wellbeing status in animals and the human beign.

CONFLICT OF INTEREST

The authors confirm that they have no conflict of interest to declare for this publication.

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2. ELICITATION: A TOOL FOR ENRICHING THE BIOACTIVE COMPOSITION OF FOODS

(Annex 2)

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Review

Elicitation: A Tool for Enriching the Bioactive Composition of Foods

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Abstract: Elicitation is a good strategy to induce physiological changes and stimulate defense or stress-induced responses in plants. The elicitor treatments trigger the synthesis of phytochemical compounds in fruits, vegetables and herbs. These metabolites have been widely investigated as bioactive compounds responsible of plant cell adaptation to the environment, specific organoleptic properties of foods, and protective effects in human cells against oxidative processes in the development of neurodegenerative and cardiovascular diseases and certain types of cancer. Biotic (biological origin), abiotic (chemical or physical origin) elicitors and phytohormones have been applied alone or in combinations, in hydroponic solutions or sprays, and in different selected time points of the plant growth or during post-harvest. Understanding how plant tissues and their specific secondary metabolic pathways respond to specific treatments with elicitors would be the basis for designing protocols to enhance the production of secondary metabolites, in order to produce quality and healthy fresh foods.

Keywords: elicitor; phytochemicals; health; phenolics; glucosinolates; activity

1. Introduction: Secondary Metabolites in Plants, Foods and Human Health

Plant-based nutrients and phytochemicals present in vegetable foods include proteins, lipids, carbohydrates, vitamins, minerals, and bioactive compounds, including phenolic compounds and

glucosinolates, that confer additional advantages to plant cell adaptation capacity to the surrounding environment, and act as precursors of molecules involved in the plant defense systems such as antibiotics, antifungals, and antivirals. Therefore, secondary metabolites are able to protect plants from pathogens (phytoalexins) [1] and insects [2], as well as constituting important UV-radiation absorbing compounds, thus preventing serious leaf damage [3]. The content of secondary metabolites in vegetables also confers a relevant role as health-promoting compounds and therefore contributes to their economic importance of foods [4]. Phenolic compounds contribute significantly to imparting specific flavours and colours to various plants widely utilized in foods and beverages. Examples includes capsaicin, responsible for the pungent properties of the red peppers, alkylphenols, responsible for the characteristic taste and odour of clove oil, tannins, which add a distinct bitterness or astringency to the taste of certain foods, and the anthocyanin pigments, such as the pelargonidins, the cyanidins and the delphinidins (responsible for red, blue and purple colours) [5]. The glucosinolates, characteristic of cruciferous foods, also add bitter taste (progoitrin) and aroma intensity (total glucosinolates) to vegetables [6].

The relevance of phenolic compounds [7] and glucosinolates [8] for human consumption has been associated with a protective effect against oxidative processes in relation to cardiovascular and central nervous system health, and neurodegenerative diseases, and with a reduced risk for cancers of the gastrointestinal tract, lung, colon, bladder, pancreas, skin, breast and prostate [9]. Optimizing the composition of fruits and vegetables would be a very cost-effective method for improving nutrition and disease prevention, since diet-induced health improvements would not represent any added costs for the health sector, even more it might help to reduce these costs [10–12].

The phytochemical composition of plants foods vary according to genetics (family, species, cultivar, *etc.*), physiological (organ, maturity and age) and agronomical factors (photoperiod, saline stress or fertilization) [13–19]. These factors are grouped as biotic (genetics, physiological determinants, pests and diseases) and abiotic (environment and agronomical conditions) and can be used to enhance valuable metabolites in foods and ingredients, in a year-round production [16,17,20]. Specific treatments, including precursor feeding and elicitor application can be used to increase metabolite production in the plant and to enhance its qualitative value for fresh produce, enriched food, or as a raw ingredient for feed/food and pharmaceutical products [21,22].

2. Elicitors

2.1. Concept and Classification

Elicitors are substances which induce physiological changes in the plant. Plants respond to these stressors by activating an array of mechanisms, similar to the defense responses to pathogen infections or environmental stimuli, affecting the plant metabolism and enhancing the synthesis of phytochemicals. The first biotic elicitors were described in the early 1970s [23]. Since then, numerous publications have accumulated evidence for pathogen-derived compounds that induce defense responses in intact plants [24,25] or plant cell cultures [22,26]. The use of elicitors as a tool to enhance the phytochemical content in plants, applied alone or in combinations at selected time points of the vegetable growth,

should not be confused with those administered during the plant production cycle or pre-harvest, such as conventional fertilization.

Elicitors could be classified as biotic and abiotic compounds, also plant hormones (salicylic acid (SA), jasmonates, *etc.*) may be considered as elicitors (Table 1) [27,28].

Table 1. Elicitor classification based on their origin.

