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Maria Concetta Di Bella

ENHANCEMENT OF THE DIVERSITY OF BRASSICACEAE FOR THE SUSTAINABILITY OF CROPS AND PRODUCT INNOVATION

Ph.D. Thesis

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Never stop believing in yourself

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1. Sintesi

Le colture che afferiscono alla famiglia delle *Brassicaceae* hanno un ruolo di fondamentale importanza sia dal punto di vista economico, poiché sono coltivate e conosciute in tutto il mondo, che dal punto di vista nutraceutico, perché presentano un profilo fitochimico ricco di antiossidanti, e pertanto vengono considerati dei veri e propri: '*functional food'*. Si stima che la popolazione mondiale raggiungerà i 9 miliardi entro il 2050 per cui l'umanità dovrà affrontare diverse sfide e trovare soluzioni a problematiche legate alla necessità di aumentare le produzioni di cibo e di energia dalle colture di almeno il 70% e dovrà farlo utilizzando un'agricoltura più sostenibile e senza trascurare le implicazioni connesse ai cambiamenti ambientali e climatici a cui stiamo già oggi assistendo. La presente tesi di dottorato di Ricerca in 'Biotecnologie' è rivolta allo studio di colture resilienti, efficienti e sostenibili coltivate secondo i metodi e le tecniche colturali dell'agricoltura biologica. Essa si inserisce nell'ambito del progetto europeo H2020 BRESOV (*Breeding for Resilient, Efficient and Suistainable Organic Vegetable, G.A. n. 774244*) per il quale sono state effettuate diverse prove sperimentali rivolte all'*organic breeding and farming* di tre diverse colture: *Brassica oleracea* L. (cavolo broccolo, cavolfiore, cavolo da foglia, cavolo cappuccio, cavolo rapa), *Solanum lycopersicum* L. (pomodoro) e Phaseolus *vulgaris* L. (fagiolino). In quest'ambito sono state effettuate delle prove sperimentali su alcune colture afferenti a *Brassica oleracea* L., in particolare su *B. oleracea* L. var. *acephala* (cavolo da foglia), var. *botrytis* (cavolfiore), var. *capitata* (cavolo cappuccio), var. *gemmifera* (cavoletti di Bruxelles), var. *gongylodes* (cavolo rapa), var. *italica* (cavolo broccolo), var. *sabauda* (cavolo verza), e altre *Brassica* complex species (n=9), *Brassica incana* Ten., *Brassica villosa* Biv., *Brassica trichocarpa* Brullo, Giusso & Ilardi, *Brassica macrocarpa* Guss., *Brassica rupestris* Raf. coltivate presso i campi sperimentali del Dipartimento di Agricoltura, Alimentazione e Ambiente dipartimento (Di3A) dell'Università di Catania in collaborazione con gli altri partner internazionali e non di tale progetto. La ricerca è stata svolta durante un triennio ed ha preso in considerazione i genotipi provenienti dalla banca del germoplasma del (Di3A) e da altre banche del germoplasma presenti in altre parti del mondo, sottoponendoli sia ad una analisi fenotipica, mediante tecniche di rilievo bio-morfometriche e l'utilizzo di descrittori internazionali, che ad una analisi genotipica effettuata in diversi laboratori. Queste indagini hanno permesso di selezionare accessioni/genotipi che, sottoposti a stress abiotici, hanno indicato la loro resistenza e/o sensibilità a tali stress. In seguito, sono state effettuate delle ricerche più approfondite per studiare i meccanismi di resistenza, avvalendosi di tecniche di laboratorio analitiche, chimico-fisiche (HPLC), analisi enzimatiche, diagnosi molecolari (QTL, SSR) e trascrittomica. Tra i genotipi individuati è stato preso quale modello di studio la cultivar locale siciliana di cavolo broccolo *sprouting "*Broccolo nero", che è stata utilizzata per la produzione di nuovi prodotti orticoli, quali i germinelli, i *micro-greens,* i *baby-leaves*, ed i loro trasformati, quali il succo. I succhi estratti da questi prodotti hanno fatto evidenziare una elevata valenza nutraceutica sia in termini di composti fitochimici contenuti che per la presenza di miRNA capaci di controllare *in vitro* la diffusione delle linee cellulari cancerogene prese in considerazione.

1. bis Abstract

The crops belonging to the *Brassicacea*e family have a role of fundamental importance both from an economic point of view, since they are cultivated and known all over the world, and from a nutraceutical one, because they have a phytochemical profile rich in antioxidants, and therefore are considered real: 'functional food'. It is estimated that the world population will reach 9 billion by 2050 for which humanity will have to face various challenges and find solutions to problems related to the need to increase the production of food and energy from crops by at least 70% and will have to do so using a more sustainable agriculture and without neglecting the implications connected to the environmental and climatic changes that we are already witnessing today. This PhD thesis in 'Biotechnology' is aimed at the study of resilient, efficient, and sustainable crops grown according to the methods and cultivation techniques of organic farming. It is part of the European project H2020 BRESOV (Breeding for Resilient, Efficient and Suistainable Organic Vegetable, GA n.774244) for which various experimental tests were carried out aimed at the organic breeding and farming of three different crops: Brassica oleracea L. (broccoli, cabbage, cauliflower, kale, white cabbage, kohlrabi), Solanum lycopersicum L. (tomato) and Phaseolus vulgaris L. (green bean). In this context, experimental tests were carried out on some crops belonging to the *Brassica oleracea* L. species, in particular on *B. oleracea* L. var. *acephala* (kale), var. *botrytis* (cauliflower), var. *capitata* (cabbage), var. *gemmifera* (Brussels sprouts), var. *gongylode*s (kohlrabi), var. *italica* (broccoli), var. *sabauda* (savoy cabbage), and other *Brassica* complex species (n = 9), *Brassica incana* Ten., *Brassica villosa* Biv., *Brassica trichocarpa* Brullo, Giusso & Ilardi, *Brassica macrocarpa* Guss., *Brassica rupestris* Raf. grown in the experimental fields of the Department of Agriculture, Food and Environment Department (Di3A) of the University of Catania in collaboration with other international and non-international partners of this project. The research was carried out over a three-year period and took into consideration the genotypes coming from the germplasm bank of (Di3A) and from other germplasm banks present in other parts of the world, subjecting them to both a phenotypic analysis, using bio- morphometric and the use of international descriptors, which to a genotypic analysis carried out in different laboratories. These investigations made it possible to select accessions / genotypes which, subjected to abiotic stress, indicated their resistance and / or sensitivity to these stresses. Subsequently, more in-depth research was carried out to study the mechanisms of resistance, using analytical, chemical physical (HPLC) laboratory techniques, enzymatic analyses, molecular diagnoses (QTL, SSR) and transcriptomics. Among the genotypes identified, the local Sicilian cultivar of broccoli sprouting “Broccolo nero” was taken as a study model, which was used to produce new horticultural products, such as sprouts, micro-greens, baby-leaves, and them processed, such as juice. The juices extracted from these products showed a high nutraceutical value both in terms of phytochemical compounds contained and for the presence of miRNAs capable of controlling *in vitro* the spread of the carcinogenic cell lines taken into consideration.

2. Keywords and abbreviation

Brassica Crops; Broccoli; Kale; Kohlrabi; Landraces, Organic Farm; Breeding; Phenotyping; Genotyping; MiRNA; Water Stress; Biochemical Analysis; Transcriptomics; Innovation Products; Juice; Innovation Process; Sprouts; Microgreen; Baby-Leaf; Functional Food, Antioxidant; Nutraceutical compounds; Enzyme analysis.

List of Abbreviation

APX **Ascorbate peroxidase**

BRESOV **Breeding for Resilient, Efficient and Sustainable Organic Vegetable Production**

C **Control**

CAT **Catalase**

CC **Core Collection**

CWR **Crop Wild Relatives**

DM **Dry matter**

EC **European Commission**

ETC **Evapotranspiration crops**

GLSs **Glucosinolates**

HPLC **High Performance Liquid Chromatography**

IFOAM **International Federation of Organic Agriculture Movements**

LAPs **Leaves adult plants**

MiRNA **Micro RNA**

MRL **Maximum Residue Levels**

QTL **Quantitative Traits Locus**

SSRs **Simple Sequence Repeats**

TPC **Total Polyphenols Content**

PRO **Proline**

MDA…… **Malonildialdehyde**

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4. Introduction and purposes

The present Ph.D. thesis describes the study and the research activities carried out during the three-year period (2018-2021) of the XXXIV Ph.D. course on "Biotechnology", curriculum "Agro-food", of the University of Catania. This period of studies concerns the strategies to be implemented to support the innovation of the vegetable productions in organic farming and to improve them for agronomic traits of the plant and for organoleptic/nutraceutical ones of the products.

The activities were carried out at the Vegetable and Flower section of the Di3A, of the University of Catania, in the frame of the EU H2020 BRESOV project which since its starting has paid particular attention to the enhancement of the rich genetic heritage that distinguishes Sicilian vegetable productions.

The approach of our studies was based on the relationship among some abiotic stresses, the genotypes considered and the processes of growth and development of the plant. Consistent was also the wide range of the methodologies and of the equipment utilised for characterising and for evaluating the BRESOV *Brassica* core collection.

The increasing attention paid by consumers, for new and healthy vegetable products, and by growers, for resilient cultivars, has focused our attention on the rich vegetable heritage, consisting of a wide range of species, often neglected or underutilised, which can support the process of innovation of the vegetable production food chains by several landraces.

The tradition to grow and to use a great diversity of vegetable products in Italy helps us in this task as it is rich in minor species, used especially in the past, and in wild relative species, that occasionally in addition to being collected and consumed by local communities, are of great interest for genetic improvement and for breeding.

The research activities carried out pointed to the process and products innovation by using the great diversity offered by *Brassica oleracea* complex species (n=9) widespread in Europe expressed by the *Brassica* core collection of the H2020 BRESOV (Breeding for Resilient, Efficient and Sustainable Vegetable productions – G.A. 774244) project dealing with the organic breeding of broccoli (*Brassica oleracea*), and of the related *B. oleracea* crops, snap bean (*Phaseolus vulgaris*) *and* tomato (*Solanum lycopersicum*).

The *B. oleracea* crops are usually grown by amateurs in home gardens in all Europe, observing a great diversity of morphotypes among and within them, which in most of the cases are supported by propagation materials self-produced by urban amateurs and growers.

The *Brassica oleracea* crops are represented by the followed varietal morphotypes: i) broccoli (*B. oleracea* var. *italica*); ii) Brussels sprouts *(B. oleracea* var. *gemmifera*); iii) cabbage (*B. oleracea* var. *capitata*); iv) cauliflower (*B. oleracea* var. *botrytis*); v) kale (*B. oleracea* var. *capitata*); vi) kohlrabi (*B. oleracea* var. *gongylodes*); vii) Savoy cabbage (*B. oleracea* var. *sabauda*).

This species assumes a particular interest in Europe as in the articulated environmental context expressed by the different countries has been ascertained a wide genetic variability such as to consider it native to the same Mediterranean environment, which represents its centre of origin and diversification (Vavilov, 1926).

The wide polymorphism expressed by *B. oleracea* in Sicily, seems to play a determining role on the origin and diversification of kale, broccoli, and cauliflower and on their related domestication process. The combined presence of different *Brassica* wild relatives (n=9) populations and of *B. oleracea* crops at the same time in several Sicilian agro-ecological niches continues to facilitate the gene flow between several wild and grown genotypes, determining a plurality of gene recombination that support the wide diversity observed in the Island.

It is therefore considered very likely that in the Mediterranean the first phases of the domestication process of *Brassica oleracea* have been initiated and kale represents the first result of the selection process of man which has subsequently allowed the origin and the diversification of broccoli before and of cauliflower after. This hypothesis seems to be supported by the enormous variability that is recorded in this region for these crops that find feedback and support in culinary traditions for the specificity of the corresponding products.

These crops, represented in Sicily by several landraces, are still today reproduced by some growers and they often express a significant agronomic difference that satisfies the requirements of the local grower, but they often do not fit with the needs of the globalised markets. Moreover, this discrepancy prevents the use of products by the processing industry. On the other hand, the placing of the products on the local or niche markets is of greater interest for the local farmers in the current economic situation conditioned by the COVID-19 pandemic, and a particular appreciation was underlined for the traditional (local/territorial) fresh vegetable products.

Vegetable product innovation is mainly based on the identification of minor and/or wild species occasionally gathered in the countryside or introduced in home gardens, or on scarcely widespread subspecific entities of species already widely used. The product innovation shall be supported also by new processing technologies which allow the full exploitation of species/landraces which provide products unfit for handling and preservation as they are traditionally addressed for fresh consumption by the local markets.

In recent decades, the food technology of the fourth gamma products (ready to eat/ready to cook) has allowed to improve the maintenance of product quality characteristics during the post-harvest phase of many species and minor cultivars not adequately valued for fresh consumption.

Among the *B. oleracea* crops considered in the present Ph.D. thesis some supply products are represented by vegetative organs (cabbage, kale, kohlrabi) that for organographical characteristics are subject to very intense metabolic activities that determine the rapid reduction of the cellular turgor and the rapid increase of the fibre, and some by reproductive ones (broccoli and cauliflower) which are developed in relation to the cold requirements requested by the plant for flower induction and differentiation. The quality of the reproductive organs could be affected by the high thermal fluctuation consequent to the climate change in acts.

The wide BRESOV *Brassica* core collection analysed has provided a wide range of variability for the several agronomical, bio-morphological, biochemical, organoleptic and nutraceutical traits studied consenting to individuate accessions and genotypes of interest

for their resistance to biotic and abiotic stress. In this frame our main interest was devoted to water stress which is one of the main constraints for vegetable crops in Mediterranean countries and islands, such as in Sicily.

We have studied the characteristics of the several morphotypes expressed by the BRESOV *Brassica* core collection paying special attention to the characteristics of the root system of the plant in addition to its *habitus,* stems and leaves. The several morphotypes expressed a great level of diversity among and within each *B. oleracea* crop, and among the crops and the *Brassica* wild relatives (n=9) compared.

The rusticity characteristics of the *B. oleracea* crops allow us to hypothesise their cultivation in different environments through the adoption of growing methods and techniques with low environmental impact and with modest contributions of chemical means.

It is therefore possible for all the ***B. oleracea*** crops by organic growing methods and techniques that allow to better enhance the health characteristics of the product avoiding the use of pesticides. In this frame we have evaluated the adaptability of some accessions of kale and of kohlrabi to water deficiency technique by the analysis of the variation of the main bio-morphological, biochemical, physiological, and genetic traits in comparison to the plant ordinary irrigated replacing all the amount of water lost by the crop by the evapotranspiration process.

The organic product offered by these two crops would also make it possible to satisfy an interesting market niche that could identify a particularly suitable production area in Sicily. Based on these considerations, it was considered important to identify, to characterise and to improve the rich germplasm of *B. oleracea* crops traditionally cultivated in Sicily in family and suburban gardens, to contribute for the development of the corresponding production chains in different areas of the island.

With reference to the new products proposed, such as sprouts, microgreens and baby-leaves, the attention has been placed not only on different crops and cultivars, but also on the growing seasons. For the Sicilian sprouting broccoli landraces “Broccolo nero” were analysed the characteristics of the products of the first stages of the plant growth, such as sprouts, microgreens and baby-leaves, compared with the leaves of adult plants (LAPs), both for fresh consumption and for producing juices. The crescent interest of the beverage industries to use nutraceutical matrixes for satisfy the consumers requested pointed our attention not only to the main antioxidant compounds (glucosinolates, polyphenols, vitamins) but also to some microRNA (miRNA) contained in these products and in their related juices.

4.1. Organic farming and ethics

According to the definition given by the International Federation of Organic Agriculture Movements (IFOAM - Organics International), organic agriculture is "a system of production that supports the health of the soil, ecosystem and people based on ecological processes, biodiversity and for different local conditions".

The aim of the use of Organic agriculture is to combine tradition, innovation, and science, because that the environment benefits from its benefit and to promote correct relationships (www.ifoam.org/growing). Main points of organic agriculture are: i) well-being (health): that supports and promotes the health of the soil, plants, animals, humans, and the planet like an indivisible whole; ii) ecology (ecology): based on systems and cycles living ecological. iii) equity (fairness): develops relationships that ensure fairness and solidarity towards the necessity of life; iv) precaution (care): is managed as a precaution and responsible, to protect the health and well-being of present and future generations (IFOAM).

The term organic agriculture refers to an alternative agricultural system, capable of adopting cultivation strategies and techniques that do not use chemicals, thus respecting the regular balance that regulates the natural life and seasonality of each crop (Gomez & Thivant, 2017).

Figure 4.1. IFOAM.

The first forms of organic farming are to be referred to the first applied forms of production, in fact, the agriculture could be defined as organic since its appearance dating back to 10,000 years ago; only in the last century did synthetic chemicals make their appearance in the agricultural field, making substantial changes (Martucci, 2005).

Organic farming is considered as a natural farming system therefore exempt from the use of chemical fertilisers or pesticides (Srutek & Urbn, 2008). The concept of naturalness, which is often associated with organic farming, also refers to the fact that often to obtain this type of production, the best varieties are selected that best fit the system of naturalness (Van Bueren et al., 2002; Verhoog et al., 2003).

In fact, the main objective of organic farming is to use techniques capable of replacing chemicals with natural components, therefore, to ensure adequate stability of the ecosystem (Palaniappan & Annadurai, 2018). Organic farming has its own production system based on a concept of farm which must be understood as an agro system that aims mainly at including biotic diversity in its production (Altieri, 2018).

The basic principles for obtaining a good organic production concern the fertility of the soil and the management of different components, including the chemical ones which

therefore concern the quantity of nutrients dissolved in the soil and the pH, the physical ones which concern the soil structure and retention water, and biological ones directly linked to the soil microbiota (Power & Prasad, 1997; Stockdaleet al., 2002; Fernández & Hoeft, 2009).

Despite the spread of organic farming, the latter was often supplanted by an agriculture that mainly focused on the use of chemical products, able to offer a defence against the attack of microorganisms and insects, able to lose much part of the economic resources that could derive from the sale of the same (Verhoog et al.,2003; Lamine, 2011; Morgan & Murdoch, 2000).

The current objective is to use agricultural production systems that aim to avoid the use of GMOs, fertilizers, pesticides, or intensive indoor farming, but which on the other hand can optimize the objective of sustainability through techniques completely organic (FAO, 2017).

Figure 4.2 FAO (Food and Agriculture Organization).

In this way, it will be possible to define the main differences from a technical and operational point of view between organic and conventional agriculture (Morgan & Murdoch 2000).

The main differences between organic and conventional agriculture mainly concern: the conventional one concerns a systematic exploitation of the land and all the natural resources available, while the organic one tries to base its production and agricultural system in a state of maximum harmony and balance (Morgan & Murdoch 2000).

Considering the need to ensure good soil fertility, often following the principle of respecting the environment itself and maintaining it and not impoverishing it unlike the conventional, which, on the other hand, aims to follow industrial principles (Power & Prasad, 1997; Stockdale et al., 2002; Fernández & Hoeft, 2009; Monteduro, 2013).

Therefore, it is essential to use substances that are valid alternatives to chemical ones, and it will be necessary to implement different techniques in defence of the ecosystem, such as the use of the so-called biological control capable of guaranteeing a non-polluting pest control (Bale et al., 2008).

From the point of view of the quality of the productions, it is well known that a lot of properties for compounds in food crop, depend on genotype evaluated, part of the crops analysed, year of practices (Johansson et al., 2014).

Cultivation techniques about seems are also an important parameter in terms of content of nutritionally important compounds in a crop, these make the main differences between organic cultivation and conventional (Johansson et al., 2014).

Based on the research that highlights the substantial differences between plants obtained through organic farming and conventional agriculture, there are studies already cited in the literature concerning the characteristics relating to the genotype and phenotype of the plants taken into consideration (Seufert et al., 2012; Sangiorgio et al., 2021).

Considering the current studies, what has been highlighted is that the quantity of micronutrients is slightly increased, especially as regards zinc and iron in cultivars obtained through organic farming, rather than conventional ones (Erika et al., 2020; Sangiorgio et al.,2021).

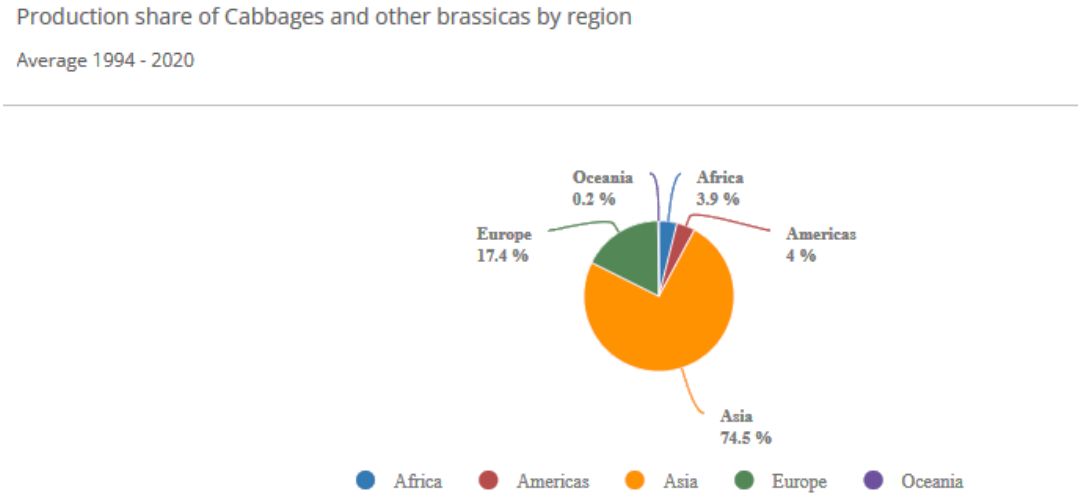

Figure 4.3. Faostat 2021.

To highlight the main qualitative characteristics of the products, a lot of studies have been made, in fact, the literature reports information about the content of various compounds in organic and conventional crops (Rembiałkowska, 2007; Lairon, 2010).

Numerous researchers argue that more attention needs to be pain in towards of research, especially about on genotypes suitable for organic production, avoiding studies comparing conventional bred genotypes in conventional and organic systems (Watson et al., 2008).

In Vrcek et al. (2014), more attention was paid on differences due to farming system applied. Especially, difference about the methodology selected for the comparison and variation of i) content in micronutrient compounds including iron and zinc; ii) contents between organically and conventionally grown crops.

The study was based in the cultivar of a lot of crops as like haricot beans, strawberries, wheat flour, tomatoes, cotton, wheat, etc., the results shown a higher content of iron and zinc in the organically produced crops compared to the conventionally grown ones (Vrcek et al., 2014).

Moreover, other comparative studies, have evaluated several organic fertilizing strategies and have been able to differentiate certain organic fertilization strategies as resulting in increased levels of iron and zinc in the crops (Citak et al., 2009). Other studies

said about other factors like type of crop, year, place, environment, harvest timing, etc., seem to be of higher importance than organic cultivation (Liliane & Charles,2020). However, important evidence is the high mineral content that has been found in organically grown wheat with a certain genetic background and grown in a certain environment (Hussain et al., 2010).

Many studies are available comparing levels of total phenolics as well as of individual phenol compounds trough organic and conventional system for many crops like peach, pear, apple, kiwi, tomatoes, leaf lettuce, collards, maize, oats, potatoes, berries, strawberries, blueberries (Blair, 2012; Zheng et al., 2019).

There have been many studies that have brought to light a genetic variation so important in determining the various bioactive compounds of a crop (Kaushik et al., 2015; Egorova et al., 2021).

Several important compounds present in food crops depend on the evaluated genotype, location, year, and cultivation practices of the crop analysed (Johansson et al., 2014). Furthermore, there are many authors who, on the contrary, argue in their studies that the only difference is the cultivation system and not variation in cultivation locations, attributes of the soil, eventual irrigation, whether, crop varieties, harvesting conditions, storage methods (Lester et al., 2011).