Biotic Elicitors	
Lipopolysaccharides [27]	
Polysaccharides: Pectin and cellulose (cell walls) [28]; chitosan [21,28], chitin and glucans (microorganisms) [28], alginate, arabic gum [29], guar gum, LBG [27], yeast extract [27].	
Oligosaccharides: Galacturonides, guluronate, mannan, mannuronate [27,30].	
Proteins: Cellulase [31], cryptogein [32], glycoproteins [27], oligandrin [27], pectolyase, fish protein hydrolysates[33], lactoferrin [33].	
Complex composition: Fungal spores, mycelia cell wall, microbial cell wall [27].	
Pathogen toxin: Coronatine [34].	
Oregano extract [33].	
Abiotic Elicitors	
Chemical	Physical [35]
Acetic acid [21]	Altered gas composition
Benzothiadiazole [36]	Chilling
Silicon [36]	CO_2
Bioregulator prohexadione	Drought
Ethanol [37]	Extreme temperature shock
Ethene [37]	High pressure
Inorganic salts: mercuric chloride (HgCl2), copper sulfate (CuSO4), calcium chloride (CaCl2), and vanadyl sulfate (VSO4) [28]	High or low osmolarity
	UV irradiation
	Saline stress
Metal ions: Co^{2+}, Fe^{2+}, Al^{3+}, Ag^{2+}, Ag^{+}, Mn^{2+}, Zn^{2+}, Cu^{2+}, Pb^{2+} and Cd^{2+} [28,38]	Wounding
	Ozone
Plant Hormones	
Jasmonic acid, methyl jasmonate [39], methyl salicylate, salicylic acid, ethylene [21,40], cytokinin, gibberellin GA_3 [37]	

Biotic elicitors (chitosan, alginate, cellulose, *etc.*) have biological origin, often originated as a result of fungi, bacteria, virus or herbivore infections (exogenous elicitors), and in some cases are released from the attacked plant by the action of enzymes of the pathogen (endogenous elicitors) [27]. Often complex biological preparations have been used as elicitors, where the molecular structure of the active ingredients is unknown. Examples of such elicitors are yeast extract and microbial cell-wall preparations [27]. Yeast extract contains several components that can elicit plant defense responses, including chitin, N-acetylglucosamine oligomers, β-glucan, glycopeptides and ergosterol.

SA and jasmonates (jasmonic acid (JA), methyl jasmonate (MeJA)) are widely known to elicit a wide range of compounds by inducing the expression of plant genes for various biosynthetic pathways, and are also defined as "hormones" because they induce cellular responses at low concentrations distant from their site of synthesis, and can be applied to plants in a variety of ways. For instance, MeJA may be applied to plants as a gas in an enclosed environment, on a liquid form to a hydroponic

solution, or by jasmonate sprays [39]. The treatment of young red and black raspberry fruits with 0.01 mM or 0.1 mM MeJA increased their anthocyanins and phenolic compounds [41]. Analogs of MeJA or JA have physiological activity. For instance, N-propyl dihydrojasmonate (PDJ) increased the abscisic acid (ABA) and anthocyanin content of apples [42]. Abiotic elicitors are produced by factors responsible for environmental stress. These factors can be of chemical (inorganic salts, metal ions and others which disturb the membrane integrity) [28] and physical origin (UV irradiation, wounding, saline stress, ozone *etc.*) [35] (Table 1). For instance, exposure of alfalfa, broccoli and radish 3-old-day sprouts to high light intensity (700 $\mu mol \cdot m^{-2} s^{-1}$ for 1 day) or chilling (4 °C and and 120 $\mu mol \cdot m^{-2} s^{-1}$ for 1 day) resulted in higher total phenolic content and antioxidant capacity compared with controls, by 20% in alfalfa and 40% in broccoli, and showed a 25% increase of phenolic content and 40% of higher antioxidant capacity in radish [43].

Apart from the classification of elicitors according to their nature, they can also be classified upon their interaction with the host plant, as "general elicitors", such as carbohydrates, cell wall proteins, oligosaccharides *etc.*, which induce non-specific mechanisms for the induction of defense response in different plant cultures, and "specific elicitors" from fungal, bacterial, viral or plant origin, which affect only a specific host cultivar since the presence of its corresponding resistance gene in the host plant is directly associated with the resistance against a specific gene pathogen [4,44].