However, the same studies, said that among all the micronutrients and bioactive compounds discussed above, phenolic compounds seem to be more influenced by the farming system used, but there isn't significant difference in amounts or composition between crops grown conventionally vs. organically (Lester et al., 2011).

Other studies concerning the difference between traditional and organic method mainly concern the evaluation of the quantity of phenolic compounds in strawberries, red fruits, pears, peaches, apples, kiwis, lettuces, and tomatoes.

Regarding phenolic compounds, there are evidence that very often the content of phenolic compounds is influenced by the farming method used (Fernandes et al., 2021).

The fact that organic products give a lower yield, compared to those obtained through traditional agricultural technologies, is mainly since organic farmers often use 50 if not 80% less nitrogen than conventional farming techniques (Seufert et al., 2012; van Bueren & Struik, 2017).

Figure 4.4. Drought stress.

Therefore, the amount of nitrogen released in the fertilizers will come out gradually and will have a lesser impact on the finished product (Diacono & Montemurro, 2011).

Furthermore, other reasons linked to the low yield, as regards organic products, are linked to the inadequate ability to manage weeds or parasites, or even to be able to resist cultivar diseases (Seufert et al., 2012; van Bueren & Struik, 2017).

To help consumers, a lot of pesticides are approved in organic agriculture, because they are of low toxicological concern. In fact, they represent part of the diet of human nutrients (iron, potassium, bicarbonate). Plant protection practises developed in and for organic agriculture may be of health to the entire agriculture system (Van Bruggen et al., 2016).

It's important to say that in organic food production, there is the many advantages to have a low residue level in foods and lower pesticides exposure for consumers, in fact studies of EFSA say that pesticides residues below Maximum Residue Levels (MRL) in 43.7% of all 13.8% in organic food (EFSA; 2015).

The population's exposure to several pesticides can be measured by analysing blood and urine samples, as is routinely done in the US, and Germany, Spain, Belgium, Poland, and Denmark (CDC; 2013).

A general observation has been higher urinary concentrations of pesticide metabolites in children compared to adults, most likely reflecting children's higher food intake in relation to body weight and maybe also more exposure prone behaviours (Hyland et al.,2019). A larger consumption of fruit and vegetables is positively correlated with pesticide excretion (Ye et al., 2015), and consumption of organic produce is associated with lower urinary pesticide concentration (Curl et al., 2015).

Also, the risk assessment of pesticides, is comprehensive of toxicological effects, and increased risk of some diseases including Parkinson's disease, and certain types of cancers including non-Hodgkin lymphoma (Schinasi et al., 2014), and childhood leukaemia or lymphomas, e.g., after occupational exposure during pregnancy (Ntzani et al., 2013) or residential use of pesticides during pregnancy (Van Maele- Fabry et al., 2013).

Studies show that the consumption of organic food can reduce the risk of allergic diseases, overweight and obesity (Seufert et al., 2012).

As far as legislation is concerned, organic production is already recognized as a valid alternative to traditional agriculture in 1985, this recognition envisaged by the green paper of the European Commission coincides, among other things, with an increased interest in organic farming by consumers.

Nevertheless, attention to human health, together with increasing consumer demand, are the reasons why several governments have set goals as to how much organic agriculture should increase in their respective countries.

In fact, here are different examples of food policies related to development of organic production as: i) the Food, Conservation and Energy Act of 2008 in the USA, with a mandatory five-fold funding increase for organic research programs and cost-share assistance for farmers and handlers; ii) the European Action Plan for Organic farming from 2004, that focus on strengthening information and research as well as improving production standards and streamlining public support.

From the data processed by Ismea in 2011, the horticultural supply chain represents a very important set of products that today are made all over the world and which

sometimes in relation to the soil and climatic needs of the environment, need to be imported and exported to third countries.

The first year's farmers in fact tended to use organic mineral fertilizers and different rotations so that biodiversity could be guaranteed even if, in a rudimentary way, then when around agriculture started to create a real business and therefore the invested capital was increased and the means used, a modern conventional agricultural system was generated that gradually shifted intensive cultivation towards new techniques that are increasingly avant-garde the use of mechanization to carry out the main operations and processes in the short term.

It therefore allowed to improve the fertility of clayey soils to increase labour productivity to create an ideal habitat for plants and thus for their root system, as well as used much of the relative time to any operations that could on the other hand have negative effects such as erosion or alteration of weeds (Ferrari, 2003).

Furthermore, among the systems used to carry out integrated control against microorganisms or weeds, according to the "Legislative Decree 14 April 2012 n° 150", the goal is to try to make the use of pesticides sustainable, for which a fundamental role in the chemical fight and for chemical weeding.

It appears to be given to the farmer's ability to fight the problems associated with it, such as the reduction of biodiversity, a decrease in microorganisms able to help the plants themselves, a reduction or almost a disappearance of useful organisms, with a consequent increase in harmful organisms and an increase in genetic resistance phenomena.

Organic farming represents a cultivation technique based mainly on sustainability and eco-compatibility. In fact, this has the objective of knowing how to safeguard the environment as much as possible, protect it and at the same time guarantee the stability and health of consumers (Ferrari, 2003).

According to the provisions of Regulation 2082/91, in the action plan for organic farming, the council and the European Parliament invited the commission to reconsider the regulatory framework on organic farming to make it easier to understand, especially considering all the member countries of the European Community, the legislation on organic production has increasingly adapted to the evolution of agricultural markets characterized by production increases.

At the same time the European Union itself has shown itself sensitive to the perception that the consumer must have for organic production, the main objective is therefore to be able to create a system capable of managing agriculture in a sustainable way to obtain high quality food and products in respect of human health and the environment.

Currently, the regulation places greater attention on animal welfare and food safety as regards not only feed and farming methods but and above all the phases of plant production and aquaculture and feed obtained through a biological technique.

The article n° 9 of the regulation confirms to avoid as much as possible the use of chemical substances and the absolute prohibition of using genetically modified organisms for organic production is clearly expressed even if a general limit of 0.9 can be taken into account % due to a casual presence of authorized GMOs in food and feed.

Figure 4.4. Cabbage and others brassica market.

Nowadays, the mentioned food policies, is practiced in more than 160 countries. A total of 37 million hectares of land were grown organically in 2010, but large increases are still seen in most of the European countries in terms both of increase of agricultural land for organic production, organic market size and per capita consumption.

However, more than 80% are found in the developing countries, where the largest share of emerging markets is also present. The main importers of such products are the United States, Germany, and France (Willer et al., 2012).

Furthermore, aspects of environmental sustainability, such as biodiversity and greenhouse gas emissions, may also be affected by the agricultural production system and may affect human health food security (Reganold et al., 2016).

Some recent studies have concerned the association between consumption of organic food and health. These studies are generally based on a little representative sample of the populations and short durations, thus limiting statistical power and the possibility to identify long-term effects (Dangour et al., 2010).

What emerges from the studies, is a radical change in the consumers' point of view, who regularly buy organic food tend to choose more vegetables, fruit, wholegrain products, and less meat, and tend to have overall healthier dietary patterns (Torjusen et al., 2014).

Furthermore, a diet based on this kind of diet characteristics is associated with a decreased risk for mortality from or incidence of certain chronic diseases (Abete et al., 2014).

The important objective of being able to support sustainable food security and the common well-being of society in general is taken into consideration when studying the genomic and genetic profile of the cultivars to be cultivated.

Following ethical and socio-economic criteria, it is possible to evaluate which are the main characteristics to be considered, to guarantee integrity in the life of future generations (IFOAM). Among the ethical criteria, it is essential to respect the cell and the genome of the plants taken into consideration, without however forgetting the economic profile, which basically wants to evaluate the possibility of using the germplasm to obtain

pollinated varieties that have greater value from a sustainable agriculture point of view (Nuijten et al., 2020).

4.2. *Brassica oleracea* crops: origin, domestication process and innovation

Brassicaceae or *Cruciferae*'s family includes herbaceous plants distributed in all continents, as they can easily adapt to any environmental condition; in fact, they are found in regions characterised by extratropical climates as well as in mountainous or arctic regions.

The term *Brassicaceae* was proposed by the Italian botanist Teodoro Caruel (1830-1898), from the Celtic brassica ("cabbage"), from which probably also the verza and cabbage of Spanish and Portuguese and the Romanian varza. The highest centre of biodiversity for this family, in terms of the number of species, it is the Mediterranean basin. *Brassicaceae*'s family belongs, according to the Angiosperm Phylogeny Group, to the class Magnoliopsida (Dicotyledons) and to the order Brassicales. Classifications older (es. System Cronquist) assigned this family to the order Capparales, now no longer recognized as valid.

They are closely related to the *Capparaceae* (the family to which the caper belongs), so recently it is inclusion in the *Brassicaceae* has been proposed. The *Brassicaceae* include over 300 genera and almost 4000 species, some of which have great economic importance (Bianco and Pimpini, 1990). This family usually includes plants with multi-year cycle, biennial or annual, characterised by alternate leaves, with often engraved lamina or pinnate.

The name Crucifere (term accepted by the International Code of Botanical Nomenclature) comes from the appearance of the flower, typically composed of calyx formed by 4 sepals and a corolla with four petals, arranged in the shape of a cross. A special feature of this flower is the presence of 6 stamens, of which 4 cross like the petals and 2 shorter outer ones (Figure 4.5).

Figure 4.5. Cabbage was harvested in the 15th Century, as depicted in this illustration from the book "Tacuinum Sanitatis," a medieval handbook on health.

The ovary is super and bicarpellary. The pollination is entomogamous. The fruit, a main character for the determination at species level, is dry and dehiscent. We can consider a particular type of bicarpellary capsule, called siliqua or siliquetta.

The fruit is characterised by two valves which, once opened, leave the seeds uncovered. The siliqua may present some bottlenecks that at maturity determine the rupture of the capsule in articles containing seeds, this is mainly the case for the genus Raphanus. The seeds, rather small and globular in shape, have an embryo, oleaginous and are devoid of endosperm.

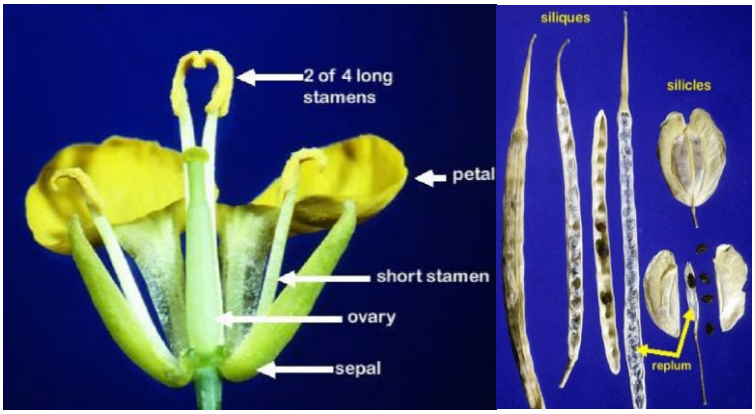

Figure 4.6. Brassica flower and seeds.

The species of horticultural interest belonging to the family of the Brassicaceae are the following, according to the most accredited classification (Spooner et al., 2003; Mcneill et al., 2006; Hammer. et al., 2013):

Brassica oleracea L.

- var. *acephala* (DC.) (kale).
- var. *botrytis* (L.) (cauliflower).
- var. *capitata* (L.) (cabbage).
- var. *gemmifera* (DC.) (Brussels cabbage).
- var. *gongylodes* (L.) (kohlrabi).
- var. *italica* Plenck. (broccoli).
- var. *sabauda* (L.) (savoy cabbage).

In this classification is also possible to distinguish morpho-types:

var. *acephala* (DC.) (kale).

The morphotypes presents different types of leaf morphology and plant architecture, ornate leaves, extended vegetative phase.

var. *botrytis* (L.) (cauliflower).

with different inflorescence, colour. leafy heads, enlarged inflorescences and stems.

var. *capitata* (L.) (cabbage) and var. *sabauda* (L.) (savoy cabbage)

with different and colour, form Round-headed or flat-headed.

var. *gemmifera* (DC.) (Brussels cabbage).

var. *gongylodes* (L.) (kohlrabi)

with different forms and colours from green to purple. or cabbage morphotypes with their typical leaf-heading trait, diversification into heading and tuber-forming.

var. *italica* Plenck. (broccoli).

with different inflorescence type sprouting and not sprouting that presents an enlarge inflorescences.

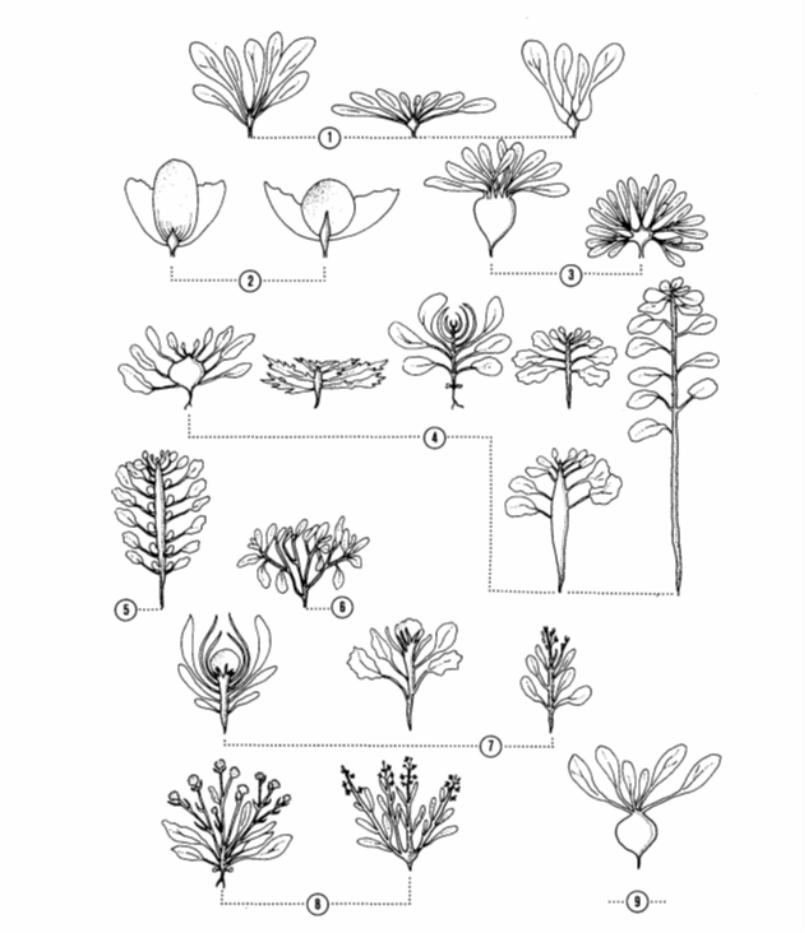

Figure 4.7. IBGR.

Brassica napus L. var. *napobrassica* (L.) (Rutabaga).
Brassica rapa L. subsp. *rapa* Thell. (Turnip).
Brassica rapa L. subsp. *sylvestris* L. Janch. var. *esculenta* Hort. (broccoli raab).
Raphanus sativus L. (radish).
Raphanus sativus L. var. *niger* Mill (DC) (winter radish).

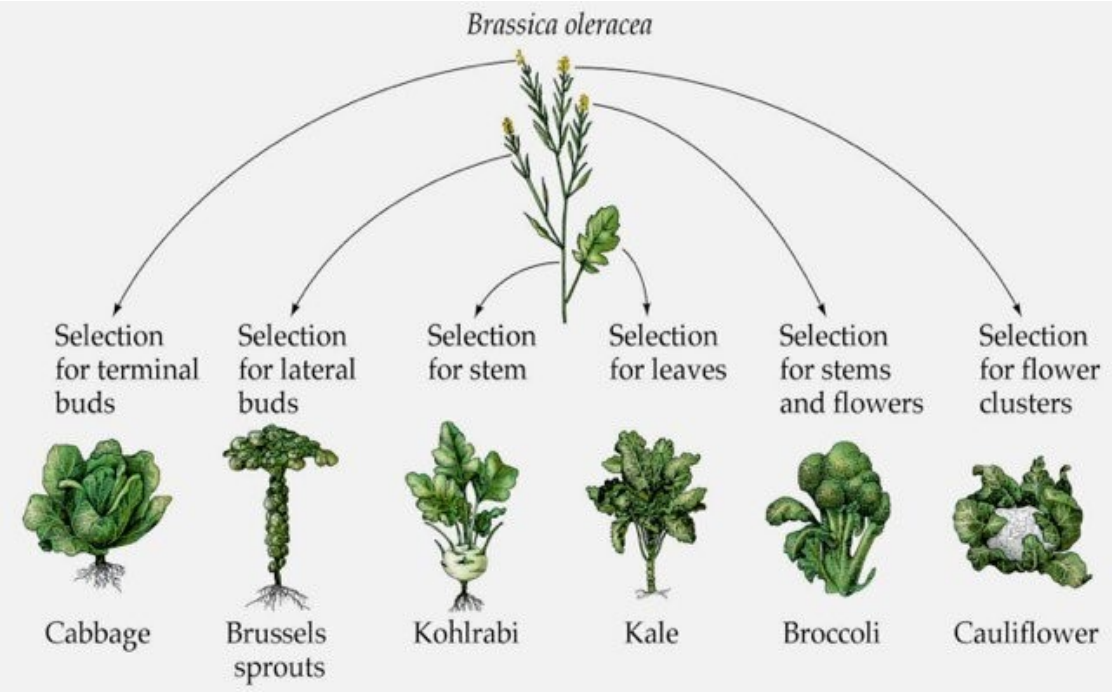

Figure 4.8. Brassica Domestication.

The classification reported on the Multilingual, Multiscript Plant Name Database replaced the varieties of *B. oleracea* and *B. napus* listed above with as many groups of the same name and introduced the Radicula Group for radish.

The edible *Brassicaceae* are about 200 and in Italy, are particularly important crops belonging to Brassica and Raphanus (Bianco and Pimpini, 1990; Branca et al., 2018).

Brassicaceae is a case study of relevance for the close interaction between the different genomes of the different related species, which have played an important role in the process domestication. This process has allowed the evolution of different crops also within individual species, as occurred for *Brassica oleracea*.

The high bio-morphological diversity that show the species of Brassica, and more generally the other species of the family, offers important opportunities to increase not only knowledge about plant growth and development, but also on molecular and genetic biology (Branca and Cartea, 2011).

The widespread presence in the Italian peninsula of spontaneous and cultivated *Brassicaceae* and allogamy, consequence of auto-incompatibility mechanisms, are the cause of the wide inter and intra variability specific to this family (Branca and La Malfa, 2001).

The genus Brassica, within the family, is the most important one. The genus belongs to the *Brassicinae* subtribe, one of the nine subtribes of the *Brassicaceae* tribe, which share with 18 other tribes a large genetic pool, which over time has been used to improve different cultures. A long series of investigations on chloroplast-DNA (cp-DNA) and restriction sites (Pradhan et al. 1992; Warwik and Sauder 2005) show how many species belonging to subtribe *Raphaninae* and *Moricandiinae* are closely related to *Brassicinae*.

Taxonomic and cytogenetic studies were carried out to determine the number of chromosomes and chromosome mating in interspecific hybrids of Brassica (Prakash and Hinata, 1980).

In the U Triangle the cytogenetic relationship between the main species is represented of the genus Brassica (Figure 4.9.). In the triangle, the three diploid species in the vertices:

Brassica nigra (L.) Koch (n = 8);

Brassica oleracea L. (n = 9).

Brassica rapa L. (n = 10).

These species have originated, thanks to their crossing, three amphidiploid species *Brassica carinata* A. Braun (n = 17), *Brassica juncea* (L.) Czern. (n = 18) and *Brassica napus* L. (n = 19).

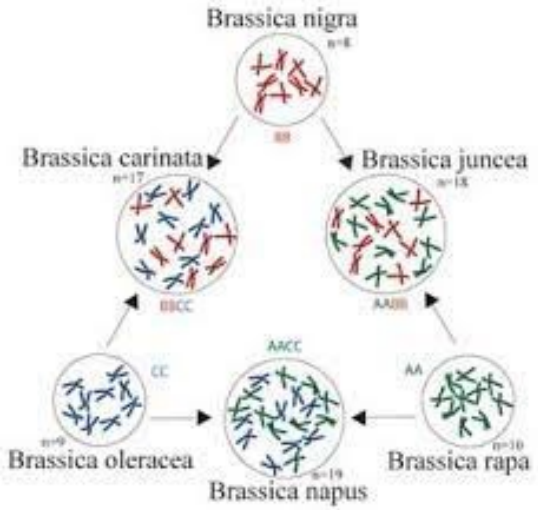

Figure 4.9. U Triangle.

The divergent selection has enriched the diversity of the corresponding cultivars, during the domestication process of each species.

Natural hybridization events laid the foundations on the evolution of the genome of the genus Brassica and on the interspecific crossings that have allowed the gene exchange and have contributed significantly to gender differentiation.

The divergent selection, during the domestication process of each species, has enriched the diversity of the corresponding cultivars and crops. Natural hybridization was at the base of the evolution of the genome of Brassica, moreover, the interspecific crossings have allowed the exchange and have generated new types or species (Branca and Cartea, 2011).

Brassica oleracea L. is represented by different varieties, which have given rise to different crops between them both for growth habits and for morphology.

Brassica oleracea L. var. *acephala* (kale), between the varieties of *Brassica oleracea* L., seems to be the evolutionary bridge for other varieties of the species.

Brassicaceae are very versatility, they are widely used, and they can also be used to produce innovative foods since the most important challenges of the future are to guarantee food and water to the growing world population and to protect the planet's biodiversity.

These challenges are essentially aimed at the world of agriculture, which is constantly evolving precisely to meet the most basic needs of mankind (Di Gioia et al., 2015).

Horticulture, among the sectors of vegetable production, is the one that expresses the greatest agrobiodiversity. The high richness of species, varieties, and products, beyond the agroecosystems, in horticulture is also linked to the food use of different parts of the plant, even of the same species.

New natural food products containing high levels of bioactive compounds are being researched and studied today.

Sprouts, microgreens, and baby-leaf represent a growing market segment within vegetable products, consumed mainly raw for their high nutritional value and sensory characteristics.

The most used species currently belong to the *Brassicaceae* families (for example cauliflower, broccoli, Chinese cabbage, kale, savoy cabbage, turnip greens), *Asteraceae, Chenopodiaceae, Lamiaceae, Apiaceae, Amarillydaceae* (garlic, onion), *Amaranthceae* (amaranth, chard, spinach) and *Cucurbitaceae* (melon, cucumber, pumpkin).

The demand for seeds and sprouts of broccoli and other species belonging to the *Brassicaceae* family, in recent years, has become increasingly popular among consumers interested in improving and maintaining their health by changing their eating habits. The little buds were probably used for the first time in China starting from 3000 a.C.; the use in Italy dates to the first post war period (Renna et al., 2016).

Sprouts, according to European Commission Regulation 208/2013 are 'products obtained from the germination of the seed and its growth in water or other culture medium, collected before the development of true leaves and intended to be consumed entirely, including the seed' and therefore represent from the biological point of view, the very first phase of formation of a seedling after the partial or complete germination process.

In this phase, the seeds begin to produce enzymes that convert nutrients into simpler molecules and energy, essential factors in the early stages of the vegetative cycle when photosynthesis has not yet started.

Microgreens or micro-vegetables, widespread first in North America and then in Northern Europe, Asia, and Oceania, are increasingly used and sought after by consumers (Treadwell et al., 2010).

Microgreens indicate young and tender edible seedlings produced from the seeds of various species of vegetables, herbaceous crops, aromatic herbs, and wild plants. These seedlings, depending on the species, have a production cycle of 1-3 weeks from the germination of the seeds (Di Bella et al., 2020) and are characterised by the presence of two true leaves.

Microgreens are harvested by cutting the individual seedlings at the base when they reach a variable height of 3 to 9 cm, excluding the rootlets. The edible portion is represented by the stem, by the cotyledonary leaves and by the true leaves, and in some cases also the integuments of the seeds that remain attached to the cotyledons, if small and tender, can become part of the edible portion (Renna et al., 2016).

Microgreens represent a new category of vegetables, with characteristics quite distinct from those of sprouts, and from those of the now very common small-sized fresh-cut leaf vegetables, known as baby-leaf; moreover, they are distinguished from " mini

vegetables " (also known as " miniature vegetables ", " mignon" or " baby "), which are obtained through special cultivation techniques.

Sprouts, microgreens, and baby-leaf have the advantage of being able to be marketed with all the growing substrates. This marketing method represents a great innovation as it guarantees longer shelf-life and ensures high quality both in terms of freshness and nutritional value (Di Gioia et al., 2015).

These vegetables are grown in greenhouses or in open air, on soil or on alternative substrates, in the presence of light, unlike sprouts, consisting of sprouts and rootlets deriving from fully or partially sprouted seeds, generally produced in the dark and in water, with a production cycle between 5 and 7 days.

Furthermore, the terms microgreens and baby-leaf do not have a legal definition but are used to describe two categories of products (Treadwell et al., 2010).

Sprouts, microgreens, and baby-leaf represent an innovation process because they can be easily grown in small urban gardens and in the home garden, as well as on the farm, and thanks to the short growth cycle, they can be produced on soil or with soil-less cultivation systems, all the year and in an economic and sustainable way, even without the use of fertilisers and pesticides (Ebert et al., 2014).

The possibility of self-production, therefore, can help increase the availability and accessibility to food of the poorest sections of the world population, and improve the quality of food, increasing the availability of fresh foods rich in nutrients essential for human health.

One aspect that makes them very interesting is the possibility of using species and varieties characterised by a wide range of shapes, colours (green, yellow, red, purple), textures and flavours; therefore, they can be proposed as a new ingredient capable of enhancing and enriching drinks, salads, appetisers, first and second courses, soups, sandwiches, and desserts (Di Gioia et al., 2015).

One of the reasons for the success of these products is represented by the reduction and less waste of time in the preparation phase. Sprouts, microgreens and baby-leaf are eaten raw, avoiding the loss of nutrients or the degradation of thermolabile vitamins and are also proposed as "super food" or "functional foods" for the content in micronutrients and bioactive compounds capable to improve some functions of the organism and / or reduce the risk of diseases (Di Bella et al., 2020), and they are also called ‘‘biogenic foods’’, a term which indicates that they are foods rich in vitamins, minerals, proteins and numerous bioactive compounds with beneficial and preventive action against numerous diseases (Renna et al., 2016).

Sprouts, microgreens, and baby-leaf can also be considered as innovative products in terms of production processes because they can be used to produce processed products such as juice. One of the objectives of this Ph.D. thesis in fact was to evaluate the healthy properties of Broccolo nero’s juice.

Figure 4.10. Broccoli Sprouts, microgreens, and baby-leaf.

4.3. Exploiting *Brassica oleracea* L. complex species (n=9) for improving crops resilience and sustainability

Global warming, due to human activities, a phenomenon still controversial twenty years ago, has been observed on a global scale since the beginning of the 20th century, thus becoming a clearly perceptible reality (Mahalingam, 2015). This climate change results in an increase in both carbon dioxide (CO_2) emissions and global temperature (Peters and al., 2012).

Based on scenarios issued by the Intergovernmental Panel on the Evolution of Climate (IPCC), projections of the European climate have been modelled. The contrast of averages rainfall between northern and southern Europe is likely to increase in the next century. The average annual rainfall in southern Europe between 2080-2099 would be 4% to 27% more lower than that of the period 1980-1999.

As for summer temperatures, southern Europe would be faced, by 2080-2099, with an increase of 2.2 ° C to 5.1 ° C depending on the areas compared to the 1980-1999 average (Christensen et al., 2007).

Drought is not to be confused with aridity which is a permanent feature of weather. Drought is a temporary anomaly characterised by a relative lack of water. A region can be dry or humid, but drought occurs in each location during a period given (Kolb et al., 2016).

The increase in temperature in turn leads to evapotranspiration, which leads to increased drought and soil salinization (Zhao & Running, 2010).

Plants, at the same time, have developed specific mechanisms that allow them to detect and respond to precise environmental changes, thus minimising damage and conserving valuable resources for their growth (Atkinson et al., 2013; Suzuki et al., 2014).

It is relevant to elucidate the nature of these responses to create a targeted varietal improvement process. Plants cannot move, so they must endure stresses such as drought, salinity, and extreme temperatures.

These stressors greatly limit the distribution of plants, alter their growth and development, and reduce crop productivity. Recent progress in our understanding of the molecular mechanisms underlying the responses of plants to abiotic stresses emphasises their multilevel nature; multiple processes are involved, including sensing, signalling, transcription, transcript processing, translation, and post-translational protein modifications.

This improved knowledge can be used to boost crop productivity and agricultural sustainability through genetic, chemical, and microbial approaches (Zhang et al., 2021). Stress is, in biology, the set of reactions of an organism subjected to pressures or constraints of the environment (stressors).

Stress situations and plant reactions may differ. Stresses can be usual or exceptional, transient, or irreversible, and then create life in extreme conditions. It is a balance between the constraints of stress and the processes of adaptation which makes that "it passes, or it breaks", that the plant comes out of it or dies.

According to Selye's theory of Dynamics, we can observe the succession of phases depending on the “forces” of stimulation and inhibition. When a constraint arrives at the

cell, the alarm phase begins, it begins with the destabilisation of a certain number of structures, especially membranes, and functions. Then the resistance sets in.

Repair processes, restoration of the initial state and synthesis of protective molecules appear, this is the recovery phase.

The state returns to the initial stage. If the stress continues, the plant accentuates its protective processes. But, if the stressor intensifies or lasts too long, there is the phase of exhaustion with great damage, due for example to the attack of parasites which take advantage of the weakness of the plant, and the latter dies (Viner, 1999).

In general, a plant that is under stress is a disabled plant. This has repercussions on its growth and development, and ultimately on the yield and quality of the harvest.

On the nature of the stressful element, there are two types of stress: biotic and abiotic. The most common stresses are those related to predation by herbivores, attack by viruses, insects, parasites, heat stress and water/salt stress.

Biotic stress is stress resulting from the harmful action of a living organism on another living organism such as an attack by a pathogen. It differs from abiotic stress exerted by a change of environment.

Biotic stress in plants is caused by living organisms, especially viruses, fungi, bacteria nematodes, insects, weeds, and arachnids. The agents causing biotic stress directly to deprive their host of its nutrients can lead to death of plants.

Biotic stress can become major because of pre- and postharvest losses. Despite lacking the adaptive immune system plants can counteract biotic stresses by evolving themselves to certain sophisticated strategies.

Plants ``recognize" microorganisms thanks to "signal" molecules included in the walls of the latter. Certain microorganisms are beneficial and symbiotic (mycorrhizae, rhizobium), others are pathogenic and responsible for disease. When a pathogen "attacks" a plant, the latter will trigger a cascade of defence reactions within the cell.

Following this attack, the plants respond in several ways:

By "cell suicide": at the site of infection to block the pathogen, the plant sacrifices cells.

By strengthening the mechanical barrier by thickening the cell wall.

By the production of metabolites with anti-microbial activity, phytoalexins (Langcake and Pryce, 1976).

By the production of enzymes which degrade the wall of pathogens such as glucanase and chitinase (Van Loon, 1997).

It should be noted that, often, the "immune response" has a systemic characteristic (throughout the plant) and no longer localized (like cell suicide). This RSA (Acquired Systemic Response) is based on the activation of genes that will maintain the entire plant in a state of resistance against a broad spectrum of pathogens.

Brassica virus diseases represent a major health concern for the brassica cultivation sector under cover or in the open. Because of their diversity, the inexistence of direct means of treatment but also because of their modes of propagation which are always difficult to control, viruses constitute a component serious biotic stress.

The means of control are resolutely oriented towards the selection of tolerant or resistant varieties, sanitary prophylaxis against materials and equipment as well as the

fight against vector insects. The two most common viruses that affect brassicas are the Cauliflower Mosaic Virus and Turnip Mosaic Virus.

The viruses can occur as individual diseases or together in a plant. When both Cauliflower Mosaic Virus and Turnip Mosaic Virus are present severe spotting will occur. Cauliflower Mosaic Virus causes unusual growths on the veins of the lower leaf surface.

When plants are stored, they show black colourings with spots on leaves inside the head. Turnip Mosaic Virus causes black spots that start as yellow rings on leaves making them fall off early. In stored cabbage, black sunken spots develop on the leaves throughout the head. The spots are larger than those caused by Cauliflower Mosaic Virus. Few control methods are available in the case of a viral infection and above all, no curative treatment can be applied.

The means of controlling viral diseases can be broken down into two main categories. The first involves the prophylactic strategies which make it possible to limit or reduce to a minimum the arrival of the virus on the plot and the second relates to the strategies which tend to limit the effects of the virus on the plant, once infected, by means of varietal resistance.

Health authorities benefit from the development of a large panel of detection tests based on techniques of immunology (Dalmon et al., 2009), molecular hybridization (Crespi et al., 1991), qualitative PCR (Lefeuvre et al., 2007) or quantitative PCR (Papayiannis et al., 2010).

Water stress is a form of abiotic stress that occurs when the moisture of plant tissues is reduced to suboptimal levels. Water stress occurs in response to atmospheric and soil water availability when the transpiration rate exceeds the rate of water uptake by the roots and cells lose turgor pressure. Moisture stress is described by two main metrics, water potential and water content (Chappelka and al., 1995).

The water potential defines the state of drought of the plant. It is expressed as the potential chemical of water in a system compared to the chemical potential of pure water at temperature and identical atmospheric pressure (Lambers et al., 2008).

To determine the water potential, the pressure chamber is a reliable method (Scholander et al., 1965). It allows to measure the values of the basic leaf water potential (Ψ base). When the transpiration of the plant is weak, as during the night, the water potential of the plant is in equilibrium with that of the soil. The more the ground is dry, the more the water potential of the soil and therefore of the plant will be negative.

In some plants, the first response to water stress is to slow down its growth by stopping cell division (Cramer et al., 2013). The leaf surface is reduced, and the formation of new leaves is interrupted in the event of prolonged stress (Lovisolo & Schubert, 1998).

This makes it possible to limit the water evaporation surface. Closing the stomata reduces water loss through transpiration and, on the other hand, net photosynthesis (Cramer et al., 2013) resulting in 39% to 55% lower carbon gain via photosynthesis (Escalona et al., 2012).

Drought tolerance comes down to a plant's ability to survive and produce under conditions of water stress. Plants that grow on soils with little water reserve use two strategies: avoidance and tolerance. Moisture stress influences stomatal opening, mainly

causing a closure in stomata as to reduce the amount of carbon dioxide assimilation (Hand et al., 1982). Closing of the stomata also slows the rate of transpiration, which limits water loss and helps to prevent the wilting effects of moisture stress (Freeman, 2014). This closing can be trigged by the roots sensing dry soil and in response producing the hormone ABA which when transported up the xylem into the leaves will reduce stomatal conductance and wall extensibility of growing cells.

This lowers the rates of transpiration, photosynthesis, and leaf expansion. ABA also increases the loosening of growing root cell walls and in turn increases root growth to find water in the soil (Lambers et al., 2008) (Figure 4.11.).

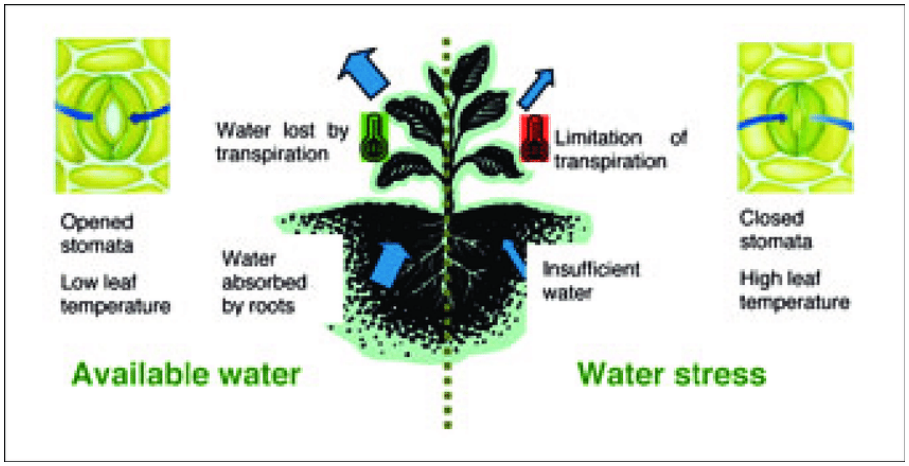

Figure 4.11. Effect of water stress (Erena et al, 2012).

Phenotypic response of plants to long-term water stress was measured in corn and showed that plants respond to water stress with both an increase in root growth both laterally and vertically (Singh and al, 2020).

In all Droughted conditions the corn showed decrease in plant height and yield due to the decrease in water availability (Weaver, 1926). Under these conditions, the availability of water can be improved in several ways. First by facilitating the leaching of salts accumulated in the roots, then by improving drainage systems and finally by using irrigation systems adapted to cultivated plants.

Currently, the challenge is to increase plant production, in this case the useful biomass per hectare, in an increasingly unfavourable climatic and pedological context. Given the difficulties inherent in a better distribution of agricultural resources and in the absence of adequate solutions to desalinate the soil as well as the irrigation water, it is from the biological point of view that the ways of tolerance will be orchestrated. to water stress. Pathways involving a complex network of transcriptional, translational, enzymatic, and metabolic regulatory systems (Marco et al., 2015).

It is with this in mind that researchers have been trying for several years to take advantage of new approaches for the global analysis of gene expression in phenotypes such as transcriptomics (Kreps et al., 2002), proteomics (Bae et al., 2003) and more recently, metabolomics (Sévin et al., 2016).

While each of these levels of analysis is itself a complex function of numerous cellular determinants, the metabolome is particularly integrated because it results from

events and regulations taking place at the genetic, transcriptomic, and proteomic levels (Parvaiz et al., 2013). It is also a transversal level of organization that integrates multiple environmental and developmental influences at the cell and whole plant level.

The plant perceives and responds to water stress by perception and transduction of signals (Pandey, 2015). Attempting to improve tolerance in this way requires the regulation of induced responses, both at the level of the mechanisms of perception of stress itself and of the triggering of effector responses (Marco et al., 2015).

Stress responses are organised into blocks or regulons placed under the control of a limited number of perceptual and regulatory mechanisms. Thus, it would be theoretically possible to circumvent the multigene and systemic nature of tolerance to water stress by manipulating these control points, generalised, and located upstream.

Perception of the stress signal activates receptors upstream of a series of cascading mechanisms, which transmit, amplify, and ultimately trigger cellular responses opposing the harmful effects of such stress.

Stress signals are first perceived by membrane receptors belonging to different families. In Arabidopsis, the AtHK1 protein (Histidine kinase) functions as an osmotic sensor which transmits the stress signal downstream to a cascade of MAPK (Mitogen-activated protein kinases) (Urao et al., 1999).

The G-Protein Coupled Receptor (GPCR) protein, coupled to G proteins, also perceives stress signals, activating a signalling pathway involving heterotrimeric G proteins (Tuteja, 2009). RLKs (Receptor-Like Kinases) are a family of transmembrane proteins with approximately 600 members (Shiu & Bleecker, 2001). In potatoes, StLRPK1 (Leucine-rich Repeat Receptor-like Kinase) is a membrane receptor induced in leaves in response to environmental stresses.

The second messengers are small molecules or intracellular signalling ions, located in the cytoplasm of a cell and whose quantity varies according to the response to a signal perceived by a membrane sensor. They spread by diffusion and regulate the activity of proteins, helping to distribute and amplify the received signal. The second messengers include Ca2+ (Mahajan & Tuteja, 2005), reactive oxygen species, ROS (Suzuki et al., 2014), cyclic nucleotides, and polyphosphoinositides (Tuteja & Sopory, 2008).

Phytohormones, on the other hand, activate signaling pathways independently or in synergy with other stress triggered pathways (Marco et al., 2015). Next to abscisic acid (ABA), a hormone widely studied in the stress response scale, are cytokinins (CK), salicylic acid (SA), ethylene (ET), jasmonic acid (JA), nitric oxide (NO) as well as sugars and polyamines (PAs) playing a substantial role, direct or indirect, in the response to water stress (Tuteja & Sopory, 2008) (Figure 4.12.). Protein kinases and phosphatases are a fundamental pillar in the coordination between the activity of several signal transduction pathways.

These proteins control the phosphorylation state of their target proteins by catalysing several reversible phosphorylation processes. They are separated into several categories according to their structural and functional characteristics, interact with each other, thus forming a complex network whose purpose is the modification of target proteins having an enzymatic or structural function to lead to rapid physiological responses (Marco et al., 2015).

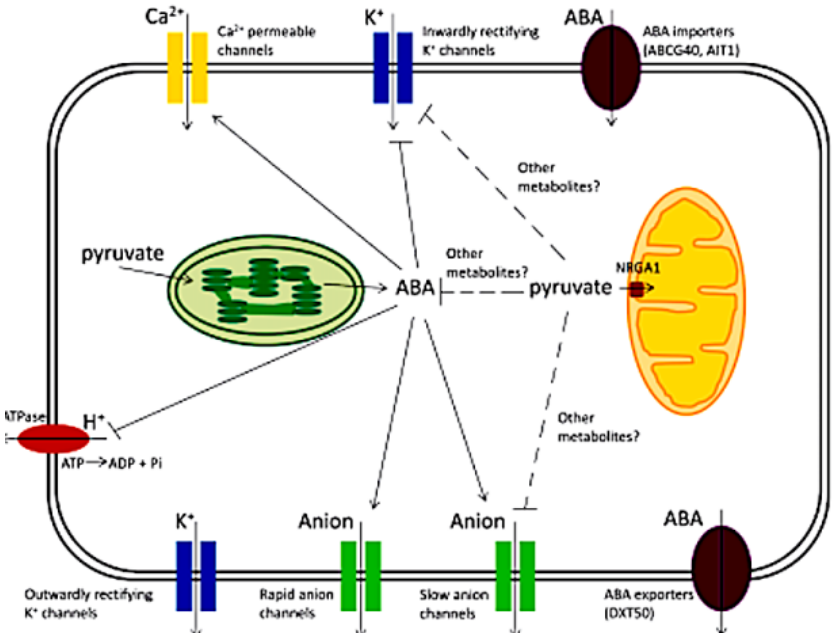

Figure 4.12. Ion Channels and Transporters in ABA Biosynthesis, Transport, and Signaling in Guard Cells. (Yu et al., 2014).

Signalling pathways result in the production of new proteins through transcription factors (FTs), which in turn induce the expression of target genes. FTs are classified according to the presence of known DNA binding domains (Marco et al., 2015).

Stress-related FTs include members of APETALA2 / Ethylen responsive factor (AP2 / ERF), basic helix-loop-helix (bHLH), basic leucine zipper (bZIP), homeodomain leucine zipper (HD-ZIP), myelocytomatosis (MYC) myeloblastosis (MYB) and the NAC and WRKY families (Lindemose et al., 2013).

Transcriptome studies revealed that genes induced by signalling cascades triggered by abiotic (water) stress could be divided into two major groups depending on the function of their products (Seki et al., 2002):

Functional proteins: This first group encodes many proteins with defined enzymatic and structural functions such as membrane proteins which help restore cellular homeostasis (HKT, SOS, and NHX), osmoprotectants (proline, betaine, etc), ROS detoxification enzymes (GPOX, CAT, APX ...) and other proteins involved in the protection of macromolecules (LEA, HSPs and mRNA-binding proteins) (Marco et al., 2015).

Regulatory proteins: The second group encodes a series of regulatory proteins (transcription factors, protein kinases, receptor protein kinases, etc.) and signal transducer proteins (phospho-esterases and phospholipase C) involved in the regulation of the cascade signals controlling the expression of additive genes, the products of which may belong, in turn, to each of the two groups (Fujita et al., 2007).

Apart from FTs, the modulation of gene expression, in response to water stress, can be accomplished at other levels including the involvement of alternative splicing events (Seo et al.., 2013), post-translational modifications (miRNA and ubiquitination) (De Lima et al., 2012) or epigenetics (Marconi et al., 2013).

The concerted actions of these control mechanisms ensure that the expression of genes taking place following the perception of stress, can be carried out in adequate spatial and temporal configurations. These responses adjust, to each stressful situation, a new balance between the growth, development, and survival of the plant (Xu & Yang, 2013).

Ionic and osmotic stress can create secondary stresses or derived stresses such as the accumulation of unwanted toxic compounds (Mittova et al., 2015). Among these is oxidative stress, which is a major constraint (Sevilla et al., 2015). There are basically four types of ROS in the cell: singlet oxygen (1O2), superoxide radical (O2 -), hydrogen peroxide (H2O2) and hydroxyl radical (HO-) (Uzilday et al., 2015).

The main sites of ROS production are the mitochondria, chloroplasts, peroxisomes and the apoplast. These ROS are involved in cell signalling and the production of toxic compounds (Uzilday et al., 2015).

Under optimal conditions, the radicals (O2-) and (OH-) are generated by the normal metabolism of the different subcellular compartments for use as signalling molecules and by products of different metabolic pathways (Lai et al., 2012). However, abiotic stresses can, through excessive concentrations of ROS, damage DNA, RNA, proteins, chlorophylls, and membrane functions (Miller et al., 2010).

The plant must constantly deploy defence mechanisms to overcome oxidative damage. This is how they provide themselves with antioxidants of a non-enzymatic nature such as phenolic compounds, flavonoids, anthocyanins, and ascorbic acid (Uzilday et al., 2015).

The fact remains that plants also involve a wide range of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), glutathione S-transferase (GST) and glutathione peroxidase (GPX) (Uzilday et al., 2015). SOD can eliminate the superoxide radical by catalysing the disproportionation reaction of O2– to H2O2, which however remains a toxic intermediate whose concentration can be regulated by APX, CAT or GPX (Parent et al., 2008) (Figure 4.13.).

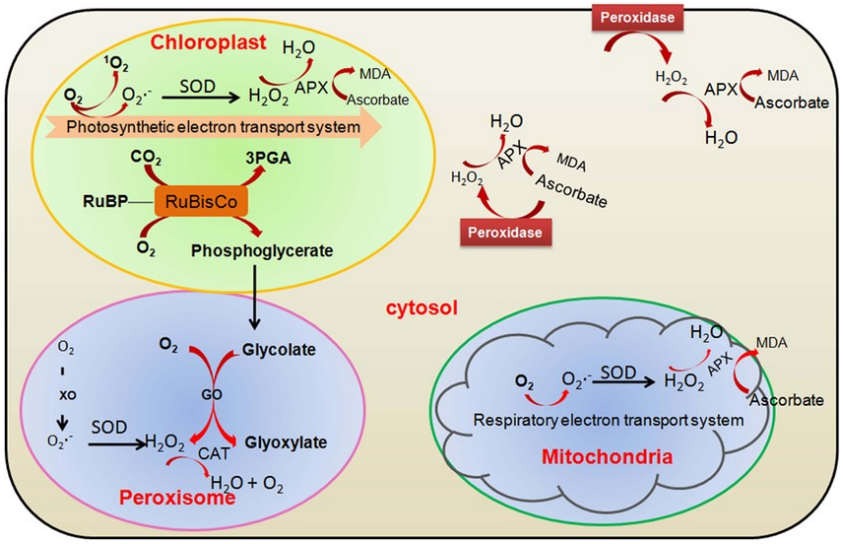

Figure 4.13. Major sites of reactive oxygen species (ROS) production in photosynthetic cells and involvement of major antioxidative enzymes (Das et al, 2015).

4.4. Nutraceutical profile of the products

New consumer needs and growing market interest for innovative product types have determined in recent decades a particular attention of research on the improvement of characteristic technological aspects of agricultural production. Among the characteristics sought by the consumer in agricultural products emerge those that support the health virtues of the same. Epidemiological studies have highlighted the association between a diet rich in vegetables and diseases such as cardiovascular (Dauchet et al., 2006; He et al., 2006).