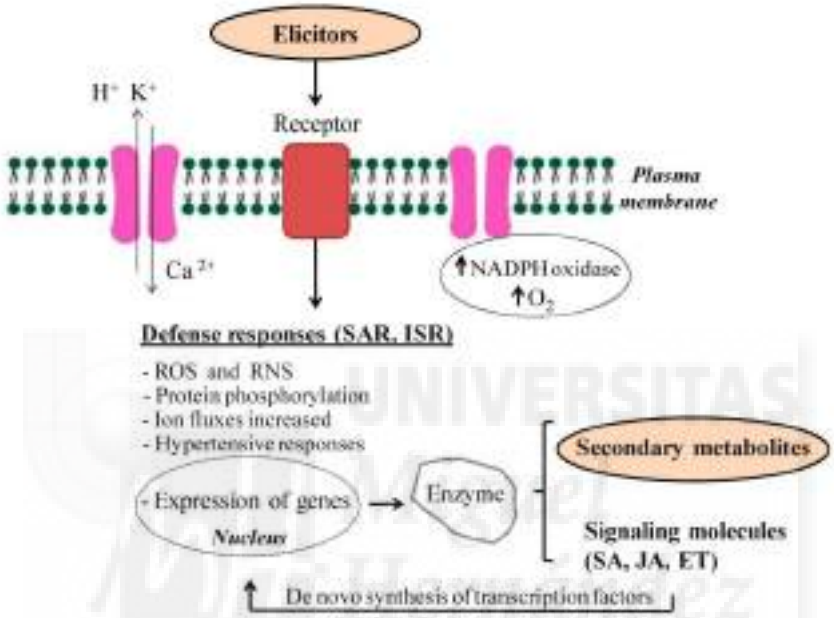
2.2. Mode of Action of Elicitors

In plant defense systems each cell has acquired the capability to respond to pathogens and environmental stresses and to build up a defense response. Plant response is determined by several factors, mainly depending on their genetic characteristics and physiological state. In the majority of cases, plant resistance to diseases is known to be genetically controlled by plant resistance (R) genes and pathogen avirulent avirulence (Avr) genes (gene-for-gene interaction concept) [45]. However, triggering resistance is not always due to specific Avr products which activate defense responses in cultivars possessing the matching R genes but, instead, proceeds from the action of general elicitors, able to activate defenses in different cultivars of one or many species [45]. First step in the response of plant against elicitors is the stimulus perception by receptors localized in plasma membranes of the plant cell (Figure 1), like protein kinases, which represent one of the most important in pathogen perception for a number of fungal elicitors [46], or could be localized within the cell to initiate signaling processes that activate plant defenses, as for certain bacterial elicitors, which initiate signaling processes that activate plant defenses [47].

The elicitor signal transduction is an important subject of investigation. In this sense, several authors have described that plants respond to elicitors by activating an array of defense mechanisms on the surface of the plasma membrane (Figure 1), including induction of pathogenesis-related proteins and enzymes of oxidative stress protection, hypertensive responses, characterized by rapid cell death in the immediate vicinity of the point of exposure to the pathogen [45], the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS), the activation of defense-related genes, changes in the potential of plasma membrane cell and enhanced ion fluxes (Cl^- and K^+ efflux and Ca^{2+} influx), rapid changes in protein phosphorylation, lipid oxidation, and structural defensive barriers, such as reinforcement and lignification deposition inn cell wall, *etc.* and the activation and the *de novo*

biosynthesis of transcription factors, which directly regulate the expression of genes involved in secondary metabolites production [48–50] (Figure 1).

Figure 1. General mechanism after elicitor perception. Abbreviations: SAR (systemic adquired response), ISR (induced systemic resistance), ROS (Reactive oxygen species), RNS (reactive nitrogen species), NADPH (nicotinamide adenine dinucleotide phosphate), SA (salicylic acid), JA (jasmonic acid), ET (ethylene) [48–50].



2.3. Preharvest Elicitation: Priming Seeds and Edible Plants

Preharvest elicitation could be done as seed priming [33,51], soaking seeds in a water solution with the elicitor, or after seedling, applying exogenous spraying treatment over the leaves [52] or in a hydroponic system [53].

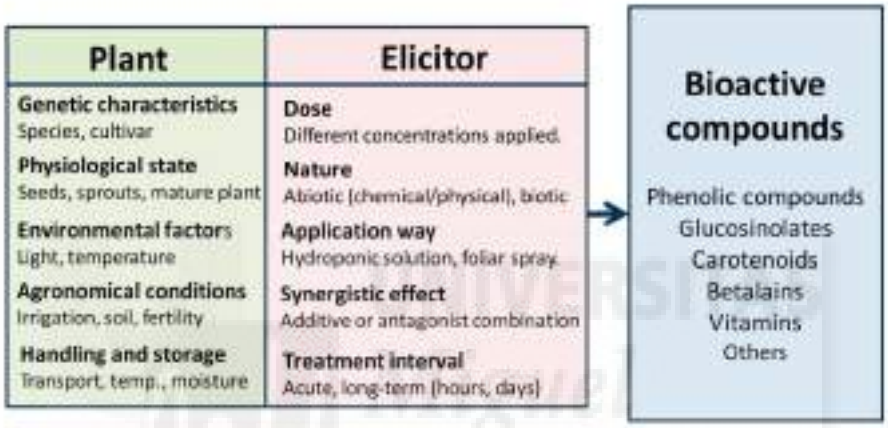
Elicitor nature, doses and time of treatment strongly affects the intensity of the plant response (Figure 2). Elicitors can stimulate different classes of secondary metabolites and affect in a different way the concentration of these compounds, being more dependent on plant genetics (species and cultivars) than on the elicitor nature.

A MeJA elicitation, applied daily by exogenous spraying at 10 µM, resulted in a 31%, 23% and 22% increase of total flavonoid, phenolic and glucosinolates concentration, respectively, in 7 day old broccoli sprouts [25]. Also a MeJA sprayed treatment (10 mM) at the beginning of veraison in grape (*Vitis vinifera*) increased anthocyanin and flavonols content up to 81% and 131%, respectively [54].