The compounds, such as vitamins, polyphenols, and glucosinolates, take great interest today because they express properties antioxidants, inhibit the activation or propagation of chain reactions oxidation of free radicals, inhibiting the initiation chains or breaking the propagation chains, overall reducing oxidation processes in human body (Namiki, 1990).

These compounds are present in the species afferent to the family of the *Brassicaceae* (Krinsky, 2001; Shi et al., 2001) that have common characteristics, specifically organic substances with low molecular weight responsible for the colour and organoleptic characteristics of plants, which possess preventive and protective action for human health.

Glucosinolates (GLSs) are secondary metabolites that comprise more than 130 compounds; they are mainly present in the cruciferous family, and especially in the *Brassicaceae* (Fenwick and Heaney, 1983).

GLSs are molecules consisting of a common glycone and a side chain aliphatic, indolic or aromatic, derived from a corresponding α-amino acid, such as methionine, phenylalanine, tyrosine, and tryptophan (Carratù and Sanzini, 2005).

The glucosinolates, following the cut, or the laceration of the vegetal tissues contained in the cells, are rapidly hydrolysed by the enzyme myrosinase (present both in the cells of the plant and in the intestinal microbiota), to unstable intermediates that spontaneously rearrange themselves leading to the formation of several volatile compound characteristic such as isothiocyanates, nitriles, sulphides (Figure 4.14.).

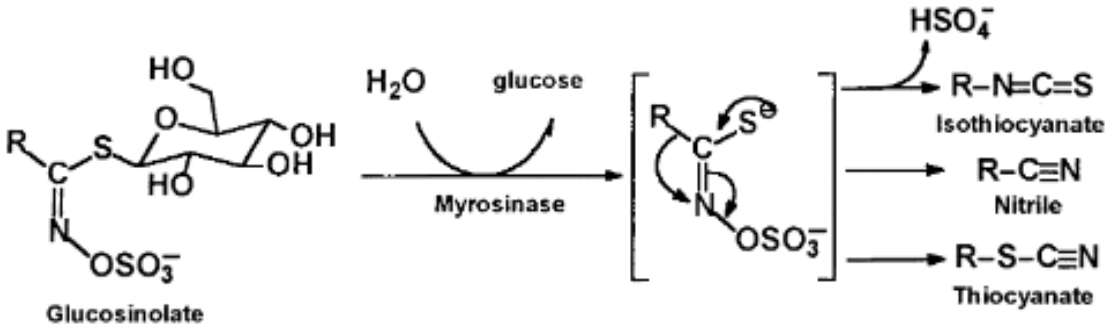

Figure 4.14. GLSs hydrolysis.

The conversion is influenced by several factors such as temperature, pH and ferrous ions (Fe2+). The main GLSs, are glucoraphanin (GRA), glucoiberin (GIB), glucoerucin (GER), 4-OH-glucobrassicin (4-OH-GBS), glucobrassicin (GBS), 4-methoxy-glucobrassicin (4 OM-GBS), neo-glucobrassicin (NEOGBS), sinigrin and progoitrin.

Glucobrassicin (glucosinolate indole), sinigrin and gluconapin (glucosinolates aliphatic), in the specific case of *Brassicaceae*, give a better aroma to the product, positively influencing the choice of the consumer.

Glucoraphanin is one of the main glucosinolates studied because of the innumerable beneficial properties due to its isothiocyanate, sulforaphane (4-methylsulfonyl-ethyl isothiocyanate), considered a powerful inductor of the phase II enzymes involved in the carcinogenic detoxification; this isothiocyanate is mainly present in broccoli, cabbage, and cauliflower with pigmented curds (Figure 4.15.) (Velasco et al., 2007; Korus et al., 2007).

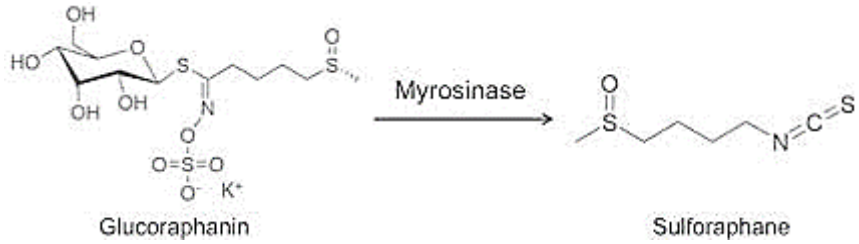

Figure 4.15. Glucoraphanin and sulforaphane.

In several white cauliflower cultivars, however, was found the presence, in a small quantity, of the sinigrin and its isothiocyanate, allyl isothiocyanate, toxic for many animal organisms (Branca et al., 2008).

A recent screening on glucosinolates present in different varieties of kale, moreover, from various parts of the world found great differences depending on the area of cultivation and even between individuals of the same variety (Hahn et al., 2016).

The content of GLSs depends on the environmental factors, the part of the plant examined, the phenological stage of plant growth and damage by insects, and the total concentration of glucosinolates in the leaves of *Brassica oleracea* increases with the age of the plant (Velasco et al., 2007).

The interest for GLSs is mainly due to the correlation, found by several studies (Shapiro, 2001; Talalay and Fahey, 2001), between the consumption of *Brassicaceae* and the reduced risk of chronic diseases and cancer.

Isothiocyanates derived from aromatic and aliphatic glucosinolates show chemo-preventive activity in animal models, as important agents blocking chemical carcinogenesis. Glucosinolates are of great interest because they have antioxidant properties, in particular those properties are explained by their ability to inhibit the propagation of free radical oxidation chain reactions.

GLSs reduce oxidation processes in the human body. Most glucosinolates contribute to the aromatic characteristics of *Brassicaceae* and protect the plant from attacks of insects, bacteria, nematodes, and fungi; some.

However, such as nitrites and isothiocyanates, have toxic or anti-toxic effects when included in animal feed (Branca et. al, 2008). *Brassicaceae*, compared to other vegetables, because of the high content of glucosinolates, are defined although some studies have shown that an abuse of these foods can cause harmful effects for the consumer, because the action of thyroid hormones is inhibited; in particular, this action is inhibited by progoitrin (Felker et al., 2016).

Polyphenols, other phytochemicals found, represent a large class of organic compounds that include several aromatic rings associated with different phenolic groups.

Phenols range from simple, single-loop, low molecular weight aromatic compounds to large and complex tannins (Di Bella et al., 2020), and are classified according to the number and arrangement of their carbon atoms in flavonoids (flavanols, flavones, flavan-3-oils, anthocyanins, flavonoids, isoflavones) and not flavonoids (phenolic acids, hydroxymethyl, stilbenes).

Polyphenols are often produced in response to biotic or abiotic stress (Di Bella et al., 2020) and act as reducing agents, hydrogen antioxidants and singlet oxygen quencher.

The multifunctionality of polyphenols is due to their distribution in different tissues and organs of plants at different concentrations; they act as antioxidants, flavourings, and food dyes.

The groups of polyphenols most common and differentiated in the Brassica species are flavonoids (mainly flavanols but also anthocyanins) and hydroxamic acids.

Anthocyanins and carotenoids, on the other hand, characterise only the violet corymbs, being absent in those with white or green colouration, and carry out antioxidant activity blocking the free radicals involved in the oxidative processes of the metabolism of animals and man (Figure 4.16.) (Branca et al., 2008).

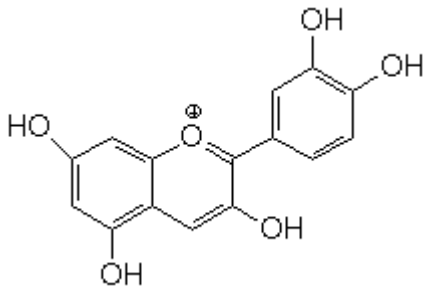

Figure 4.16. Chemical structure of anthocyanins.

Minerals are inorganic compounds that perform essential functions for human life, specifically participate in the formation of bones and teeth, in the regulation and activation of many metabolic cycles and are important factors for the development of organs and tissues.

Mineral salts cannot be synthesised by living beings but are assimilated through water and food.

In relation to the daily requirement, they are divided into microelements and macroelements.

The microelements are present in the organism in small traces, the requirement in this case goes from micrograms to milligrams; the macroelements are, however, present in good quantities, in this case the need is around the order of the grams.

Among the minerals mainly present in the kale are mentioned: calcium, magnesium, and potassium.

Vitamins are organic compounds essential for the human organism, for growth, for cellular integrity and to allow the normal development of metabolic processes. These

compounds have no energy value and act in minimal doses and have specific action. (Ragusa, 2011).

In general, vitamins are divided into two groups: water-soluble vitamins (B vitamins and vitamin C) and fat-soluble vitamins (vitamins A, E, D, K).

Kale is an excellent source of vitamins, especially vitamin K (Kim et al., 2017).

Among the vitamins of group B are mentioned:

- Vitamin B1 (Thiamine);
- Vitamin B2 (Riboflavin);
- Vitamin B3 (PP or Niacin).
- Vitamin B5 (Pantothenic acid).
- Vitamin B6 (Pyridoxine).
- Vitamin B8 (H or Biotin).
- Vitamin B9 (Folic acid).
- Vitamin B12 (Cobalamin).

Vitamin B1 is present both in foods of plant origin, especially in cereals and legumes, as well as in foods of animal origin, such as eggs, pork, and yeast. As there is no reserve of thiamine in the human body this vitamin must be introduced daily with the diet and its deficiency can cause chronic diseases such as, for example, beriberi.

Vitamin B2 is present in milk, eggs, and meat, and is synthesised by many plants and various microorganisms. Riboflavin deficiency is rare.

Vitamin B3 is present in foods of animal origin and what is very important can be synthesised by the human organism from tryptophan, its precursor.

Vitamin B5 is a precursor of Coenzyme A. This vitamin is found in meat, eggs and cereals and its deficiency can cause serious gastrointestinal disorders.

Vitamin B6 is present in many foods such as meat, kidney, brain, fish, fruit, and vegetables. Its deficiency is rare.

Vitamin B8, present in yeast, legumes, meat, and egg yolk, has the function of constituting the coenzyme of decarboxylase and transcarboxylase enzymes.

Vitamin B9, mainly found in broad-leaved vegetables, is indispensable in certain physiological states of the human organism such as pregnancy, as it protects the fetus from malformations of the neuronal tube.

Vitamin B12 is only found in foods of animal origin, although small amounts are also found in fresh vegetables. (Cappelli and Vannucchi, 2005).

Vitamin C, which includes ascorbic acid and its oxidation product (dehydrocarbon acid), participates in various biological activities in the human organism (Block et al. 2004).

More than 85% of vitamin C in human diets is supplied by fruits and vegetables (Davey et al., 2000; Lee and Kader, 2000).

Epidemiological studies show that dietary intake of 49 grams of vitamin C per day leads to a 21% reduction in all types of cancer that can affect men; however, no significant effects have been observed in women (Borek, 2005).

Ascorbic acid (L-ascorbate) is a cofactor for many enzymes, acts as a scavenger of free radicals and can recover superoxide and hydroxyl radicals, as well as to regenerate α-tocopherol (Davey et al., 2000).

Among the subspecific entities of Brassica oleracea the content of Vitamin C varies greatly, especially environmental conditions can determine variations in the content of ascorbic acid in plant tissues (Howard et al., 1999).

Vitamin A (retinol) is a long-chain alcohol that is found, in nature, in an esterified form not synthesised ex novo by animal organisms. It is an important vitamin for sight and for the proper functioning of the immune system.

Vitamin D, divided into Ergocalciferol (Vitamin D2) and Cholecalciferol (Vitamin D3) is present in blue fish, milk, eggs, and mammalian liver; among its main tasks is the regulation of calcium metabolism (Cappelli and Vannucchi, 2005).

Vitamin E (tocopherol) is mainly found in foods of plant origin, particularly broad-leaved vegetables, oilseeds, and oleaginous fruits.

Vitamin K in vegetables, meat and intestinal flora is divided into Phylloquinone (Vitamin K1), Prenilmenaquinone (Vitamin K2) and Menadione (Vitamin K3) (Cappelli and Vannucchi, 2005).

One aim of this Ph.D. was aimed at the search for some miRNAs of health interest in the juice of four stages of growth of the local cultivar of Broccolo nero.

MicroRNA (miRNA) are non-coding RNA molecules that have less than 200 nucleotides (sRNAs). (Li et al., 2021)

The mechanisms of miRNA gene transcription and maturation have been mainly studied in *Caenorhabditis elegans*, *Drosophila melanogaster* and human cell lines (Li et al., 2021).

In animals, miRNA synthesis begins with the transcription of a transcript several kilobases long known as the primary microRNA (pri-miRNA) followed by its cleavage by a complex of microprocessors located in the nucleus into a precursor microRNA 60-120 nt long (pre-miRNA).

The catalytic subunit of the microprocessor complex consists of a class II ribonuclease enzyme, DROSHA, and a double-stranded RNA-binding protein (dsRNA), the critical 8 protein of DiGeorge syndrome (DGCR8) (Ha & Kim, 2014).

The pre-miRNAs are exported from the nucleus to the cytoplasm through the Exportin 5 protein (XPO5): Ran GTPase complex where they are processed into miRNA: miRNA by class III DICER RNase (Sundaram, 2019).

The miRNA is then loaded onto the RNA-induced silencing complex (RISC) where the guide strand is selectively retained, and the passenger strand is degraded. The recruitment of the RISC complex together with the guide strand (mature miRNA) on the target mRNAs leads to translational repression followed by transcription degradation (Sundaram, 2019).

The biogenesis of miRNAs differs significantly between the plant and animal kingdom. In plants, unlike animals, the processing of first miRNAs into pre-miRNA and finally into miRNAs is entirely carried out in the nucleus.

Furthermore, it is DICER LIKE-1 class III RNase III (DCL1) that catalyses the conversion of pri-miRNA to pre-miRNA and of pre-miRNA to miRNA (Liu et al., 2018).

In addition to DCL1, a double-stranded RNA-binding protein called HYL1 and SE, a C2H2-type zinc finger protein, are essential in the biogenesis of plant miRNAs.

Furthermore, the protein known as the HATY gene (HST1) is the plant counterpart of XPO5 that exports the miRNA duplex from the nucleus to the cytoplasm.

Another difference between plant and animal miRNAs is that the last nucleotide at the 3 'end of the mature miRNA is 2'-O-methylated in plants, which is not observed in animal miRNAs (Figure 4.17.).

In plants, target mRNAs exhibit near-perfect complementarity to miRNAs, consequently transcription degradation is hypothesised to be the primary pathway for miRNA-mediated suppression (Sundaram, 2019).

In March 2018, miRbase counted 2654 mature human miRNAs and 1917 precursors, otherwise for *Arabidopsis thaliana*, the model organism for plant biology, 326 precursors and 428 mature miRNAs were reported (Sundaram, 2019).

From commercially important crops such as soy, rice, and wheat are also reported, possessing 684, 604, 122 pre-miRNAs and 756, 738, and 125 mature miRNAs, respectively (Sundaram, 2019).

Furthermore, there are several examples on literature of how specific miRNAs play a key role in different physiological processes such as plant development, tolerance to abiotic and biotic stresses (Liu et al., 2018; Wang et al., 2017; Liu et al., 2017), more recently several works describe their role also interactions. between plant and microorganisms (Huang et al, 2019).

Some miRNAs are known to target a specific gene, while others can regulate the expression of hundreds of genes simultaneously (Li et al., 2021).

Cross Kingdom RNAi has been reported that *Cuscuta campestris haustoria* while parasite induces many 22nt miRNAs that affect *Arabidopsis thaliana* Heynh growth and pathogenesis, influence growth and pathogenesis as some of these miRNAs could reduce mRNA accumulation, stimulate production of secondary siRNA, or promote the cleavage of host mRNA (Shahid et al., 2018).

The first to demonstrate that through food intake, the exogenous plant MIR168a can be detected in animal sera and tissues and serve as signalling molecules in intercellular communication were Zhang et al. (2012).

It was observed that after feeding mice total RNA extracted from fresh rice, or synthetic MIR168a, or synthetic methylated MIR168a, the levels of MIR168a in mouse serum and liver were all elevated 3 hours after feeding.

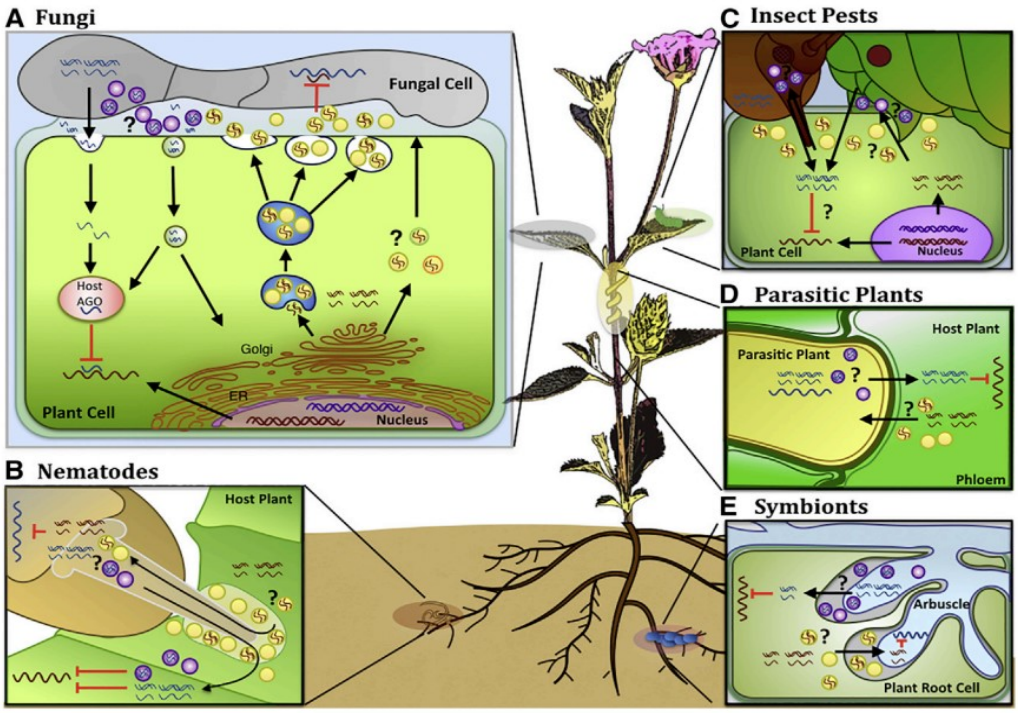

Figure 4.17. Cross Kingdom sRNA Trafficking between Plants and Interacting Organisms.

Food derived miRNA

As already explained above, the micro RNAs (miRNAs) are about 22 single-stranded non-coding RNA nucleotides, these regulate the expression of protein-coding genes by driving the protein-RNA complex called RISC towards the mRNAs (Liu et al., 2017). By convention it is believed that the expression of most genes is regulated by miRNAs in mammals, consequently the involvement of miRNAs in the pathogenesis of diseases and biological processes is foreseeable (Bartel, 2004).

In several successive studies in recent years, stable and cell-free miRNAs have been found in samples derived from the human circulating system such as plasma, urine, and saliva (Kosaka et al.,2010). Many circulating miRNAs are being studied for the possible function of diagnostic biomarkers in many diseases (Schwarzenbach et al., 2011).

Furthermore, the miRNA of the plant miR168a which regulates the human LDLRAP1 gene was found in mammalian sera (Zhang et al., 2012). Several studies have published positive and negative results of experimental tests on the possible presence of exogenous miRNAs in the animal circulatory system derived from food ingestion (Liu et al., 2017).

One study indicated that plant miRNAs can present themselves within the human circulatory system via food intake and consequently regulate human gene expression. One example is miR159 from broccoli, this has been found in human sera and has been shown to inhibit the growth of breast cancer by targeting the TCF7 gene (Chin et al., 2016).

Liu et al. (2019) identified abundant plant miRNAs sequences from 410 human plasma small RNA sequencing data sets. Among them, the plant miRNA miR2910, conserved in fruits and vegetables, was found to present in high relative amount in the plasma samples. This miRNA, with same 6mer and 7mer-A1 target seed sequences as

hsa-miR-4259 and hsa-miR-4715-5p, was predicted to target human JAK-STAT signalling pathway gene SPRY4 and transcription regulation genes (Liu, et al., 2017).

The recent increase in studies and discoveries on the characterization of miRNA has not only improved the knowledge of the pathogenic mechanisms of diseases, but also offers applicative opportunities for the treatment of various disorders.

Traditionally, active ingredients with pharmacological activities have been considered the chemical compounds in herbal or plant extracts however, recently, it has been observed that nutrients and bioactive compounds, such as flavonoids, alkaloids and diterpenoids, could regulate the crosstalk of miRNA, lncRNA and circRNA, in addition to exerting pro-apoptosis, anti-inflammatory, anti-proliferation, anti-atherosclerosis and anti-infective activities (Bruna et al., 2017; Dong et al., 2019) (Figure 4.18.).

As versatile molecular regulators, plant miRNAs not only regulate host gene expression, but when ingested exogenous plant miRNAs can also influence the physiological and pathological conditions of mammals (Li et al., 2021). For example, Panax ginseng C. A. Mey (Panax ginseng) is one of the most valuable medicinal herbs in China whose influence on the regulation of immune function, anticancer, cardiovascular protection and cognitive enhancement activities is well known.

Through the Illumina HiSeq platform, 298 known and 3,500 possible new miRNAs have been identified in Panax ginseng. Furthermore, bioinformatics analysis showed that as many as 2686 endogenous ginseng miRNAs could regulate 50992 potential human genes, involved in 296 signalling pathways (Wang et al., 2019).

The rice miR168a has been reported to specifically target the mammalian LDLRAP1 gene, and thus enable cross-kingdom RNAi between plants and mammals (Zhang et al., 2012). Another example is the discovery that a small RNA isolated from the Chinese Hong Jing Tian herbal medicine (HJT) grown at high altitude, HJT-sRNA-m7, could improve pulmonary fibrosis both in vitro and in vivo by targeting associated genes. to pulmonary fibrosis of COL3A1, α-SMA and fibronectin mice (Du et al., 2017).

Subsequent studies confirmed the finding by detecting plant miRNAs in human blood (Shu et al., 2015; Fabris and Calin, 2016; Beatty et al., 2014; Zhao et al., 2018). Interestingly, in Huang et al. (2019) they isolated small RNAs from 10 herbal traditional chinese medicine decoctions prepared according to the conventional Chinese methods of processing and performed high-throughput sequencing analysis getting millions of small RNA sequences were obtained from each herb.

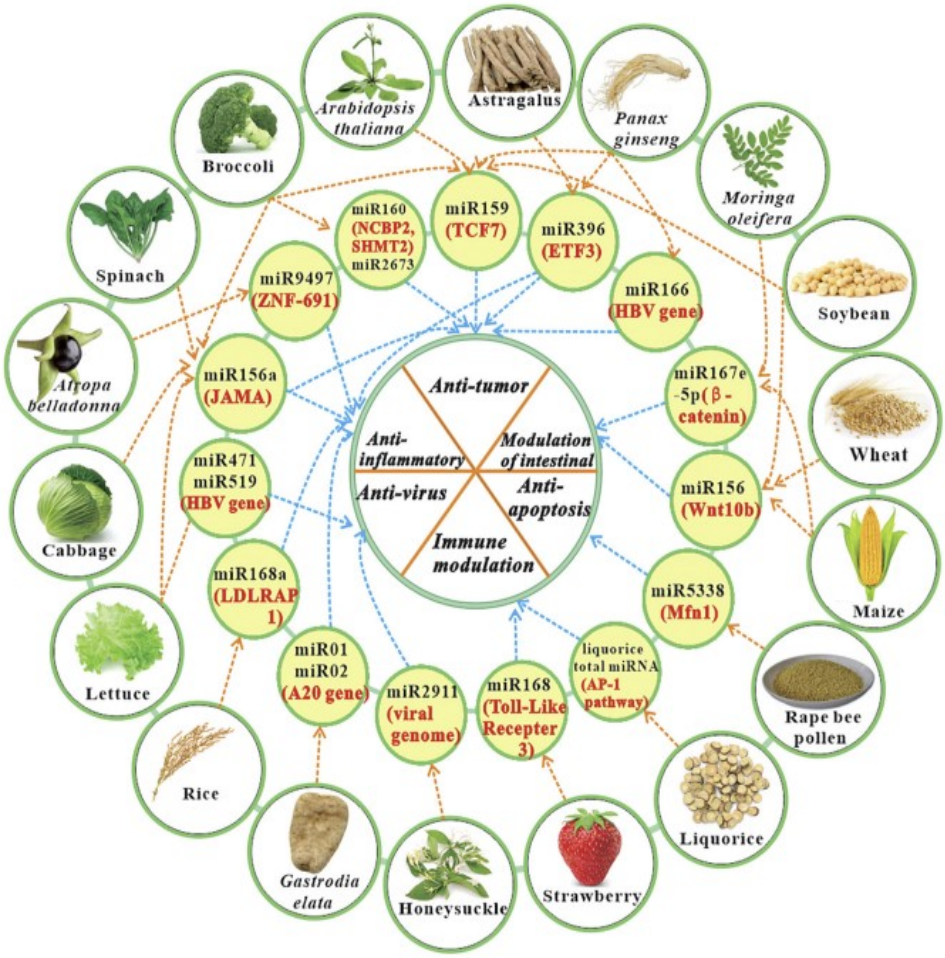

Figure 4.18. The representative plant-derived miRNAs with cross-kingdom bioactivity and their role in cross-kingdom communications. Note: The information in yellow circle contains miRNAs derived from specific plants (black text) and their main targets (red text). Some conservative miRNA family can be found in many different plant species.

5. Experimental activities

5.1. General premises and objectives

The rich vegetable heritage widespread in the Mediterranean basin, and in particular the hot spot present in our country, and the recent acquisitions relating to the nutraceutical value of the corresponding products has prompted us to focus on it to support the innovation process of vegetable productions.

In particular, the wide diversity of minor and / or neglected species, used in Italy especially in the past, continues to be appreciated above all by the local communities that traditionally use them in traditional gastronomy in the various regions of our country.

This heritage of incomparable value, object of study by various national and foreign research institutions, could represent a strong point to support the vegetable innovation process diversifying the corresponding productions.

The wide diversity of vegetable species can easily take advantage of recent scientific acquisitions aimed at improving the production of related vegetable species of greater diffusion to improve the agronomic and technological traits of the cultivars spread in cultivation.

This need is supported by the various production chains which, responding to market demands, place great emphasis on product standardisation and food safety, required for the quality certification of the corresponding productions.

Based on these considerations, our attention has been placed on some *Brassica oleracea* crops such as broccoli (*B. oleracea* var. *italica*), cabbage (*B. oleracea* var. *capitata*), cauliflower (*B. oleracea* var. *botrytis*), kale (*B. oleracea* var. *acephala*), kohlrabi (*B. oleracea* var. *gongylodes*), widespread throughout the European continent, which has diversified traits especially in the countries bordering the Mediterranean basin.

These crops express valuable nutraceutical traits and could easily take advantage of the recent genetic tools by new 'omics technologies (metabolomics, proteomics, genomics, etc.).

The broccoli, cauliflower, kale, and kohlrabi express particular interest for our region where there is a wide genetic diversity not only for the crops belonging to *B. oleracea* species but also for the candidate relative species represented by different wild species of *Brassi*ca sharing the same chromosomal set, equal to n = 9.

Recent studies are validating the hypothesis that the kale represents the first result of the domestication process of *B. oleracea* crops and that the first stage of its cultivation has been written in the Mediterranean basin.

An interesting hypothesis is that Sicily has contributed considerably not only to the domestication process of leafy kale but that this crop has contributed together with the various wild species of Brassica (n=9) to the origin and diversification of broccoli and cauliflower crops.

The importance of the corresponding gene pool widespread in Sicily has in recent decades aroused the interest of various national and foreign researchers for the importance it plays in the genetic improvement of *Brassica* crops for resistance to biotic and/or abiotic stresses and for the improvement of nutraceutical traits of the corresponding products.

This gene pool is increasingly threatened by the massive introduction into cultivation of F1 hybrids endowed with valuable agronomic and technological traits that have limited the spread of the landraces and the propagation of the corresponding biological materials normally operated by the growers themselves.

In any case, the genetic self-incompatibility mechanisms that characterise the various vegetable crops belonging to *B. oleracea* facilitate allogamous pollination and consequently the genetic pollution of the landraces with significant repercussions on the genetic drift process.

The work carried out in recent decades by various researchers has likely allowed the collection and *ex situ* conservation of genetic resources in germplasm banks, now established, and spread all over the world, has allowed the definition of diversified strategies aimed to reduce the genetic drift in acts which now affects numerous species of agricultural interest.

This static strategy of germplasm conservation has recently been flanked by some more dynamic ones, essentially represented by *on farm* conservation protocols, which place particular importance on the interaction existing between human activities aimed at the selection and propagation of local cultivars, and on the environment, with its especially climatic evolutions, which determines the evolution of gene pools over time, preserving their corresponding allelic frequencies.

The importance of safeguarding the Italian *Brassicaceae* germplasm from the home/sub-urban gardens/farms is also supported by the numerous references that attest and enhance the peculiar organoleptic characteristics of the corresponding landraces widespread in our country, paying particular attention to the nutraceutical properties of these vegetables and especially to some ones particularly rich in various antioxidant compounds.

In this frame, the interest for safeguarding and conserving the *B. oleracea* germplasm from the above-mentioned agroecosystems and the several populations of their wild relatives (n=9) widespread in our country is well understood and in activating specific research lines to improve the quality profiles of the related products.

In this direction, a program of identification, collection, *ex situ* conservation and evaluation of the *B. oleracea* germplasm widespread in Italy, and specifically in our Region, has been established in the last decades by the Vegetable and Flower Crops section of the Department of Agriculture, Food and Environment (Di3A) of the University of Catania to enhance the genetic materials and to qualify the corresponding productions.

Various accessions of this collection and of others appropriately chosen among those preserved in other European germplasm banks have been studied to define a reference framework of the diversity that is recorded for some *B. oleracea* crops in Europe.

As is well known, product innovation, in addition to being pursued through the enhancement of the large existing genetic heritage, can be supported by modern transformation technologies.

In particular, the recent diffusion of the IV gamma products (ready to eat/ready to cook) helps to improve the characteristics of the product in the post-harvest phase as

can be seen from the recent and widespread experiences aimed at enhancing minor and / or spontaneous species that arouse interest for the corresponding vegetative organs. These species have so far been the object of attention above all for local communities due to the rapid deterioration of quality due to the rapid perishability that characterises the product.

In this context, the work carried out was directed to phenotyping the *B. oleracea* complex species (n=9) core collection of the H2020 BRESOV (Breeding for Resilient, Efficient and Sustainable Organic Vegetable production) project on leafy cabbage, to evaluate the effects of the water stress to the plant phenotyping of kale and kohlrabi, to individuate genotypes belonging to the *B. oleracea* complex species (n=9) resistant to water stress, and to evaluate new products of interest for nutraceutical traits.

In the latter regard, the stimulus to launch specific research activities was supported by the growing interest of consumers regarding broccoli sprouts which have high concentrations of antioxidants and specifically of glucosinolates which have a high anti-carcinogenic activity.

The activities carried out as part of the Ph.D. course fall within the theme of product innovation for vegetable products, the enhancement of the genetic heritage of species of interest as vegetables present in our country and the activation of specific production chains to integrate modern technologies for processing vegetable products.

5.2. Framework of the research lines of activity

The study activities were promptly activated at the beginning of the XXXIV Ph.D. course and in the first year concerned the identification and study of the bibliography relating to *B. oleracea* complex species (n=9) diversity and on the origin and diversification of *B. oleracea* crops, to the biotic and abiotic stresses affect *B. oleracea* crops, to the organic farming etic and consumer requests, to the antioxidant compounds and to fresh-cut products with particular reference to sprouts, microgreens and baby-leaf, antioxidant compounds and the corresponding nutraceutical functions, and to the contribution offered by neglected landraces and/or wild species of interest for their agronomic and nutraceutical traits.

Since from the first year and during the other two years the research activities were addressed to evaluate the *B. oleracea* complex species (n=9) for their biometric, biochemical, and genetic traits of interest for improving the resistance to biotic and abiotic stresses of *B. oleracea* crops and enhance the nutraceutical properties of the correspondent products.

The important role played by vegetables in the human diet in relation to the content of bioactive compounds in controlling various human chronic-degenerative diseases was highlighted. Particular attention was paid to the health policies of developed countries aimed at controlling the main human pathologies resulting from modern lifestyles and the adoption of unbalanced diets.

The high costs incurred in these countries for health care costs and the need to contain them have determined the opportunity to prevent the main chronic degenerative diseases by adopting diets in which vegetables are present at a significant level. In this frame, the importance of the Mediterranean diet emerged as an important point of reference recently also highlighted by UNESCO which declared it a World Heritage Site. Various experimental evidence confirms the health value of vegetables that largely support the Mediterranean diet because of their daily intake.

Bibliographic research has made it possible to expand the knowledge on the nutraceutical value of horticultural products which for twenty years have already been reflected in the research activities of various working groups both internationally and nationally.

Among the various bioactive compounds, the focus was mainly on polyphenols, carotenoids, vitamins and glucosinolates. For each of them the chemical characteristics and the main scientific evidence confirm their antioxidant properties have been listed.

Particular attention was paid to the glucosinolates compounds that characterise the *Brassicaceae* species and to their mechanisms of action that allow to block the carcinogenesis processes.

These findings have stimulated the activation of specific improvement programs of the main Brassicaceae crops to increase the content of nutraceutical compounds of the corresponding products. For this topic, the specific bibliography was collected, and the analysis protocols of the antioxidant compounds were identified to evaluate the different genetic materials estimated during the work program.

The experimental activities carried out during the three years of the Ph.D. course can be traced back to four main research lines:

i) Phenotyping the H2020 BRESOV *Brassica oleracea* complex species (n=9) core collection for bio-morphometric traits.
ii) Effects of water stress on plant phenotyping of kale (*B. oleracea* var. *acephala*) and kohlrabi (*B. oleracea* var. *gongylodes*).
iii) Individuation of source of resistance in *B. oleracea* complex species (n=9) genotypes for water stress.
iv) Evaluation of new products of interest for nutraceutical traits.

5.2.1. Phenotyping of the *Brassica oleracea* L. complex species (n=9) core collection

5.2.1.1. Introduction

The *Brassica oleracea* crops are widely grown in Europe using a great plurality of different morpho-types, because of both their adaptation to diversified environmental conditions and to their different uses addressed to human and / or animal nutrition.

This great diversity allows us to lay the foundations for the bio-morphological characterization work aimed at identifying morpho-types that allow us to be able to support product innovation for vegetable production based on the needs of consumers who are increasingly pointing their attention to the quality of the product, especially as regards to its health profile.

The pressing of the environmental problems, affected by both the climate change in act and the action of agricultural practices that are often not very respectful of the integrity of the territory, suggest identifying crops that are able to mitigate their impact on the environment.

In this regard, *B. oleracea* crops seem to satisfy these needs both for the biological cycle of the plant, most of them are annual or biannual but some are perennial, as such as kale and some broccoli types, which allows an improvement of the soil fertility for their rustic features that allow even marginal land to be used for their growing.

In various home and suburban gardens in Sicily, they are often cultivated also along the slopes and on sloping lands to limit landslides thanks to the persistence over time of the robust root system that allows the state of the places.

The rapid introduction into cultivation of new F1 hybrids of different *B. oleracea* crops, requires the opportunity to preserve this diversity which is compromised by the peculiar floral biology of the species that facilitates cross-fertilization. The risk of genetic pollution is clearly very pressing in horticultural areas which at the same time present, both landraces and F1 hybrids, as it happens in Sicily.

The seed industry, increasingly concentrated in the hands of a few efficient multinational companies, is mainly committed to obtaining both F1 hybrids, deriving from a narrow range of parental lines, and of engineered varieties.

The conservation and safeguarding of vegetable germplasm are restricted only to some traditional cultivars of notoriety, while all the others are progressively abandoned. This has caused, and still causes, the rapid loss of genetic variability of genes and gene pools present in landraces.

The conservation of phylogenetic resources has for some decades been one of the most urgent objectives for the protection of the various landraces established over time by the skilful work of several farmers. Moreover, the enhancement of these morpho-types is related to the identification of genotypes of interest for modern cultivation techniques by adapting to different levels of fertilisation, integral mechanisation, protected cultivation, artificial growing substrates, and industrial transformation, etc.

The landraces perfectly adapted to their traditional cultivation environment, the obsolete commercial cultivars, the lines already used in the breeding work and currently

not used, constitute a heritage of unrepeatable genetic variability, the loss of which cannot be remedied.

The collection, characterization and conservation of genetic resources is therefore of particular importance, especially in the field of home garden plants, for some of which our country is particularly rich in variability.

The plant material can be conserved, in relation to the duration of the reproductive cycle, the gamia system, the possibility of vegetative multiplication, etc., by means of different systems: *in situ* reserves (i.e., in the same location of identification), *ex situ* reserves (in particularly suitable environments, not very anthropized), *ex situ* collections (i.e., in specialised laboratories or structures, called germplasm banks) of seeds, pollen, material propagated in vitro, etc.

A great organisational effort has been carried out internationally, especially by the International Plant Genetics Resources Institute (IPGRI) in Rome, recently called Biodiversity International, which operates within the Consultative Group on International Agricultural Research (CGIAR), while at the national level by the Institute of Germplasm of Bari of the National Research Council and by various germplasm banks of more modest size spread throughout the national territory. In the latter case, work has often been concentrated on groups of vegetable species at various research sites to maintain traditional gene pools.

The duration of the vitality of the stored materials depends on the longevity of the species, the physiological conditions of the same before the start of conservation and the environment in which they are stored.

This line of activity is part of this framework, which had the general objective of characterising the bio-morphological traits of the types of leafy cabbage common in Europe to record the existing variability and to identify the most interesting genetic materials to be used in the genetic improvement of the crop.

5.2.1.2. Material and method

The experimental trial started with the sowing of core collection (CC) of the BRESOV projects in November 2018. We took in consideration 129 accessions representing different morphotypes of different *Brassica oleracea* complex species (n=9) provided by several EU gene-banks (Table 5.2.1.2.1.) and (Figures 5.2.1.2.1 to 5.2.1.2.1.7)

In particularly the *Brassica oleracea* complex species (n=9) considered were: *B. oleracea* var. *italica* Plenck, *B. oleracea* var. *botrytis*, *B. oleracea* var. *gongylodes*, *B. oleracea* var. *acephala*, *B. oleracea* var. *gemmifera*, *B. oleracea* var. *capitata*, *B. oleracea* var. *sabauda*, *B. oleracea* var. *tronchuda, B. oleracea* var. *alboglabra*, *Brassica drapanensis*, *Brassica incana*, *Brassica villosa*.

Table 5.2.1.2.1. Accessions list.

Morphotype code	**Accession status**	**Local name**	**Accession code**	**Origin**
BA1	Landrace	Maijo N°1	HRIGRU6225	THA
BH1	Landrace	Cavolo vecchio	UNICT380	Tusa
BH2	Landrace	Cavolo nero	UNICT364	Acireale
BH3	Landrace	Couvè Portuguesa	UNICT375	Portogallo
BH4	Landrace	Rizzo	UNICT3381	Orto Gangi, Reitano Mazzarino
BH5	Landrace	Cavolo da foglia	UNICT4591	Salina
BH6	Landrace	Asa de cantaro	UNICT379	Rocalba, Portogallo
BH7	Landrace	Cavolo da foglia	UNICT4802	
BH8	Landrace	Cavolo da foglia	UNICT4538	ASA
BH9	Landrace	Cavolo da foglia	UNICT4601	Mazzarino
BH10	Landrace	Cavolo verza d'Asti Pasqualino	UNICT 4853	SAIS
BH11	Landrace	Cavolo nero di Toscana	UNICT4448	SAIS
BH12	Landrace	Cavolo d'estate	UNICT353	Lipari
BR1	Landrace	Natalino	UNICT659	Francavilla
BR2	Landrace	Bastardo	UNICT656	Favignana
BR3	Landrace	Christmas purple sprouting	HRIGRU10660	GBR
BR4	Landrace	De Cicco	HRIGRU7432	JPN
BR5	Landrace	Early autumn	HRIGRU8624	GBR

BR6	Landrace	Broccoletti neri	HRIGRU10769	ITA
BR7	Landrace	Purple sprouting early improved	HRIGRU3521	GBR
BR8	Landrace	White sprouting late	HRIGRU3558	GBR
BR9	Landrace	Improved white sprouting	HRIGRU3570	GBR
BR10	Landrace	Broculi Tardio	HRIGRU9963	ESP
BR11	Landrace	MORSES N°4638	HRIGRU8668	USA
BR12	Landrace	Sperlings sparko	HRIGRU8665	DEU
BR13	Landrace	Ramoso calabrese precoce	HRIGRU4710	ITA
BR14	Landrace	Precoce di Sicilia violetto	HRIGRU5256	ITA
BR15	Landrace	Smuzzatura	UNICT660	Santo Stefano di Camastra
BR16	Landrace	Spigariello	UNICT2711	P. Sig. Muccio
BR17	Landrace	Cavolo broccolo	UNICT613	Furnari
BR18	Landrace	Broccoletti/ Sparaceddi	UNICT4286	Contrada Mezzoiudo, Ditta Mineo
BR19	Landrace	Ciurietto	UNICT3122	Modica
BR20	Hybrid F1	Marathon	UNICT4322	Esasem
BR21	Advanced line	Limba	CRI2400004	CZE
BR22	Landrace	Charteuse-Heading broccoli	HRIGRU6702	CAN
BR23	Landrace	Early purple head	HRIGRU6703	CAN
BR24	Landrace	Cavolo cavolina Rizzo	HRIGRU5416	ITA
BR25	Advanced line	Miranda	CRI09H2400006	CZE
BR26	Advanced line	Leonora	CRI09H2400005	CZE
BR27	Landrace	Boccolo apriloto	HRIGRU2405	ITA
BR28	Landrace	Couve broculo roxo	HRIGRU8680	Portogallo
BR29	Landrace	Cavolo broccolo tardivo calabrese	HRIGRU4716	ITA
BR30	Advanced line	Vitamina	CRI09H2400001	CSK
BD1	CWR*	Cavolo di roccia	UNICT4796	Erice
BY1	CWR	Cavolo biancastro	UNICT3513	Agnone Bagni

BY2	CWR	Cavolo biancastro	UNICT4803	Castelmola
BV1	CWR	Cavolo di Bivona	UNICT3944	Marianopoli, C. da Vuzzarella
CC1	Advanced line	Inter	CRI09H1800004	CSK
CC2	Landrace	Vysocke (Frydstejn)	CRI09H1800287	CSK
CC3	Landrace	Boehmerwaldkohl - Sumavske	CRI09H1800283	CSK
CC4	Landrace	Taborske	CRI09H1800276	CSK
CC5	Landrace	Trvanlive D	CRI09H1800275	CSK
CC6	Landrace	Klokotske	CRI09H1800274	CSK
CC7	Landrace	Kvislar	HRIGRU12966	NOR
CC8	Landrace	Blatopp kvithamar	HRIGRU12965	NOR
CC9	Landrace	Cavolo cappuccio nero testa di negro	HRIGRU6215	ITA
CC10	Landrace	Zazriva 367	CRI09H1800144	SVK
CC11	Advanced line	Holt	CRI09H180011	CSK
CC12	Landrace	Zakamenne	CRI09H1800146	SLO
CC13	Landrace	Kalibos	CRI09H1800268	CZE
CC14	Landrace	Cape splits	HRIGRU6690	ZAF
CC15	Landrace	Cavolo da fiore miscuglio	HRIGRU4886	ITA
CC16	Landrace	Langedijker Bewaar Lares RS	HRIGRU5567	NLD
CC17	Landrace	Offenham Sel B G 283-Spring cabbage	HRIGRU2700	FRA
CC18	Landrace	Cavolo cappuccio	UNICT4408	Romania
CC19	Landrace	April spring cabbage	HRIGRU3681	GBR
CC20	Landrace	Tipburn resistant-Winter cabbage	HRIGRU1975	USA
CC21	Landrace	Kantorjanosi cabbage	HRIGRU4320	Ungheria
CC22	Landrace	Yalova 1	HRIGRU12479	TUR
CC23	Landrace	Zelje ljublianskim belo pozno- Pickling cabbage	HRIGRU7826	YUG
CC24	Landrace	Rodynga G S- Red cabbage	HRIGRU12994	DEU
CC25	Landrace	Velicna 1	CRIO9H1800150	SLO

CC26	Landrace	Large flat head-Cabbage	HRIGRU6178	CHN
CC27	Landrace	Cavolo verza violaceo di Verona	UNICT4633	SAIS SEMENTI
CC28	Landrace	Cavolo verza d'Asti Pasqualino	UNICT4854	SAIS SEMENTI
CC29	Advanced line	Pluto	CRI09H1800263	CSK
CC30	Landrace	Dieners fruhrotkohl-Cabbage	HRIGRU5703	DEU
CC31	Landrace	Summer cabbage	HRIGRU5696	TUR
CC32	Landrace	Parnica	CRI09H1800151	SVK
CC33	Landrace	Kralovany	CRI09H1800152	SVK
CC34	Advanced line	Polar	CRI09H1800013	CSK
CC35	Landrace	Turnovske	CRI09H1800277	CSK
CC36	Landrace	Zora	CRI09H1800003	CSK
CC37	Advanced line	Mars	CRI09H1800007	CSK
CC38	Landrace	Dita	CRI09H1800332	CZE
CC39	Landrace	Vysocke krajove (Jenisovice)	CRI09H1800319	CSK
CC40	Landrace	Babin	CRI09H1800148	SVK
CC41	Landrace	Red cabbage	HRIGRU5697	TUR
CC42	Landrace	Dobrovodskè polopozdnì	CRI09H1800008	CSK
CC43	Landrace	Krimicke	CRI09H1800083	CSK
CC44	Landrace	Velicna	CRI09H1800149	SLO
CC45	Landrace	Pourovo cervenè	CRI09H180010	CSK
CK1	Landrace	Giant Jersey kale	HRIGRU6226	GBR
CK2	Landrace	Earliest purple - kohlrabi	HRIGRU6229	GBR
CK3	Landrace	Covo - White flowered kale	HRIGRU4302	ZWE
CK4	Landrace	Stabil- kale	HRIGRU7546	DEU
CK5	Landrace	Berzacas - kale	HRIGRU9932	ESP
CK6	Landrace	Canzon- fodder kale	HRIGRU5140	GBR
CK7	Landrace	Surmoel-kale	HRIGRU6421	USA
CK8	Landrace	Furchenkohl-kale	HRIGRU7547	DEU
CK9	Landrace	Vitessa-curly kale	HRIGRU12992	DEU

CK10	Landrace	Dwarf green curled– Borecole kale	HRIGRU3592	GBR
CK11	Landrace	Couve Penca De Gondomar	HRIGRU9574	Portogallo
CK12	Landrace	Couve Penca Da Povoa – Tronchuda kale	HRIGRU4690	Portogallo
CK13	Landrace	Curly kale	HRIGRU5693	GBR
CK14	Landrace	Scotch kale 1008 – Rape kale	HRIGRU5688	GBR
CK15	Landrace	Thousand head – Fodder kale	HRIGRU3316	GBR
CK16	Landrace	Cavolo palmizio nero di Toscana	HRIGRU4887	ITA
CR16	Landrace	Cavolo rapa Vienna bianco	UNICT4447	SAIS
CR1	Landrace	Cavolo - kohlrabi	HRIGRU5389	ITA
CR2	Landrace	Cavolo rapa gigante violetto	HRIGRU2388	ITA
CR3	Advanced line	Moravia	CRI09H2200005	CSK
CR4	Advanced line	Blankyt	CRI09H2200002	CSK
CR5	Advanced line	Luna	CRI09H2200023	CSK
CR6	Landrace	Cavolo rapa *Cavulu* Settembrino	HRIGRU5428	ITA
CR7	Landrace	Cavolo rapa *Cavulu* Agosto	HRIGRU5427	ITA
CR8	Advanced line	Azur	CRI09H2200001	CSK
CR9	Landrace	Pollux RS	HRIGRU5612	NLD
CR10	Landrace	Cavolo rapa bianco	UNICT4447	Milazzo
CR11	Landrace	Goliath local – Purple kohlrabi	HRIGRU2483	YUG
CR12	Landrace	Cavolo rapa rosso - kohlrabi	HRIGRU5265	ITA
CR13	Landrace	Goliath – Fodder kohlrabi	HRIGRU8723	NZL
CR14	Landrace	King of market-kohlrabi	HRIGRU8620	IND
CR15	Landrace	Purple top- kohlrabi	HRIGRU8916	GBR
CV1	Landrace	Di Sicilia Violetto	HRIGRU5431	ITA

CV2	Landrace	April prince- Winter cauliflower	HRIGRU2840	GBR
CV3	Landrace	EMU – Autumn cauliflower	HRIGRU6253	AUS
CV4	Advanced line	Octavian	CRI09H2300023	CZE
CV5	Advanced line	Gameta	CRI09H2300022	CZE
CV6	Advanced line	Delta	CRI09H2300021	CZE
CV7	Advanced line	Beta	CRI09H2300020	CZE
CV8	Landrace	Verde romano precoce	UNICT568	Zorzi
CV9	Landrace	Cavolfiore	UNICT4855	Bavicchi sementi
CV10	Landrace	Graffiti violetto	UNICT3879	ISI- sementi
CV11	Landrace	Algromayo N° 2	HRIGRU2838	NLD
CV12	Landrace	Febbrarese Napoletano	HRIGRU4812	ITA
CV13	Landrace	Mishmar Ha'Emek 314	HRIGRU9383	ISR
CV14	Landrace	Adlerskaja Ziemniaja-Cauliflower	HRIGRU7521	SUN
CV15	Landrace	Beladi - Cauliflower	HRIGRU7367	SYR
CV16	Landrace	Hagar - Cauliflower	HRIGRU8263	ISR
CV17	Landrace	Summer wonder - Cauliflower	HRIGRU2894	NLD
CV18	Landrace	Frialora	UNICT4451	Ragusa-Aurnia

*CWR indicates *Crop Wild Relatives.*

KALE

Figure 5.2.1.2.1 Accessions selected Brassica olearacea var. acephala: A (BH1 cavolo Vecchio), B (BH2 cavolo nero), C (BH4 RIZZO), D (BH7 Cavolo da foglia), E (BH9 Cavolo da foglia di Mazzarino), F (BH5 Cavolo da foglia Salina), G (BH6 Asa de cantaro), H (BH11 Cavolo nero di Toscana), I (BH3 Couve' Portuguesa), J (BH12 Cavolo d'estate), K (BH10 cavolo d'Asti Pasqualino), L (BH8 Cavolo da foglia).