Concentration of elicitor and interval between treatment and harvest induce different responses characteristic of plant species, making necessary to find the adequate effective dose and time empirically [4]. Radish sprouts (*Raphanus sativus* L.) treated with 100 mM of NaCl increased total glucosinolates in 5- and 7-day-old sprouts, by 50% and 127%, respectively, and the phenolic contents in 3- and 5-day-old sprouts, by 20% and 40%, respectively, while with a low and moderate level of salt

stress (10–50 mM of NaCl) reduced these contents [55]. Bodnaryk showed that JA and MeJA were equally effective at high doses (>5 nmol seedling^{-1}) in increasing the concentration of 3-indolylmethyl glucosinolates (3-IMG), maybe because of the saturated effect of jasmonates, but at lower doses, JA was more potent than MeJA [56]. The dose needed to cause a doubling of the concentration of 3-IMG in the cotyledons of 7-day-old *B. napus* sprouts, in 24 hs, was 8.2 pmol for JA and 41 pmol for MeJA. The sulphur effect, as elicitor, in broccoli sprouts was dependent on the dosage (K_2SO_4 at 15, 30, and 60 mg/L) and augmented the total glucosinolates in sprouts by 14%, 18%, and 23%, respectively, 12 days after sowing [57].

Figure 2. Factors influencing bioactive compounds in plant response.



Physiological conditions also play an important role in the elicitation techniques, which achieving better results during the exponential phase of growth of the plant, when the concentration of bioactive compounds is higher [58], and in the presence of growth regulators [59].

Different studies have reported an additive or synergistic response after combination of elicitor treatments, different signal transduction pathways appear to exist in response to environmental stresses and elicitors and these pathways could antagonize or harmonize with each other, leading to negative or additive interactions, respectively [58,60,61].

2.4. Postharvest Elicitors Applications

Specific elicitor treatments has been used in postharvest practices to enhance the phytochemical content and quality composition in many fruits and vegetables, such as the application of low or high temperature treatments [62], ultraviolet (UV) [63,64] or gas combinations before commercialization [40]. In this context, it has to be mentioned that red orange fruits (*Citrus sinensis*) accumulated anthocyanins (8-fold compared to control) in their juice vesicles during cold storage at 4 °C for a period of 75 days [62]. An accumulation of phenolic compounds was also found in apple (*Malus domestica*) during cold storage which was coupled with increasing the phenylalanine ammonialyse (PAL) activity, a key enzyme in the phenylpropanoid pathway [65]. A combination of visible light and UV-B irradiation (380 nm) applied 12 h per day during a period of 10 days, increased the total phenolic compounds (127% compared to irradiation of visible light alone) in apple peel. It was assumed that UV-stress also

mediated the increase of PAL activity [64]. Ultraviolet irradiation can lead to grapes with enhanced antioxidant properties, within normal conditions of market commercialization [63].

On the other hand, phytohormones applied to tissues will increase phenolic concentration. For instance, ethylene applied to butter leaf lettuce at 10 $\mu L \cdot L^{-1}$ in a flow of humid air for 3 days at 5 °C, induced synthesis of phenolic compounds by 38%, even though wounding increased by 87% these compounds [40]. Furthermore, the authors observed that temperature also affected the concentration of phenolics, at 10 °C ethylene and wounding induced increases of 174% and 155%, respectively. The exogenous application of the phytohormone MeJA (170 µL spontaneously vaporized at 25 °C) over strawberry fruits during 7 days, induced an increase of 35%, 52% and 187%, on phenolic content, antioxidant capacity, and anthocyanins, respectively [66]. A longer storage, after 11 days, resulted in a considerable decline of total phenolic content and antioxidant capacity, detrimental of fruit quality. On the other hand, through elicitor practices also the quality of food products could be enhance, such as the improvement of the volatile profile, flavor and taste of wine after a chitosan treatment or the increase of phenolic compounds of peppermint resulting infusions after SA foliar application in the plant [67,68]. Understanding the interactions among the stressor applied and the tissue response will help to optimize the right application.

Alternatively to a hierarchical response, additive or synergistic responses can be used to selectively target the increase of bioactive compounds [21,69]. Synergistic effects have also been found for postharvest elicitors, in sorghum seedlings exposed to low moderate temperatures during 24 h before a red light irradiation by fluorescent tubes (661 nm), resulting the optimum temperature at 20 °C for enhancement of red light induced anthocyanin synthesis (185%) compared that for seedlings growth at 24 °C [70]. The use of wounding (3 mm thick disks sliced) in combination with ethylene (1000 ppm) and MeJA (250 ppm) in purple carrot (*Daucus carota* L.) increased the total phenolic content by about 176% and 210%, respectively, compared to the separate treatments [71].

3. Elicitation Effects on Primary Metabolism

Plant primary metabolism includes physical and chemical processes that fulfill the essential functions for the maintenance of plant life: survival, growth and reproduction. Photosynthesis, respiration, nutrient uptake, transport and partitioning, protein synthesis, tissue differentiation, biosynthesis of carbohydrates, lipids and the proteins involved in these processes or in structural parts are all chemical processes belonging to the primary metabolism. Biotic and abiotic stresses (variation in agronomical conditions, such as plant organ, plant competition, fertilization, pH, season, climate, water availability, light, and CO_2 [9]) are expressed in plants by a series of morphological, physiological, biochemical and molecular changes that adversely affect plant growth and productivity [72].

Gómez *et al.*, studied MeJA spray application (0.5 mM) to the foliage of tomato plants for 4 h. There was a significant decrease in the fixation of CO_2 (20%) and an increase in the export of newly acquired carbon and nitrogen (1-fold) out of MeJA-treated leaves [73]. These results showed a change in the allocation of resources after MeJA application, this may reduce the chance of resources being lost to herbivores and act as a buffer to biotic stress by increasing the potential for plant regrowth and survival after the attack.