BROCCOLI

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Figure 5.2.1.2.2 Accessions selected of brassica oleracea var. italica: A (BR4 De Cicco), B (BR1 Natalino),C (BR5 Early Autumn), D (BR Purple sprouting early improved), E (BR2 Bastardo), F (BR8 white sprouting late), G (BR3 Chriatmas purple sprouting), H (BR18 Broccoletti /sparaceddi), I (BR20 Marathon),J (BR29 Cavolo broccolo tardivo calabrese), K (BR28 Couve broculo roxo), L (BR27 Broccolo apriloto), M (BR24 Cavolo cavolina), N (BR22 Charteuse Heading broccoli), O (BR16 Spigarello) , P (BR6 broccoletti neri), Q (BR 12 Sperlings sparko), R (BR10 Brocili tardio), S (BR11 Morses n.4638), T (BR9 Improved white sprouting) ,U (BR14 precoce di Sicilia) ,V (BR15 Smuzzatura) ,W (BR17 Cavolo Broccolo), X (BR 19 Ciurietto),Y(BR13 Ramoso Calabrese), Z (BR21 Limba), AA (BR23 Early head).

WILD SPECIES

A

B

C

Figure 5.2.1.2.2 3 Selected accessions of CWR: A (BD1 cavolo di roccia), B (BV1 Cavolo di Bivona), C (BY1 cavolo biancastro).

CABBAGES

Figure 5.2.1.2.2.4. Selected accession of Brassica o. var. capitata: A (CC9 Cavolo Cappuccio nero testa di negro),B (CC18 Cavolo Cappuccio) C ,(CC 19 april spring cabbage) D ,(CC21 Kantorjanosi cabbage), E (CC 25 Veliena 1) F (CC26 Large flat head-cabbage), G (CC 31 summer cabbage),H (CC27 Cavolo verza violaceo di verona), I (CC41 Red cabbage), J (CC15 cavolo da fiore),K (CC28 Cavolo verza d'asti di pasqualino), L (CC44 Velicna), M (CC40 Babib), N (CC38 Bita),O (CC37 Mars), P (CC34 Polar), Q

(CC32 Parnica), R (CC36 Zora) ,S (CC35 Turnovske), T (CC43 Krimicke),U(CC1 Inter),V (CC4 Taborske),W (CC7 Kvislar),Y(CC11 Holt) ,X(CC20 Tipburn resistant winter-cabbage).

KOLHRABI

Figure 5.2.1.2.2.5. Selected accessions of kohlrabi: A (CR16 cavolo rapa Vienna bianco), B (CR1 Cavolo Kolhrabi), C (CR15 Purple kohlrabi), D (CR10 Cavolo rapa bianco), E (CR3 Moravia), F (CR5 Luna),G (CR11 Goliath local purple kohlrabi), H (CR4 Blankyt), I (CR6 Cavulu settembrino), J (CR8 Azur), K(CR14 King of the market),L (CR2 Cavolo gigante violetto).

CAULIFLOWERS

Figure 5.2.1.2.2.6. Accessions selected Brassica oleracea var. botrytis: A (CV4 Octavian),B (CV17 summer wonder),C (CV9 Cavolfiore Bavicchi),D (CV12 Febbrarese Napoletano, E (CV8 Verde romano precoce),F (CV18 Friarola),G (CV15 Beladi),H (CV16 Hagar), I (CV1 violetto di Sicilia), J (CV 14 Ziemnaiaja), K (CV11 Algromayo), L (CV5 Gameta) ,M (CV10 Graffiti violetto),O (CV6 Delta).

The seeds were sown in cellular trays in a cold greenhouse under natural light (4.6 to 9.2 MJ.m-²d-²) and temperature (15.4 ± 5.8 ±C°) conditions, from October to December 2018 in IAS (Istituto Agrario Sperimentale) a (South Italy, 37°31010" N 15°04018" E; 105 m above sea level (m a. s. l.) Catania CT) using organic growing practices, utilised substrate Brill® semina bio (Geotech, Italy) to fill the cellular trays with 117 holes (13x9) and treated them once by BTK ® 32 WG (Xeda, Italy) based on *Bacillus thuringiensis* sub. Kurstaki for controlling *Pieris brassicae*. Some treatments have been carried out with products allowed in organic farming to grow plants based on macro minerals and microelements such as copper and nitrogen and iron. In addition, products such as pyrethroids have been used to control pest disease. All the products were provided by ITAKA CROP SOLUTIONS, a partner of the project. The plants were transplanted on

27 December 2018 in a cold greenhouse (36°51'13.3" N 14°29'32.0" E – Contrada Randello, Ragusa) (Figure 5.2.1.2.7.).

For each accession three plants were transplanted and analysed. During the first two weeks from transplanting the plants were irrigated reintegrating the 100 % ETc and till the end of the growing cycle 100% (Figure 5.2.1.2.2.).

The data were registered of the plant phenological phases, inflorescence appearance and of the full flowering, and the main morphological descriptors for brassica (IBPGR and UPOV) (Table 5.2.1.2.2.). After 95 days from transplanting, we scored (0 = high number of wilt leaves; 1= medium number of wilt leaves; 2= no withered and wilted leaves) to select the accessions resistant and sensible.

The plants of the selected resistant accessions were eradicated at the harvesting stage of each crop, and for kale and wild crop relatives at the inflorescence appearance, and we registered the fresh and dry weight of the epigeous and hypogeous portion of the plant, plant height, basal stem diameter, and also the roots were analysed using shavelomics board, number and fresh/dry weight of the leaves, and the fresh and dry weight of the produce (broccoli and cauliflower curd, kohlrabi stem, cabbage and savoy cabbage head, leaves for kale and wild brassica relatives) and the first trial finished at the end of June 2019.

The Leaf and the edible portion of the selected accessions were stored at -80 C° for further analysis for biochemical traits such as glucosinolates.

After all the data obtain were elaborated by a software IBM (SPSS) for the correlation phenotyping.

Figure 5.2.1.2.7. Brassica experimental field.

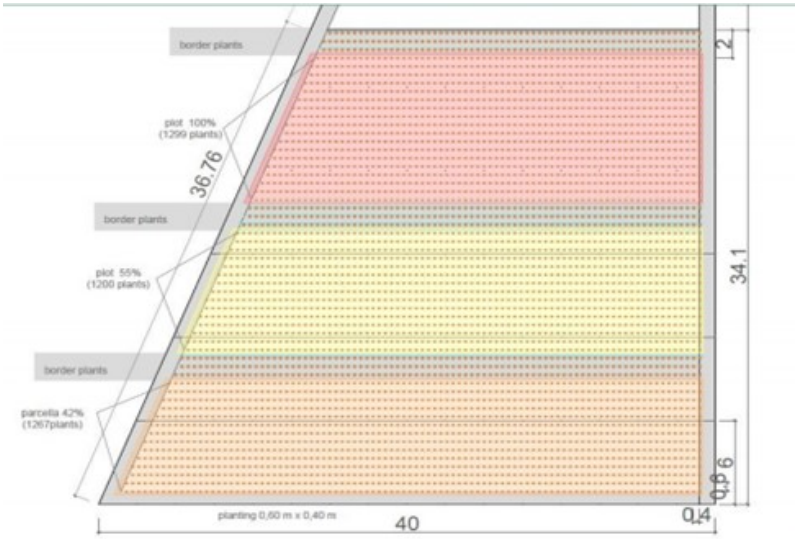

Figure 5.2.1.2.8. Greenhouse map.

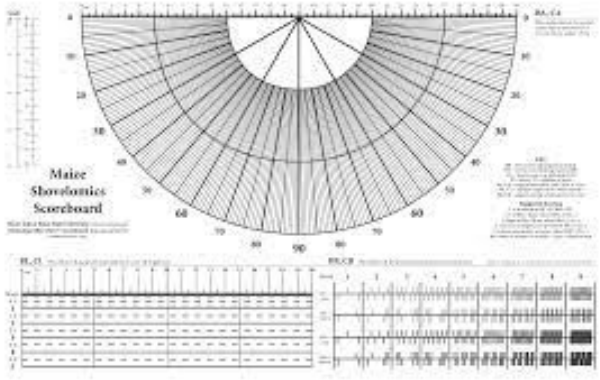

Figure 5.2.1.2.9. Shovelomics scoreboard

Table 5.2.1.2.2. Morphological descriptors and corresponding scores.

Index	**Descriptors**
IA	Inflorescence appereance
PB	Branches in the plant (0-7)
PS	Plant shape (1-5)
HH	Plant grown habit (1-9)
NL	Number leaves per plant main stem (n)
SL	Vegetative stem length (cm)
LA	Leaf area (cm^2)
LL	Leaf length (cm)

LW	Leaf width (cm)
RLA	Petiole length (cm)
RRA	Petiole width (cm)
LA	Root left angle
LAN	Root right angle
RD	Basal root diameter (mm)
MRD	Main root diameter (cm)
MRL	Main root length (cm)
LR	Lateral root diameter (cm)
RA	Root area (cm^2)
RW	Root weight (g)
DM	Dry matter (%)

5.2.1.3. Result and discussion

Principal component analysis (PCA): It is a multivariate analysis allowing to convert the starting variables corresponding to molecular markers or morphological variables into synthetic variables or axes.

The PCA provides a matrix of coordinates and degrees of contribution between the variables and the axes and thus makes it possible to define the markers that best contribute to the description of the variability and to obtain a graphic representation of the distribution of individuals or populations.

According to their similarities in the planes generated by the axes of the PCA taken 2 to 2. Thus, PCA can provide a structure based on a minimum level of genetic differentiation dependent on the number of markers incriminated and the individuals considered.

Kale

Principal Component Analysis (PCA) was performed for Kale (BH) accessions based on the first three axes PC1, PC2 and PC3 which absorb more variability. The first axis absorbs 25.8% of the total variation while the second absorbs 17.71% while the third axis absorbs 16.76% of the total variability (Table 5.2.1.3.1.).

According to the matrices provided, the first axis 1 is strongly correlated with the variables SL, LL, PL, PW, RLA and RRA.

On the other hand, axis 2 is strongly correlated with the parameters BRD, MRL, LRD, RA and negatively with LW. While the third axis is strongly correlated with the variables IA, NL and negatively with LA and RDM (Table 5.2.1.3.2).

The results of the PCA based on these morphological variables and the dispersion in three axes show the formation of five groupings, the first group A with the BH2 accession, the 2nd group B with the BH8 accession, noting that these two accessions are divergent compared to other accessions. Group C contains the 2 accessions BH7 and BH9, group D contains the 4 accessions BH10, BH12, BH5 and BH1, indeed the last group E contains the rest of the accessions.

The two groups A and B are positively correlated with the three axes PC1, PC2 and PC3. The two groups C and D are positively correlated with the axis PC1 and negatively with the two axes PC2 and PC3. While the group E is correlated negatively with the three axes PC1, PC2 and PC3 (Figure 5.2.1.3.1.).

This distribution leads to the conclusion that the variables RA and RW contribute to the definition of group A, while the variables MRL and BRD together contribute to the definition of group B. Group C is defined by the variables PW, RRA and SL. group D is defined by the variables MRD, PB, RDM and LW. Indeed, the last group E is defined by the rest of the variables by IA.

The varietal dendrogram dispersion of the BH accessions revealed a particular profile, essentially breaking down into two large distinct groups without overlapping. The first large group A is made up of two subgroups: on the one hand, A-1 encloses the accessions BH3, BH6 and BH12, and on the other hand, A-2 with the accessions BH4, BH10, BH7 and BH11.

While the second large group B is made up of the rest of the accessions, also breaking down into two sub-groups, on the one hand B-1 who groups the accessions BH1, BH5, BH8 and BH2, and on the other hand, B-2 with the accession BH9 which diverges from the other accessions.

This group (B) is characterized by accessions which have important values concerning the length and width of leaves and roots, especially the BH9 accession which explains their divergence from other accessions (Figure 5.2.1.3.1).

Table 5.2.1.3.1. – Percentage of variance of the main components identified by factor analysis among the kale accessions evaluated.

Components	% Of variance	% Cumulative
PC1	25,807	25,807
PC2	17,714	43,521
PC3	16,762	60,283
PC4	12,232	72,514

Table 5.2.1.3.2. – Correlation coefficient of the individual parameters with the main components among the kale accessions evaluated.

Parameters	Components		
	PC1	PC2	PC3
IA	-,148	-,187	,737
PB	,060	,049	-,092

NL	,013	,125	,812
SL	,786	-,245	,133
LA	,249	,128	-,435
LL	,535	-,349	-,223
LW	-,026	-,392	,081
PL	,537	-,013	,291
PW	,815	-,038	-,441
RLA	,841	,210	,179
RRA	,767	-,125	-,109
BRD	,376	,697	,272
MRD	,217	,356	,321
MRL	-,148	,458	-,475
LRD	-,337	,274	,222
RA	-,236	,920	-,066
RW	-,137	,936	-,137
RDM	-,582	-,106	-,657

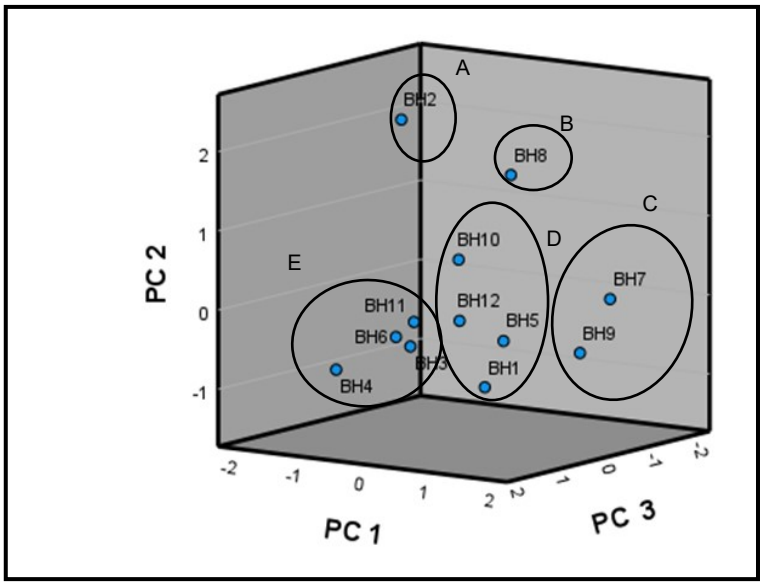

Figure 5.2.1.3.1. – Distribution of materials studied in the space described by the first three components among the kale accessions evaluated.

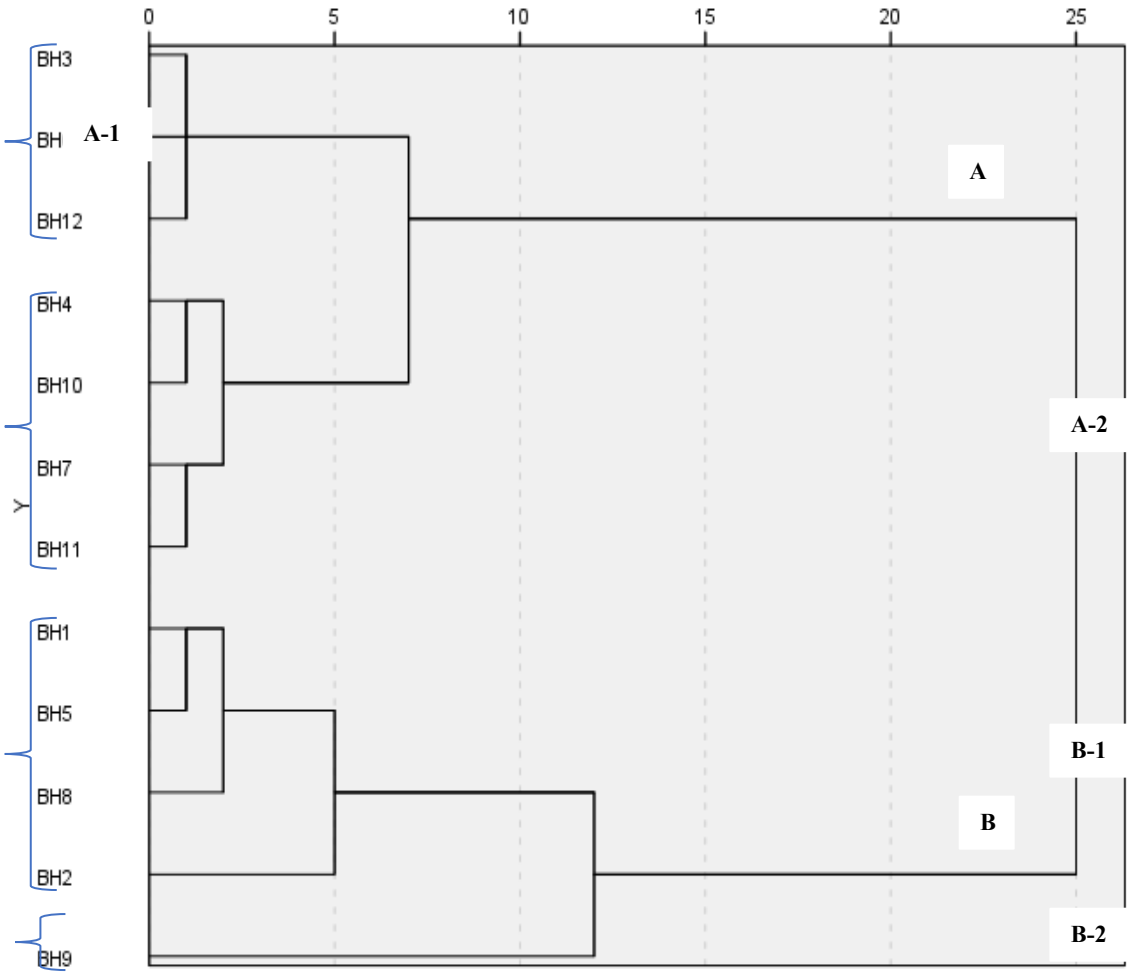

Figure 5.2.1.3.2. - Cluster classification of analysed among the kale accessions evaluated.

Broccoli

The principal component analysis was carried out for *Broccoli* (BR) accessions, the results show that the first three axes 1, 2 and 3 absorb more of the total variability (52.6%), the first axis 1 absorbs 25%, the second axis 2 absorbs 16% while the third axis 3 absorbs 11.6% of the total variability.

The parameters LL, PL, BRD, RA, RW and RDM contribute the most for the definition of axis 1, while the variables PB, PS, NL, SL, LA, and RLA contribute to the definition of second axis 2, while the variables IA, MRD, MRL and LRD contribute to the definition of axis 3 (Table 5.2.1.3.3.).

The results of the PCA based on these morphological variables and the dispersion in three axes show the formation of four groupings, the first group A groups together the accessions BR7, BR6, BR15, BR11 and BR27. The 2nd group B contains the accessions BH8, BR9, BR16, BR10, BR3 and BR14. Group C contains accessions BR1, BR2, BR12, BR13 and BR19. Group D contains the rest of the accessions (Table 5.2.1.3.4).

Group A positively correlated with axis 2, and negatively with axes 1 and 3. Group B is positively correlated with axis 1 and 3 and negatively with axis 2. Group C is positively correlated with axis 1 and negatively with axes 2 and 3. While group D is negatively correlated with the three axes 1, 2 and 3 (Figure 5.2.1.3.3).

This distribution allows us to conclude that the variable SL contributes to the definition of group A, while the variables NL and PS contribute together to the definition of group B. Group C is defined by the variables BRD, RA, RLA and RW. On the other hand, group D is defined mainly by the variables RDM and IA.

The hierarchical classification based on these different variables shows the formation of four groups, the first group A is subdivided into two subgroups: A-1 (BR2, BR18, BR11, BR21) and A-2 (BR1, BR15, BR7, BR29 and BR25), this group is characterised mainly by morphotypes with late appearance of inflorescences compared to other accessions. The second group B is made up of the two accessions BR6 and BR10, these two accessions are characterised by an important stem length (SL) and larger leaves (LA) compared to the other accessions.

The third group C is made up of two subgroups: on the one hand C-2 with the accessions BR22 and BR24 and on the other hand C-1 with the accessions BR12, BR14, BR4, BR28 and BR30. These accessions are characterised by a low total number of leaves compared to the rest of the accessions.

While the fourth group D is also made up of two subgroups when D-2 groups together the two accessions BH19 and BH20 and the other subgroup D-1 groups the rest of the accessions. This group contains accessions with a short stem (SL) and lightweight roots (RW, RDM) compared to other accessions (Figure 5.2.1.3.4).

Table 5.2.1.3.3. – Percentage of variance of the main components identified by factor analysis among the broccoli accessions characterized.

Components	% Variance	% Cumulative
PC1	24,910	24,910
PC2	15,964	40,874
PC3	11,604	52,479
PC4	9,533	62,012
PC5	7,361	69,372
PC6	6,968	76,340

Table 5.2.1.3.4. – Correlation coefficient of the individual parameters with the main components among the broccoli accessions characterized.

Parameters	Components		
	PC1	PC2	PC3
IA	-,175	-,163	,596
PB	,029	,155	,047
PS	,197	,568	-,024
NL	,282	,674	-,292
SL	-,046	,771	-,074
LA	,022	,416	,261
LL	,534	-,096	-,247
LW	,082	-,184	,079
PL	-,408	,273	-,031
PW	-,123	,139	-,149
RLA	,039	-,781	,135

RRA	-,019	-,316	,070
BRD	,847	-,054	-,004
MRD	,483	-,188	,722
MRL	,262	-,058	,709
LRD	,523	-,004	,758
RA	,796	,188	,322
RW	,933	,066	,170
RDM	-,756	-,111	-,103

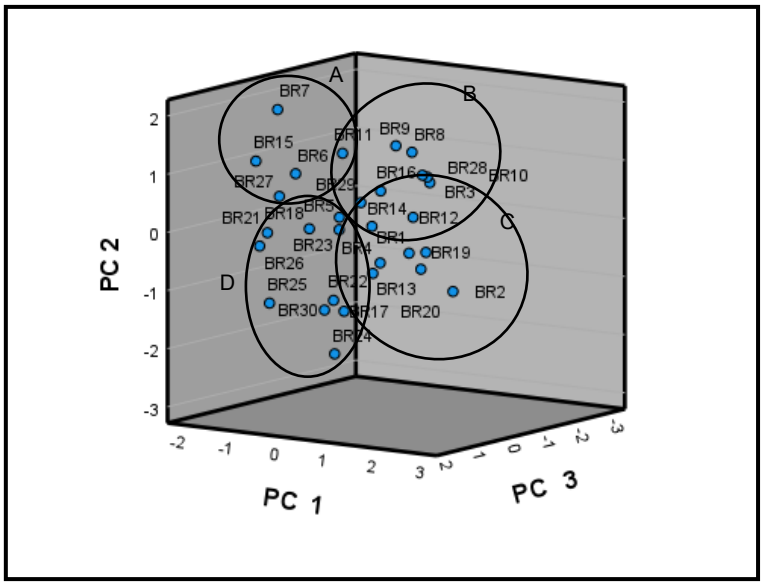

Figure 5.2.1.3.3. – Distribution of studied materials in the space described by the first three components among the broccoli accessions characterized.

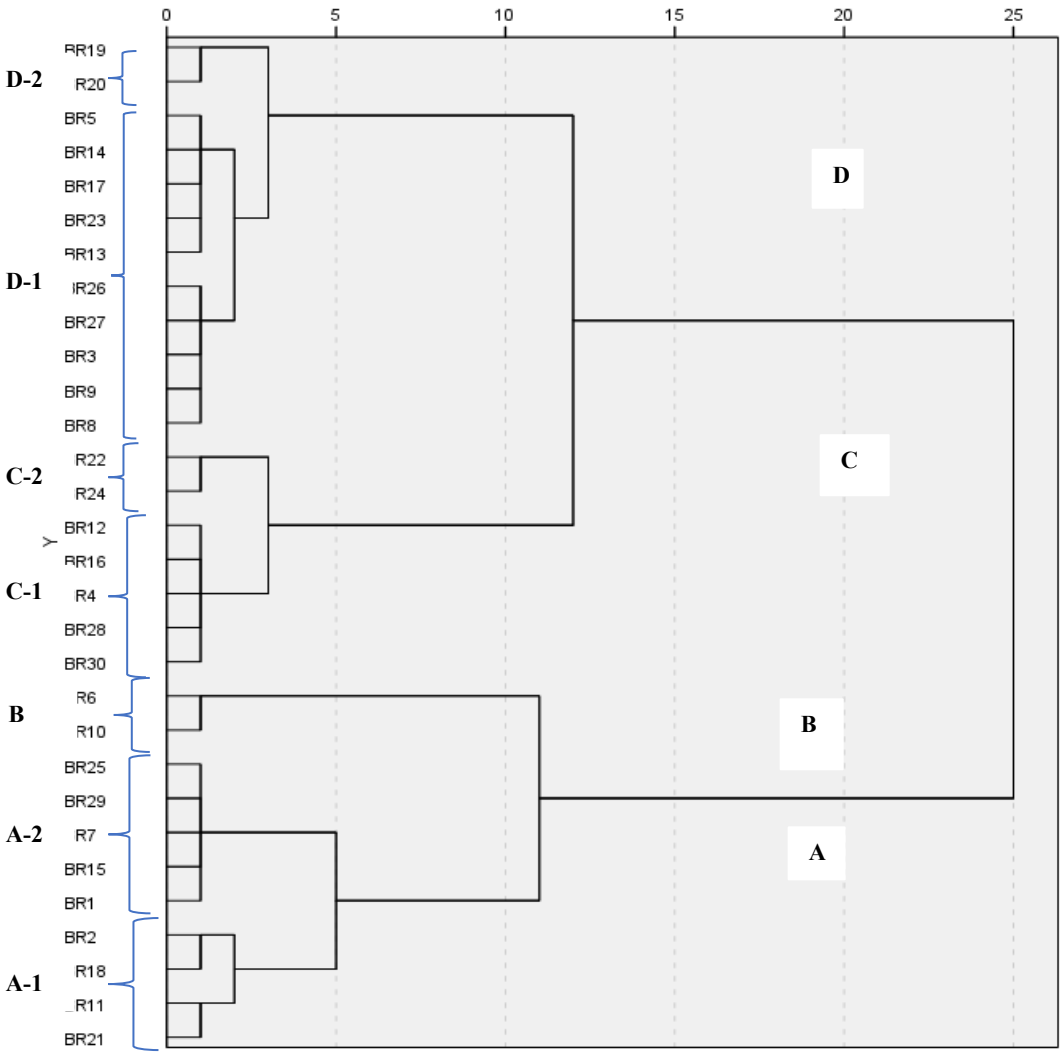

Figure 5.2.1.3.4. - Cluster classification of analysed materials among the broccoli accessions characterized.

Brassica crop relatives

Principal Component Analysis (PCA) was conducted for Brassica crop wild accessions (BD, BI1, BI2, BV) based on three axes1.2 and 3. The first axis absorbs 43.39% of the total variation while the second absorbs 32.55% and the third axis absorbs 24.04% of the total variability (Table 5.2.1.3.5.).

According to the matrices provided we can say that the first axis 1 is strongly correlated with the parameters LA, LW, LL, NL, LRD and BRD.

On the other hand, axis 2 is strongly correlated with the parameters SL, RA, RW and RDM. While the third axis is strongly correlated with the variables PB, PS, RLA, RRA, MRD, MRL, PL and PW (Table 5.2.1.3.6).

The results of the PCA based on these morphological variables and the dispersion in three axes show the dispersion and the divergence of these 4 accessions, the first group A contains the accession BV. The 2nd group B contains the accession BD. Group C contains accession BI1. While group D contains accession BI2.

The two groups A and B are positively correlated with the three axes 1, 2 and 3. However, group D is negatively correlated with these last three axes. Regarding group C,

it is positively correlated with both axes 1 and 3 and negatively with axis 2 (Figure 5.2.1.3.5).

This distribution leads to the conclusion that the variable RLA contributes to the definition of group A, while the variables LRD and NL contribute together to the definition of group B. Group C is defined by the variables MRL, PL and PW. However, group D is mainly defined by the PS variables.

The varietal dendrogram dispersion of CRWs accessions revealed a particular profile breaking down essentially into two distinct groups without overlap. The first group A is composed by the two accessions BI2 and BV which are characterised by broad and long leaves (significant values of LL, LA and LW), while the second group B is composed by the accessions BI1 and BD which are rather characterised by a high level of RLA (roots left angle) (Figure 5.2.1.3.6).

Table 5.2.1.3.5. – Percentage of variance of the main components identified by factor analysis among the *Brassica* wild relative populations characterised.

Components	% Variance	% Cumulative
PC1	43,394	43,394
PC2	32,558	75,953
PC3	24,047	100,000

Table 5.2.1.3.6. – Correlation coefficient of the individual parameters with the main components.

Parameters	Components		
	PC1	PC2	PC3
PB	,548	-,092	,832
PS	-,325	-,381	,866
NL	,915	,374	,150
SL	,067	,994	,084
LA	,661	-,517	-,543
LL	,979	-,059	,195
LW	,992	,123	-,034
PL	,189	-,938	,289
PW	,267	-,909	-,319

RLA	-,347	,362	,865
RRA	,303	,344	,889
BRD	,863	,422	,277
MRD	,548	-,092	,832
MRL	,619	-,472	,628
LRD	,999	-,003	-,044
RA	,194	,971	,138
RW	,489	,870	-,059
RDM	,467	,871	-,153

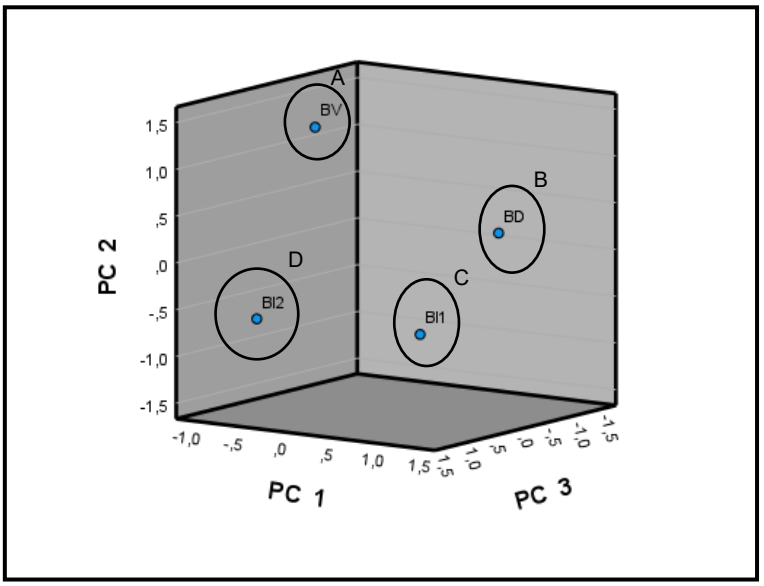

Figure 5.2.1.3.5. – Distribution of studied materials in the space described by the first three components among the Brassica wild relative populations characterized.

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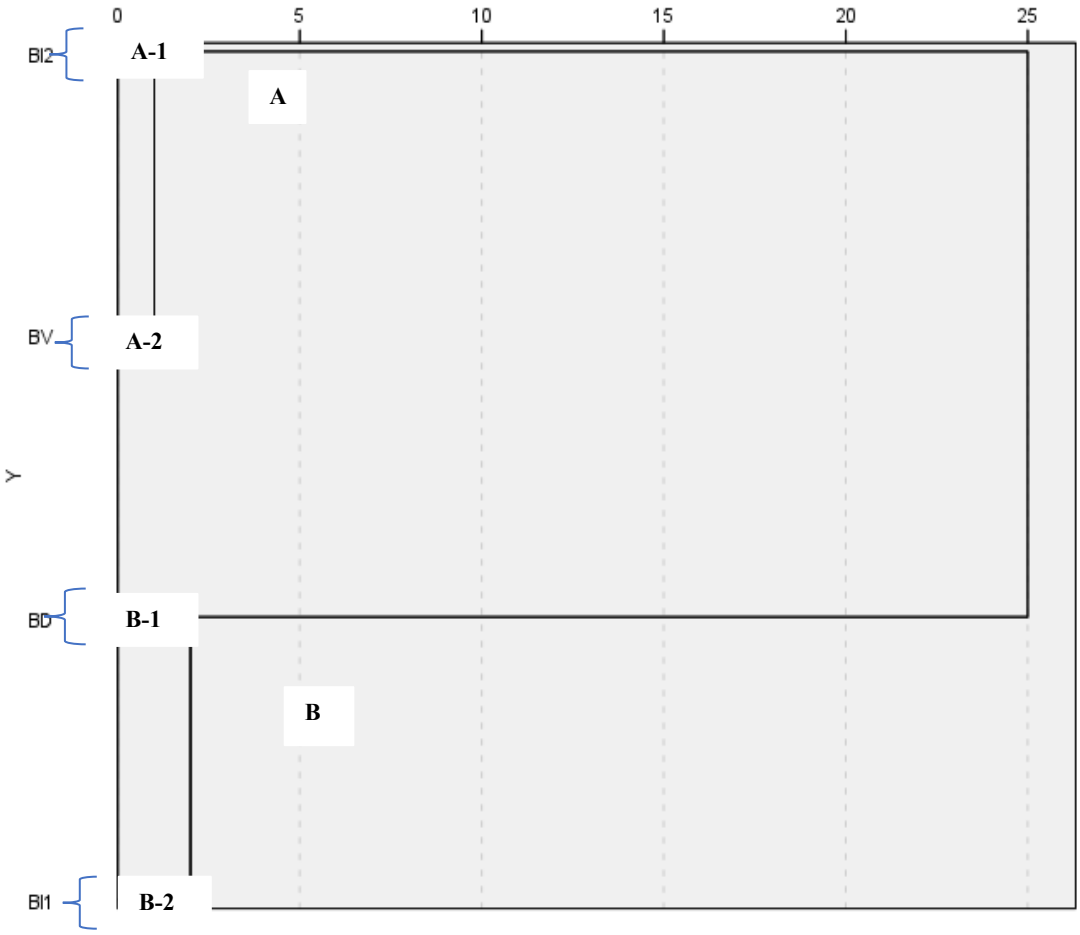


Figure 5.2.1.3.6. - Cluster classification among the Brassica wild relative populations characterized.

Cabbage

The principal component analysis was also carried out for Cabbage (CC) accessions, the results show that the first three axes 1, 2 and 3 together absorb 47.76% of the total variability, the first axis 1 absorbs 23.29%, the second axis 2 absorbs 14.25% while the third axis 3 absorbs 9.58% of the total variability (Table 5.2.1.3.7.).

The variables BRD, MRD, MRL LRD and RA contribute the most for the definition of axis 1, while the variables IA, LA, LL and PW contribute to the definition of second axis 2, while the variables PS, NL and SL contribute to the definition of axis 3 (Table 5.2.1.3.8.).

The results of the PCA based on these morphological parameters and the dispersion in three axes show the formation of five groups, the first group A contains the accession CC4. The 2nd group B contains the accessions CC (6,2,17,21). Group D groups the CC accessions (4, 44, 24, 16). Group E contains only the CC20 accession. While group C contains all the rest of the accessions (Figure 5.2.1.3.7).

In this distribution, we note that all these five groups are positively correlated with the three axes 1, 2 and 3. This distribution leads to the conclusion that the variables LL, LA and PW contribute to the definition of group A, while the variable RRA contributes to the definition of group E, which explains the divergence and isolation of these two

CC4 accessions (which has the large values in length and leaf area) and CC20 (which has the large values in right root angles).

Group B is defined by the variables RA, MRD and LRD. However, group D is defined by the two variables PS and SL. Indeed, group C is defined by the rest of the variables studied.

The hierarchical classification based on these different variables shows a continuous variability and the formation of the four groups (A, B, C, D).

Noting the divergence of the fourth group (D) which contains the two accessions CC4 and CC20, this divergence is in fact made because these two accessions are characterized by leaves that are wider in area (LA and LL) for CC4, and RRA for CC20 (Figure 5.2.1.3.7.).

Table 5.2.1.3.7. – Percentage of variance of the main components identified by factor analysis.

Components	% Variance	% Cumulative
PC1	23,929	23,929
PC2	14,251	38,180
PC3	9,587	47,767
PC4	8,729	56,496
PC5	7,549	64,045
PC6	6,014	70,059

Table 5.2.1.3.8. – Correlation coefficient of the individual parameters with the main components.

Parameters	Components		
	PC1	PC2	PC3
IA	-,155	,279	-,237
PS	-,181	-,121	,615
NL	,124	-,060	,806
SL	,011	-,120	,616
LA	,125	,848	-,147
LL	,049	,876	,004
LW	,147	,021	,116
PL	,005	,083	,215
PW	-,145	,714	-,107
RLA	,085	,163	,330
RRA	-,364	,120	-,043
BRD	,832	,052	,135
MRD	,865	-,031	,119
MRL	,692	-,105	-,020
LRD	,755	-,190	-,216
RA	,872	,104	-,117
RW	,810	,228	,016
RDM	-,129	-,078	,059

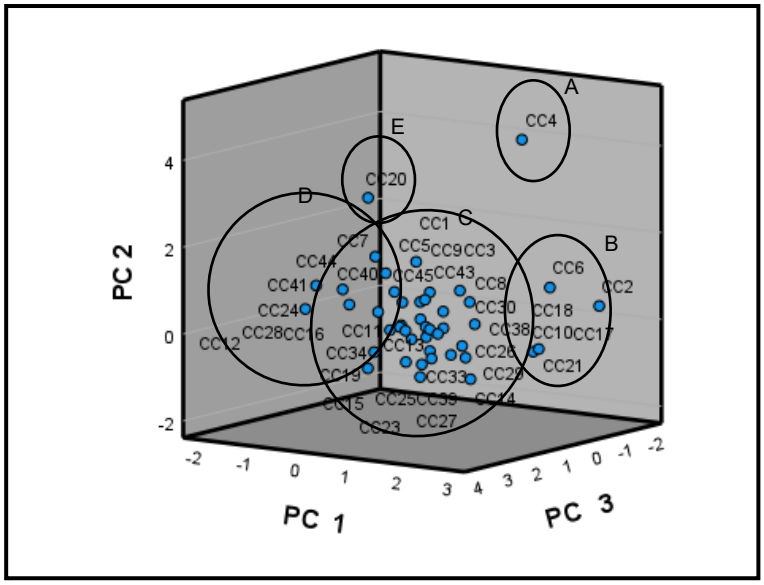


Figure 5.2.1.3.7. – Distribution of Cabbage accessions studied in the space described by the first three components.

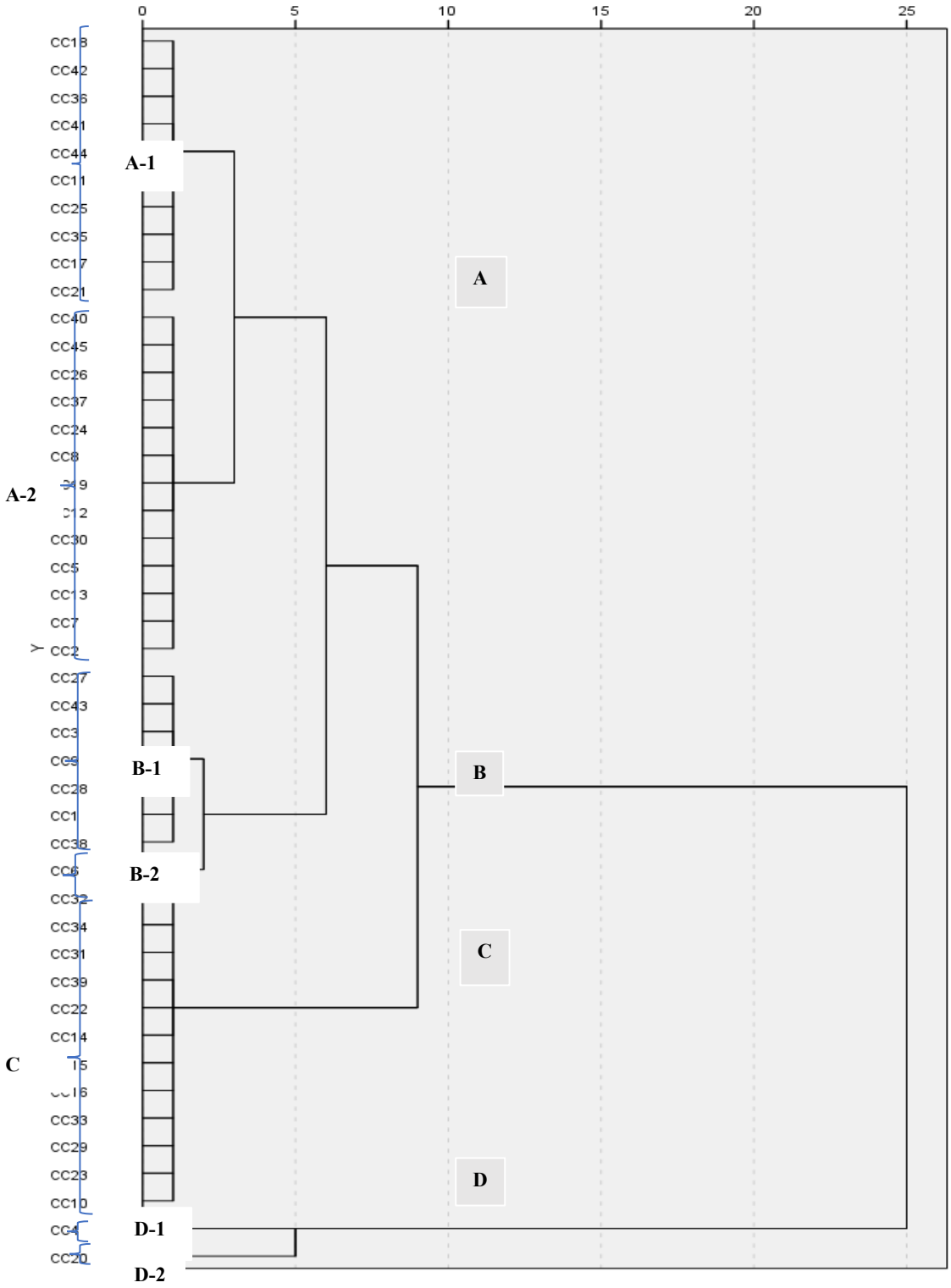


Figure 5.2.1.3.8. - Cluster classification of Cabbage accessions analysed.

Chinese Kale

The principal component analysis was carried out in the same way for the Chinese kale (CK) accessions, the results show that the first three axes 1,2 and 3 together absorb 58.11% of the total variability, the first axis PC1 absorbs 30.26%, the second axis PC2 absorbs 16.14% while the third axis PC3 absorbs 11.71% of the total variability (Table 5.2.1.3.9.).

The results show that the variables PS, MRD, MRL, LRD, RA and RDM contribute the most to define axis 1, while the variables SL and RW contribute to the definition of second axis 2, while the variables RLA, LA and IA contribute to the definition of axis 3 (Table 5.2.1.3.10.).

The results of dispersion of the ACP in 3D based on the morphological variables studied show a great variability with the formation of four groups, the first group A contains the accessions CK (1,3,11,16,7,10), this group is indeed positively correlated with the two axes PC1 and PC2, and negatively correlated with the PC3 axis. Group B contains the accessions CK (14,2,5,9,13,6) and group C contains the accession CK12. These two groups B and C are negatively correlated with the PC2 axis and positive with the PC1 and PC3 axes. On the other hand, group D contains the rest of the CK accessions (4,15,8), this group is positively correlated with the two axes PC1 and PC3 and partially positive with the axis PC2 (Figure 5.2.1.3.9).

In this analysis, we notice a great intra-population diversity, with a great dispersion and divergence of the accessions (CK4, CK15, CK8) on the one hand and the CK12 accession on the other hand. This is explained by the fact that these accessions have specific characters compared to other accessions which affects their levels of variability.

Indeed, the variables LA, RLA and RDM contribute to the definition of group D, while the variables LL, PL and LW contribute to the definition of group C. on the other hand the two other groups are defined by the rest of the variables whose group A is defined mainly by the variables SL, RW and LRD, while group B is defined mainly by the variables IA and MRL.

The hierarchical classification based on these different parameters shows a continuous type of variability with the formation of four large groups. The first group A contains three accessions CK2, CK5 and CK15, this group contains accessions with important values in (SL, LA, LL, LW, PL, PW). The second group B is subdivided into two subgroups; the first subgroup B-1 contains the accessions CK12 and CK14, while the second subgroup B-2 contains the accessions CK1 and CK4, this group (B) contains accessions with important values in (RLA, RRA, BRD, MRD, MRL, LRD, RA, RW). While the third group C contains the accessions with low values especially in LL and LA, this group also subdivided into two subgroups, the first C-1 contains the accessions CK8 and CK13, while the second contains the accessions CK6 and CK16. In fact, the last group D contains the rest of accessions, it is subdivided in two subgroups, the first one D-1 groups CK3 and CK10, while the second one D-2 groups CK7, CK9 and CK11.