The effects on the germination of alfalfa and broccoli seeds stimulated by dry smoke (by the complete combustion of *Artemisia vulgaris*) during 30 and 45 min, respectively, and aspirin solution (0.145 g/100 mL in pure water) during 10 and 30 min, respectively, showed higher growth ratio than control group (>112%) [74].

A treatment of chitosan (28 kDa), a deacetylated derivative of chitin, at 0.5% dissolved in 0.5% lactic acid, increased the total weight (12.9%), germination rate (16%) and total isoflavone content (11.8%) of sunflower sprouts [51], while a treatment in soybean sprouts with 0.05% chitosan (493 kDa) in 0.05% acetic acid solution increased the total weight (26%) and vitamin C content (14%) compared with that of the control [51,75].

Baenas *et al.*, showed an increase in biomass weight of 5 different *Brassicaceae* sprouts after 5-days spray elicitation with sucrose (146 mM), as a supply of carbon source for cell growth, and DL-methionine (5 mM), enhancing the overexpression of some genes [52].

4. Elicitors Affecting the Content of Bioactive Compounds

The most actively pursued strategies to increase the production of target natural products in plants, are the applications of chemical elicitors and the study of the signal transduction pathways and transcription factors required for the expression of genes, involved in the biosynthesis of specific bioactive phytochemicals [50].

Much effort has been put into cloning biosynthetic genes, identifying transcription factors, revealing the signal transduction steps underlying elicitor activation of plant secondary metabolism and also into the manipulation of regulatory and biosynthetic genes, to engineer plant cells and enhance the production of target secondary metabolites [76]. It is expected that a better understanding of the signal transduction pathways, linking plant cell stimulation and biosynthesis of natural compounds may help to develop new strategies to alter the production of target compounds, by either activation or suppression of certain metabolic pathways [48]. As a consequence, in plant tissues is observed the production of antioxidant molecules, compounds of technological interest in healthy foods [48]. Hao *et al.*, showed a feasible strategy to combine MeJA and SA treatment with transgenic technology for the enhancement of tanshinone, an active diterpene which is widely used in the treatment of cardiovascular diseases, in *Salvia miltiorrhiza* hairy roots [77], also SA was reported to enhance anti-inflammatory activity of *Aloe vera* by increasing its anthraquinones [78].

4.1. Phenolic Compounds

Phenolic compounds (more than 8,000 in Nature), can be classified based on the number and arrangement of their carbon atoms in flavonoids (flavonols, flavones, flavan-3-ols, anthocyanidins, flavanones, isoflavones and others) and non-flavonoids (phenolic acids, hydroxycinnamates, stilbenes and others) and they are commonly found conjugated to sugars and organic acids.

Phenolic compound contents have been associated with flavour and colour characteristics of fruits and vegetables. These compounds have additional multiple roles in plants, including attracting insects for seed dispersion and pollination and being part of the natural defense system [79]. Moreover, in recent years, phenolic compounds have been intensively investigated because of their potential health-promoting effects, such as anti-inflammatory [80], antimicrobial [81], antiallergic [82], vascular [83]

and cytotoxic antitumor activity [84], but the most cited biological activity is based on their antioxidant capacity, related with its chemical structure that confers them redox properties [85,86]. The accepted wide range of beneficial effects of phenolic compounds initiated, attempts to stimulate their accumulation in crop plants by agricultural technologies. Several reviews summarized the advantages of targeted pre- and post-harvest elicitor treatments to obtain fruits and vegetables enriched with beneficial phytochemicals [87–89]. Alfalfa three-day-old sprouts subjected to high-light (700 $\mu mol \cdot m^{-2} s^{-1}$ for 1 day) and chilling (a growth chamber at 4 °C with a light intensity of 120 $\mu mol \cdot m^{-2} s^{-1}$ for 1 day) accumulated about 2.0 and 1.5 times, respectively, significantly higher concentration of ferulic acid. Therefore, high-light seems to elicit a stronger response than chilling in enhancing the phytochemical content [43]. The largest accumulation of sinapic acid (by 83% more compared to untreated control) occurred following high-light treatment (700 $\mu mol \cdot m^{-2} s^{-1}$ for 1 day) in broccoli sprouts, similar to ferulic acid in alfalfa, however, chilling did not seem to have any effect on the sinapic acid content in broccoli sprouts [43]. Examples of biotic and abiotic elicitors affecting different groups of phenolic compounds are listed in Table 2.

Table 2. Phenolic compounds increased by elicitors.

Plant Food	Elicitor Treatment	Application	Target Compounds Class and Increase	Reference
"Fuji" apples	Ethephon (2-chloroethyl phosphonic acid) (100 mg/L)	Sprayed for 4 weeks before commercial harvest	Anthocyanins (8-fold), and flavonols (2-fold) during fruit maturation	[90]
Grape berry fruits	Ethanol (5 g/100 mL)	Sprayed for 8–9 weeks after anthesis	Anthocyanins (3-fold)	[91]
Butter Lettuce	JA 1 µM	Sprayed after 21 days of germination	Total phenolics (280%) Flavonoids (133%) Phenolic acids (360%)	[92]
Lettuce cv. "Lollo Rosso"	UV-full range (UV-A and UV-B)	Radiation during cultivation	Flavonoids (130%) and phenolic acids (200%)	[93]
Purple-flesh potatoes	Wounding (vegetable slicer)	After harvest	Total phenolics (60%)	[94]
Strawberry fruits	CO_2 (ambient + 600 µmol)	28 months	Anthocyanin and flavonols (30%–50%)	[95]
Sweet basil	MeJA 0.5 mM	Sprayed when the plants had five or six leaves	Rosmarinic acid (50%) and caffeic acid (38%)	[96]
Greek oregano	Chitosan oligosaccharides (50 and 200 mg/L)	Sprayed for 2 weeks prior to the anticipated flowering time	Phenolic acids and flavonoids (30%)	[97]
Pea sprouts	Folin acid (50 µM) and vitamin C (500 µM) solutions	Soaking seeds for 12–48 h	Total phenolic compounds (20%)	[98]

Table 2. *Cont.*

Plant Food	Elicitor Treatment	Application	Target Compounds Class and Increase	Reference
Pea sprouts	Folin acid (50 μM) and vitamin C (500 μM) solutions	Soaking seeds for 12–48 h	Total phenolic compounds (20%)	[98]
Olive trees organs	Nutrient solution "Brotomax" (0.3 g/100 mL) (urea nitrogen, copper, manganese and zinc)	Sprayed for 120 days after anthesis	Tyrosol, catechin, and oleuropein (20%)	[99]
Radish sprouts	NaCl (100 mM)	In 0.5% agar media for 3, 5 and 7 days after sowing seeds	Total phenolics (30% and 50% in 5 and 7-days-old sprouts, respectively)	[100]
Radish, chinese kale and pak choi 3-day-old sprouts	Glucose (5 g/100 mL)	Hydroponic system for 3 days after sowing seeds	Total phenolics (20%)	[53]
Broccoli 7-day-old sprouts	Sucrose, fructose and glucose (146 mM)	In 0.5% agar media for 5 days after sowing seeds	Total anthocyanins (10%)	[55]
Broccoli 7-day-old sprouts	Sucrose and mannitol (176 mM)	Hydroponic system for 5 days after sowing seeds	Total anthocyanins (40%) and phenolics (50%)	[101]

Elicitors also have been applied as a complementary treatment to fungicides, such as the exogenous application of benzothiadiazole and MeJA, increasing, at the same time, the flavonoids content (anthocyanin, flavonol, and proanthocyanidin) in grapes and showing higher color intensity and total phenolic content in wines [54].

4.2. Glucosinolates

Glucosinolates (GLS) comprise a relatively small but diverse group of over 130 nitrogen and sulfur-containing natural products found almost exclusively in cruciferous plants [102]. The glucosinolate core structure comprises a *β*-thioglucoside *N*-hydroxysulphate, containing a side chain and a *β*-D-glucopyranose moiety [14]. The structure of the side chain is highly variable and determines the glucosinolate classification as aliphatic, indolic, or aromatic [103,104] according to whether their amino acid precursor is methionine, tryptophan, or an aromatic amino acid (tyrosine or phenylalanine), respectively [14]. Glucosinolates are plant defense compounds against various pathogens and pests, and are accumulated preferentially in the organs that contribute most to the growth cycle of the plant [102]. Besides, these compounds have a potential benefit to protect humans against certain cancers, particularly lung and those of the gastrointestinal tract, and also in the reduction of risks for cardiovascular diseases [9,105,106]. However, there are still many areas that need further research to avail the full health benefits of these compounds [107]. Glucosinolates are also responsible of organoleptic properties in some plants, such as cauliflower and mustards [108].

Glucosinolates profiles can be altered by treatments with elicitors [21,109]. Exogenous application of SA, JA and MeJA have been widely studied because of the results in expression of large number of genes involved in resistance responses, among these are genes related to biosynthesis of phytochemicals in plants [110]. SA, JA and MeJA serve as signaling molecules induced by pathogen infestation [24] and mechanical wounding [56]. Treatment of *Brassicaceae* plants with these elicitors can stimulate the increase of glucosinolate content. Baenas *et al.*, (2014), reported that MeJA elicitor (25 μM) was highly effective to increase the total glucosinolates in 5 different 8-day-old *Brassica* and *Raphanus* sprouts, specially, the concentration of the health-promoting glucoraphanin and glucoraphenin by 50% [52].

The individual classes of glucosinolates respond differently to the elicitor treatment. Treatment with SA and MeJA increased the total amount of glucosinolates, particularly levels of aromatic and indole glucosinolates, in secondary roots of turnip, in contrast, SA or MeJA either reduced or did not affect the levels of aliphatic glucosinolates [111]. Kiddle *et al.* reported that JA induces mainly indole glucosinolates in leaves, and the intensity of this "induction" depended on the JA concentration applied and the age of the leaf, retaining developing leaves higher levels than mature leaves [112]. Examples of biotic and abiotic elicitors affecting glucosinolates are showed in Table 3.

Table 3. Glucosinolates increased by elicitors.

Plant Food	Elicitor Treatment	Application	Target Compounds Class and Fold Increase	Reference
Brassica 7-day-old sprouts cotyledons and leaves	JA spray (5 nmol)	Topically	3-indolylmethyl GLS (6-fold) in *B. napus*; 4-hydroxy-3-indolylmethyl GLS (9-fold) in *B. rapa*; both indole GLS (2-fold) in *B. juncea*	[56]
Turnip root exudates	MeJa (130 μM)	Added in the hydroponic system for 10 days	Indole GLS (4-fold)	[113]
Broccoli sprouts	Sucrose (146 mM)	In 0.5% agar media for 5 days after sowing seeds	Total GLS (2-fold)	[55]
Broccoli 7-day-old sprouts	1. Methionine (5 mM) 2. Tryptophan (10 mM) 3. SA (100 μM) 4. MeJA (25 μM)	Daily exogenous spraying during 3, 5 and 7 days	1. Aliphatic GLS (30%) 2. Indole GLS (80%) 3. Indole GLS (30%) 4. Indole GLS (50%)	[25]
Radish, chinese kale and pak choi 3-day-old sprouts	Glucose (5 g/100 mL)	Hydroponic system for 3 days after sowing seeds	Gluconapin (150% and 60% in Chinese kale and pak choi, respectively) Glucobrassicanapin (110-fold in pak choi)	[53]

Table 3. *Cont.*

Plant Food	Elicitor Treatment	Application	Target Compounds Class and Fold Increase	Reference
Sauerkraut (*B. oleracea* L. var. capitata)	0.5% NaCl and 0.3 mg of sodium selenite/kg	Added to fresh cabbage before fermentation	Indole GLS hydrolysis products (indole-3-carbinol and indole-3-acetonitrile in 70% and 10%, respectively)	[114]
Radish sprouts	NaCl (100 mM)	In 0.5% agar media for 3, 5 and 7 days after sowing seeds	Total GLS (50% and 120% in 5 and 7-days-old sprouts, respectively)	[100]
Brassica 8-day-old sprotuts	MeJA (25 µM) JA (150 µM) Sucrose (146 mM)	Sprayed for 5 days before harvest	Total GLS Broccoli: >50% Turnip: >20% Rutabaga: >100%	[52]
Raphanus 8-day-old sprotuts	MeJA (25 µM) SA (100 µM) Glucose (277 mM)	Sprayed for 5 days before harvest	Total GLS: > 20%	[52]
Broccoli 7-day-old sprouts	Sucrose and mannitol (176 mM)	Hydroponic system for 5 days after sowing seeds	Total GLS: > 50%	[101]
Broccoli florets	Ethanol evaporated (500 µL/L)	6 h after harvested	Total GLS: > 50%	[115]
Broccoli florets	MeJA spray (250 µM)	Acrial portions twice per week from flowering to head formation	Indolyl GLS: > 30%	[91,116]

4.3. Carotenoids and Betalains

Over the past few years, there has been a surge in interest in fat-soluble compounds, such a carotenoids, and water-soluble compounds, such as betalains, due to their beneficial effects on human health [117]. Carotenoids were initially described as playing a role in the protection against photo-oxidative processes, and they have been extensively studied for the prevention of cancers and cardiovascular diseases and for their photoprotective properties [118].

Tomato fruits cv. Liberto were subjected to UV-B radiation before harvest with an UV-B dosage of 0.075 and 0.15 Wh m^{-2} after different adaptation times of 22 and 44 h, the concentrations of carotenoids, lycopene and β-carotene, in ripe tomato fruits were higher increased by an UV-B dosage of 0.075 Wh m^{-2} after 22 h of adaptation time [119].

Betacyanins (red-violet pigments) and betaxanthins (yellow pigments) are a group of chromoalkaloids known as betalains presents in *Caryophyllales*. Interest in betalains is determined by their antiradical activity and their use as additives for food, drugs and cosmetic products. Hydrogen peroxide treatment (sprayed and infiltrated with 0.1%, 0.33% and 1% H_2O_2) led to a significant betacyanin accumulation in *Suaeda salsa* L. sprouts, the oxidative stress signal leading to betacyanin production, may be

perceived by roots initially, then was transferred to leaves and the signal transduction was performed as betacyanin accumulation induced in leaves [120]. The increase in the microelement Co^{2+} from 1–5 μM also resulted in an 60% increment on the production of betalains, however, Mo^{2+}, Fe^{2+} and Cu^{2+} presented a positive (10% increment) but less marked effect, while the increase of Mn^{2+} did not show effects on the production of betalains compared to control medium [121].

4.4. Nutrients with Biological Activity

Elicitation of plants has been studied not only to improve the nutraceutical potential of low-processed food, but also the nutritional value (content of vitamins, bioactive peptides and carbohydrates). Vitamins are vital nutrients required by organisms. Vitamin A is essential for normal cell growth, immunological functions and vision, and is found in foods in the form of provitamin-A [122]. Vitamin E, with the α-tocopherol form being the most active in humans, is considered to be one of the most potent lipid-soluble antioxidants *in vivo* [123]. Folate (a collective term used for folic acid and its derivatives) is an important component of vitamin B, which is involved in a number of cellular metabolic processes, mainly playing a role as co-factor in the synthesis of nucleic acids, amino acids, pantothenate and formyl methionine-transfer RNAs [124]. Most recent evidence from a population-based cohort study in Europe lends further support to the notion that an increased intake of folate from food sources, may be associated with a lower risk of pancreatic cancer [125]. Vitamin C, including ascorbic acid and dehydroascorbic acid, is one of the most important nutritional quality factors in many horticultural crops and has many biological activities in the human body, such as the prevention of scurvy, reduction of plasma cholesterol level and as antioxidant, reportedly reduces the risk of arteriosclerosis, cardiovascular diseases and some forms of cancer [126]. Therefore, there is an increasing interest in fortifying many foods with vitamins.

The content of vitamins in fruits and vegetables can be influenced by various factors such as genotypic differences, pre-harvest climatic conditions and cultural practices, maturity and harvesting methods, and postharvest handling procedures [26,127]. Special treatments, including precursor feeding and elicitor application can be used to increase metabolite production. Foliar application (250 μM) of MeJA and SA caused rapid 2-fold increase of folate in coriander (*Coriandrum sativum*) foliage, as well as, treated plants presented higher stability of folates than untreated foliage, during processing and storage [124]. The application of 200, 300 μM of SA and 0.01% chitosan induced increases, by 26%, 18% and 54%, respectively in the content of vitamin C in 5 days old broccoli sprouts [25]. Higher levels of ascorbic acid (in comparison with controls) have been found in 4-day-old lentil sprouts after elicitation with temperature stresses (4 °C and 40 °C for 1 h) [128]. Broccoli sprouts grown in an environment chamber with a 16 h light/8 h dark cycle were found to have much higher concentrations of vitamin C (by 83%) than those grown in the dark [19]. A considerable enhancement on the production of α-tocopherol was observed after the administration of 5 μM JA or by hypoxic conditions both in sunflower and *Arabidopsis thaliana* cell cultures [26]. Folic acid and vitamin C have been also used as exogenous growth enhancers to elicit pea (*Pisum sativum*) seedling vigour and phenolic content. Concentration of 50 μM folic acid and 500 μM vitamin C were optimum to both agronomic and biochemical seed vigour parameters, as well as, the levels of enhanced phenolic content, which were highest on days 8 and 10 of germinating seeds [98].

The starch content has been influenced in lentil sprouts after different germination conditions (elicitation by solution with 100 and 300 mM NaCl), being reduced by 50%, as well as the *in vitro* digestibility and predicted glycemic index of sprouts [129]. Also a decrease in total starch, high content of resistant starch and low starch bioaccessibility, a decrease in protein content and subsequent elevation of non-protein nitrogen fraction was reported in lentil sprouts after a elicitation treatment with H_2O_2 [130].

Food-derived bioactive peptides may have regulatory functions in the human system beyond normal and adequate nutrition (such as antimicrobial properties, blood pressure-lowering (ACE inhibitory) effects, cholesterol-lowering ability, antioxidant activities, *etc.*) [131]. As an example, some soy peptides induced the expression of defense genes implicated in phytoalexin production and pathogen defense after treatment of the aerial portion of soybean plants with hormones involved in elicitation [132].

Mineral content also could be affected by elicitation. Salicylic acid (0.5 mM) completely alleviated the negative effects of mustard plants growth under NaCl stress, increasing the uptake of major nutrients such as nitrogen, phosphorus, potassium and calcium [133]. The use of elicitation, based on natural defence mechanisms of plants, allowed the differentiation of food products and production of directed food designed for specific consumer groups (e.g., diabetics, the overweight, Alzheimer's and cardiovascular disease sufferers, among others).

5. Future Trends

The controlled short-time elicitation stresses, during the pre-harvest and post-harvest period, can be used as a tool by the fresh produce industry to obtain healthier products by enhancing their nutraceutical content. Similarly, controlled treatments can be utilized by the food processing and dietary supplement industry as tools to enhance the extractable yields of specific active compounds that have nutraceutical or other functional properties.

Interest in functional foods has been growing over the last decade as consumers become increasingly concerned with diet and nutrition. The industry continues to seek new and unique ingredient and health claims, making the idea of developing more functional food quite compelling. A special emphasis is placed on the biologically active compounds or groups of compounds responsible for the therapeutic applications, and their action mechanisms. Also, the quality and safety regulation of functional products should be established in food industry. Thus, elicitors may be a complementary strategy to breeding programs, production management, or genetic engineering activities. Understanding the interaction among stressors will make possible to find practical applications.

On the other hand, studying elicitor-activated signaling pathways with the purpose of identified signaling components, should be an efficient strategy for activating defense responses in the plant, in order to replace or reduce chemical applications to protect crops [45,110]. .

For new or enhanced plant products, it would be appropriate and unavailable the evaluation of functional properties to demonstrate the potential to obtain safe and effective non-pharmacological alternatives for human health. This may provide a new approach for disease prevention and population wellbeing monitored in clinical trials [134].

6. Conclusions

Understanding how plant tissues and their specific secondary metabolic pathways respond to different abiotic and biotic stresses, applied alone or in combinations, would be the basis for designing strategies to enhance phytochemicals in foods. The accurate determination of the effect, driven by the use of the distinct elicitors applied in selected time points of the plant growth, may allow strategies and tools to obtain tailored foods with enhanced health-promoting phytochemicals [69]. The resulting products and ingredients could be considered for functional foods or nutraceutical development that will provide benefits beyond basic nutrition and/or claims for health benefits.

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Conflicts of Interest

The authors declare no conflict of interest

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Chapter 8. References

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