We conclude that the results of the PCA and the hierarchical classification are in correspondence with each other and with our morphological data (Figure 5.2.1.3.10).

Table 5.2.1.3.9. – Percentage of variance of the main components identified by factor analysis.

Components	% Variance	% Cumulative
PC1	30,260	30,260
PC2	16,141	46,400
PC3	11,715	58,116
PC4	8,675	66,791
PC5	6,983	73,774

Table 5.2.1.3.10. – Correlation coefficient of the individual parameters with the main components.

Parameters	Components		
	PC1	PC2	PC3
IA	,301	,412	,581
PB	-,106	-,110	,126
PS	,647	-,037	,092
NL	-,122	-,082	-,287
SL	,010	,860	,023
LA	,057	,087	,925
LL	,012	-,220	,247
LW	,212	,025	,083
PL	-,067	-,128	-,349
PW	-,191	,176	-,311
RLA	,175	-,118	,599
RRA	,138	,096	,092
BRD	,445	,373	-,537
MRD	,938	-,067	,115
MRL	,601	,334	,263
LRD	,925	,132	-,038
RA	,677	,612	,005
RW	,612	,704	,104
RDM	-,627	-,449	-,015

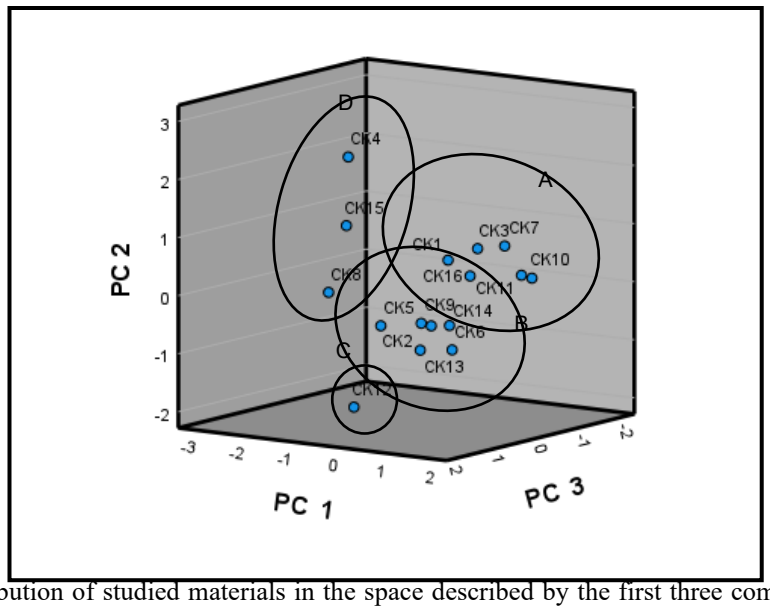


Figure 5.2.1.3.9. – Distribution of studied materials in the space described by the first three components among Chinese kale accessions characterized.

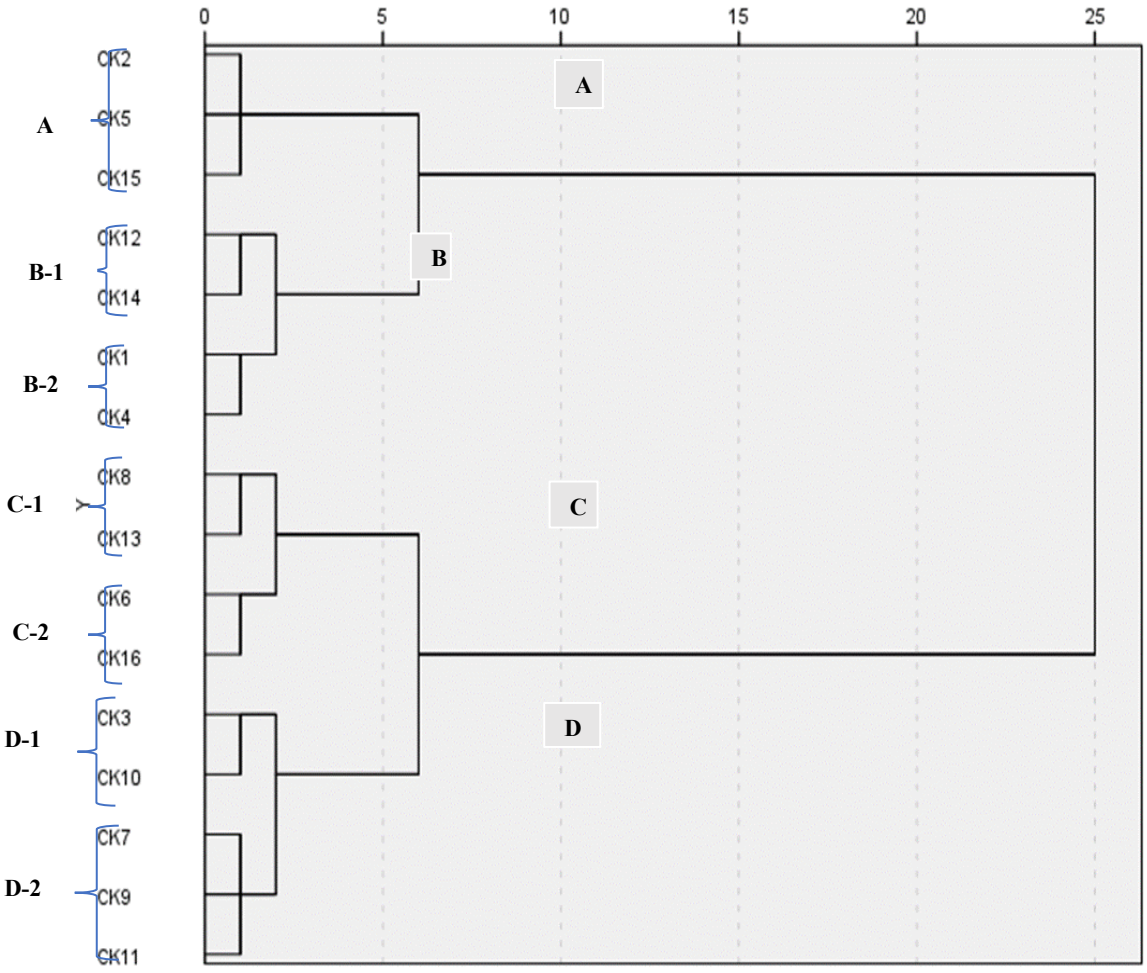


Figure 5.2.1.3.10. - Cluster classification among Chinese kale accessions characterized.

Kohlrabi

The principal component analysis was carried out in the same way for the kohlrabi (CR) accessions, the results show that the first three axes PC1, PC2 and PC3 together absorb 63% of the total variability, the first axis PC1 absorbs 35%, the second axis PC2 absorbs 16.37% while the third axis PC3 absorbs 11.6% of the total variability (Table 5.2.1.3.11.).

The results show that the variables BRD, MRD, MRL, LRD, RA and RW contribute the most to define axis PC1, while the variables LA, LL and PW contribute to the definition of second axis PC2, while the variables NL, SL and LW contribute to the definition of axis PC3 (Table 5.2.1.3.12.).

The dispersion results of the 3D PCA based on the morphological variables studied show a great variability with the formation of four groups, the first group A contains the accessions CR (1,9,16).

The second group B contains the accession CR15, these last two groups are positively correlated with the two PC1 and PC3 axes, and negatively correlated with the PC2 axis. Group C contains the accessions CR (6,10,11,12,13,14) negatively correlated with the PC2 and PC3 axes and positive with the PC1 axes. On the other hand, group D contains the rest of the accessions CR (2,8,3,5,4,7), this group is positively correlated with the three axes PC1, PC2 and PC3 (Figure 5.2.1.3.11).

In this analysis, we notice a great intra-population diversity, with a great dispersion and divergence of the accessions (CR9, CR1, CR16) on the one hand and the CR15 accession on the other hand.

This is explained by the fact that these accessions have specific characters compared to other accessions which affects their levels of variability. Indeed, the variables MRL, LRD and LW contribute to the definition of group A, while the variable BRD contributes to the definition of group B. on the other hand the two other groups are defined by the rest of the variables including group C is defined mainly by the variables NL, IA, and SL while group D is defined mainly by the variables RLA and LL. This explains this level of diversity within the selected CR accessions.

The hierarchical classification based on these different parameters shows a high variability with the formation of four large groups. The first group A is subdivided in two subgroups, the first one A-1 contains the accessions CR (9,10,13,14,8), while the second one A-2 groups the accessions CR (11,12).

The third group C contains the two accessions CR (4,5). We note the most remarkable grouping D between the accessions (CR7, CR2) and (CR8, CR3), these accessions which have the most important values in most parameters such as LA, LL, LW, PL, PW, RLA, RRA, BRD, MRD, MRL, LRD, RA (Figure 5.2.1.3.13.).

Table 5.2.1.3.11. – Percentage of variance of the main components identified by factor analysis.

Components	% Variance	% Cumulative
PC1	34,999	34,999
PC2	16,378	51,378
PC3	11,600	62,977
PC4	10,503	73,481

Table 5.2.1.3.12. – Correlation coefficient of the individual parameters with the main components.

Parameters	Components		
	PC1	PC2	PC3
IA	-,144	,065	-,182
PB	,055	,198	,049
PS	,082	-,050	,384
NL	,398	-,066	,709
SL	,171	,163	,789
LA	-,330	,888	,076
LL	-,298	,846	,290
LW	,382	-,053	-,680

PL	,123	,209	-,065
PW	-,132	,738	-,386
RLA	-,073	,507	,181
RRA	-,165	,391	-,164
BRD	,718	-,361	,400
MRD	,916	-,063	,035
MRL	,939	-,217	-,077
LRD	,920	-,057	,037
RA	,954	-,235	,042
RW	,900	-,220	,278
RDM	,236	,211	-,004

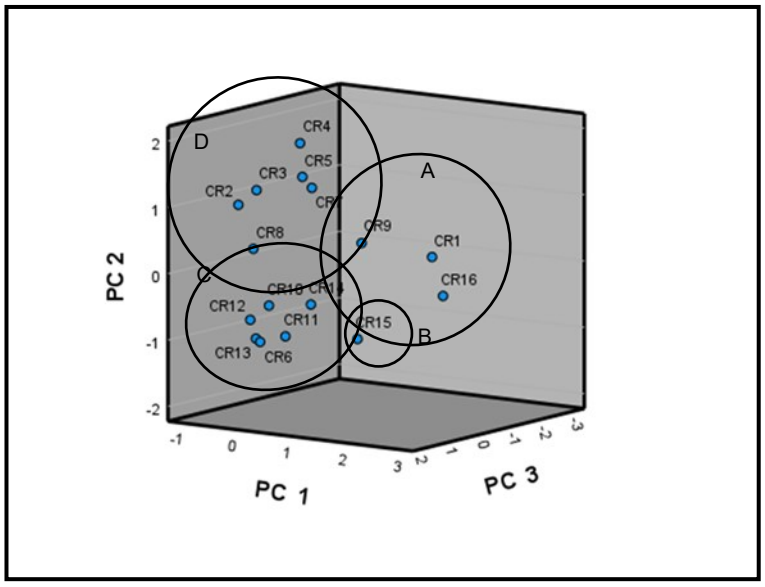


Figure 5.2.1.3.11. – Distribution of studied materials in the space described by the first three components among Kohlrabi accessions characterized.

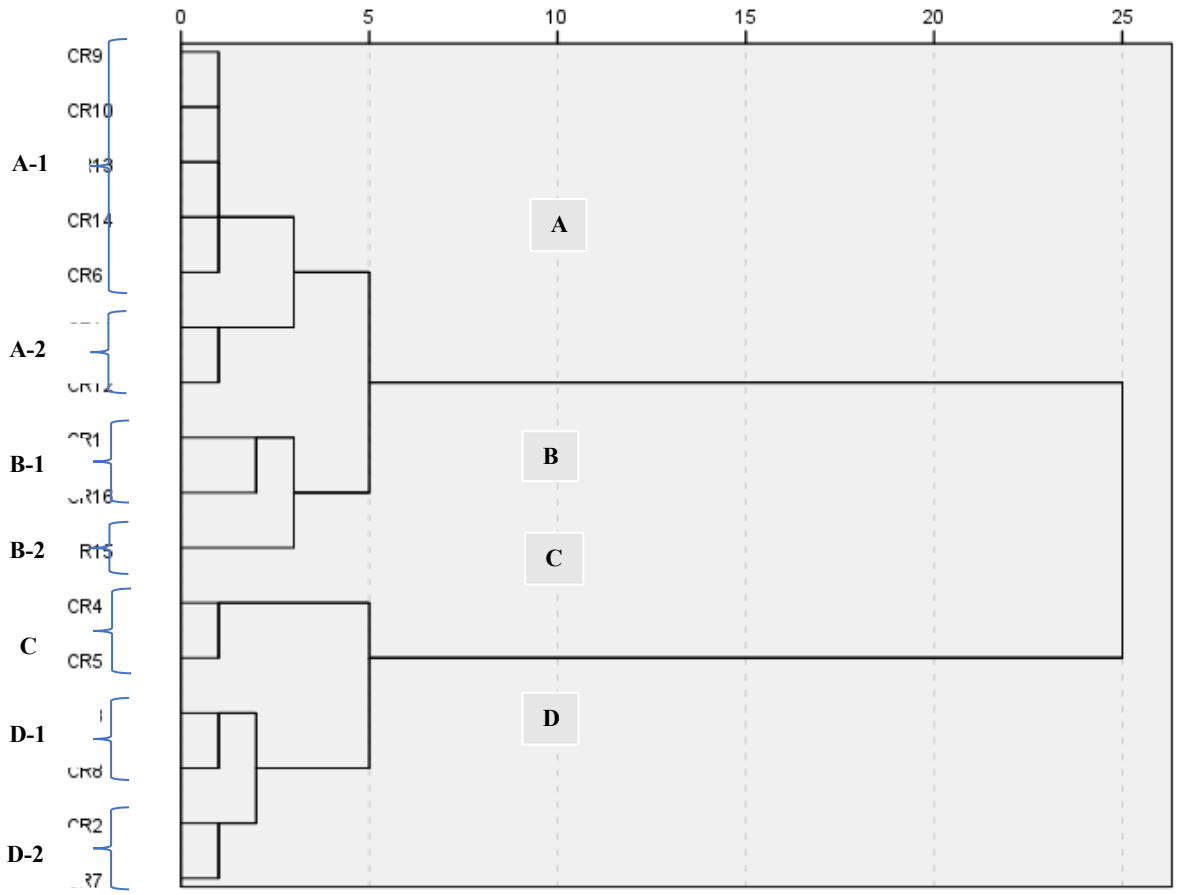


Figure 5.2.1.3.12. - Cluster classification among kohlrabi accessions characterized.

Cauliflower

The principal component analysis was carried out in the same way for the Cauliflower (CV) accessions, the results show that the first three axes PC1, PC2 and PC3 together absorb 49.76% of the total variability, the first axis PC1 absorbs 23.23%, the second axis PC2 absorbs 13.75% while the third axis PC3 absorbs 12.8% of the total variability (Table 5.2.1.3.13.).

The results show that the variables BRD, RRA, RLA, RA and RW contribute the most to define axis PC1, while the variables LA, LL, NL and LW contribute to the definition of second axis 2, while the variables IA, PS and MRD contribute to the definition of axis 3 (Table 5.2.1.3.14.).

The 3D PCA dispersion results based on the morphological variables studied show a great diversity with the formation of five groups, the first group A contains the CV accessions (2,11,14,15,18), the second group B contains the CV6 accession, and group C contains the CV4 accession, these last three groups A, B and C are indeed positively correlated with the two axes PC1 and PC3, and negatively correlated with the PC2 axis. Group D contains the accessions CV (1,3,5,7,8), this group is negatively correlated with the PC1 and PC2 axes and positive with the PC3 axes.

On the other hand, group E which contains the rest of the accessions CV (9,10,12, 13,16,17), this group is positively correlated with the three axes PC1, PC2 and PC3 (Figure 5.2.1.3.13).

From these results, we notice a great intra-population diversity, with a great dispersion and divergence of the CV6 accession on the one hand and the CV4 accession on the other hand.

This is explained by the fact that these accessions have characteristics compared to other accessions which affects their levels of variability and especially their divergence compared to other accessions. Indeed, the variables BRD and IA contribute to the definition of group A, while the variables RW and RA contribute the most to the definition of group B, group C is defined mainly by the variables LW and LRD, on the other hand the other two groups are defined by the rest of the variables of which group D is defined mainly by the variables PS and MRD, while group E is defined mainly by the variables RLA, LA and NL. This explains this level of variability within the CV accessions studied.

The hierarchical classification based on these different parameters shows a significant variability with the formation of four large groups A, B, C and D (Figure 5.2.1.3.13.). Each group is in turn subdivided into two sub-groups, noting the isolation of two accessions, CV17 (group B-2) which is morphologically particular from other accessions, because it is characterised by a large leaf area (LA), left root angle (RLA) and inflorescence which is late. compared to other accessions, also the accession CV6 (group D-2) which is characterised by an important root development which affects the average root diameter (MRD), the basal root diameter (BRD), the fresh root weight (RW) and the dry root weight (DM) (Figure 5.2.1.3.14.).

Table 5.2.1.3.13. – Percentage of variance of the main components identified by factor analysis.

Components	% Variance	% Cumulative
PC1	23,233	23,233
PC2	13,756	36,989
PC3	12,807	49,796
PC4	12,484	62,280
PC5	9,226	71,506

Table 5.2.1.3.14. – Correlation coefficient of the individual parameters with the main components.

Parameters	Components		
	PC1	PC2	PC3
IA	,005	-,033	-,737
PS	,011	-,042	,873
NL	,046	,743	,025
SL	,077	,120	,074
LA	-,215	,814	-,257
LL	,161	,731	,423
LW	,420	-,597	,051

PL	-,020	,003	-,107
PW	-,078	,170	,037
RLA	-,681	,235	,139
RRA	-,723	,104	-,182
BRD	,821	,348	-,042
MRD	,016	,044	,727
MRL	,020	,024	-,069
LRD	,224	-,423	,317
RA	,881	-,076	,031
RW	,873	-,174	,012
RDM	,007	,015	,064

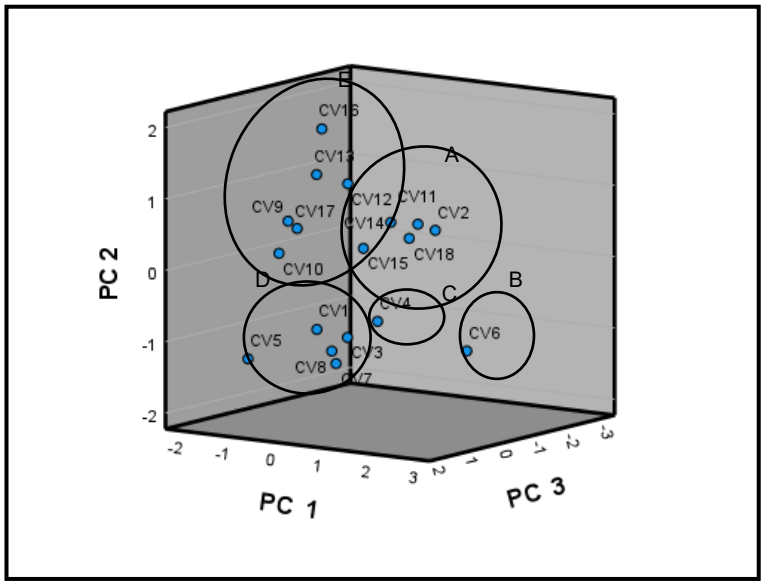


Figure 5.2.1.3.13. – Distribution of studied materials in the space described by the first three components among Cauliflower accessions characterized.

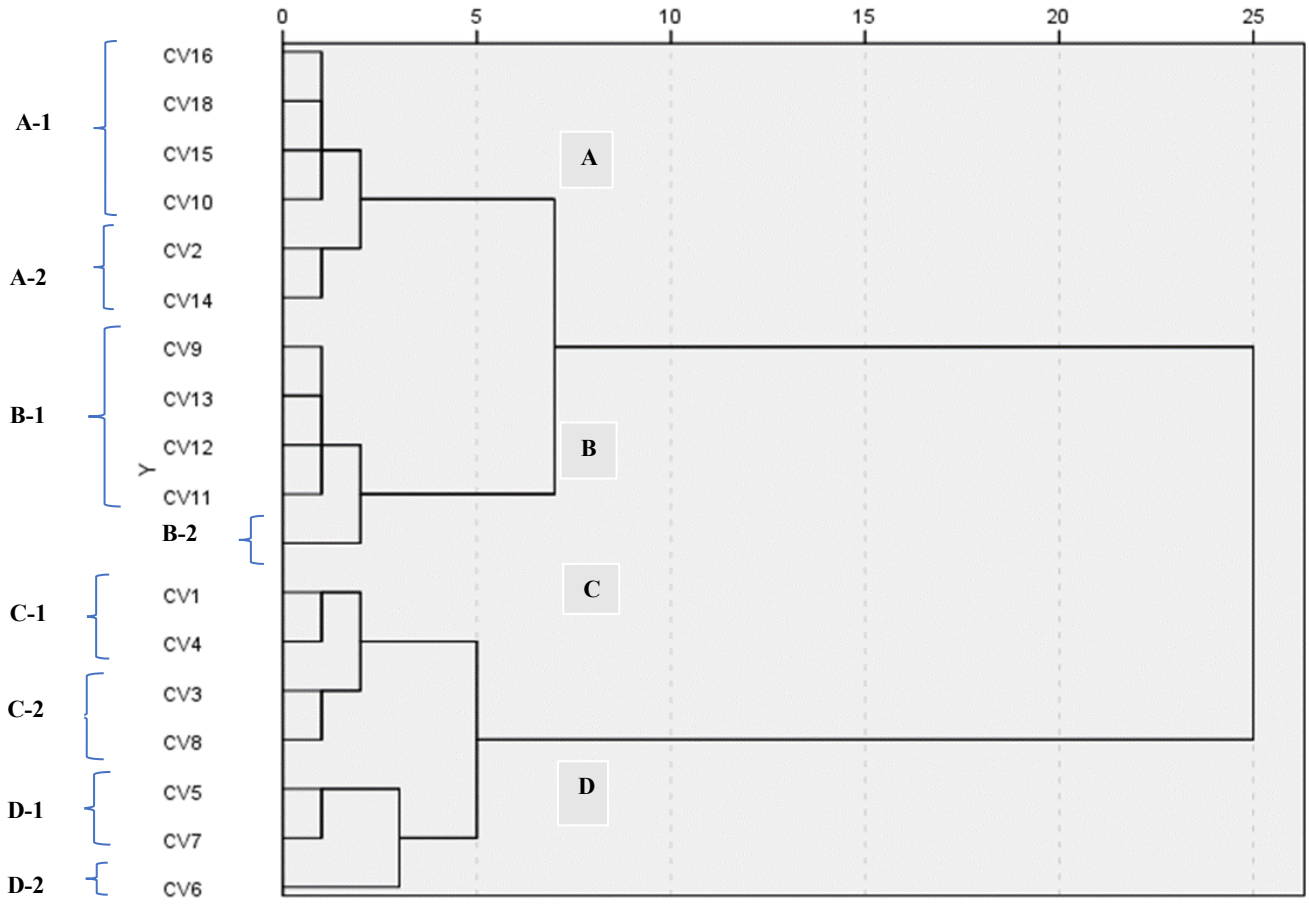


Figure 5.2.1.3.14. - Cluster classification of Cauliflower accessions characterized.

Brassica crops and wild relatives

A principal component analysis was carried out for all the accessions studied (BR, BH, BV, BD, BI, CC, CK, CV, CR), the results show that the first three axes PC1, PC2 and PC3 successively absorb 23.25%, 9.73% and 9.01% of the total variability. These three axes therefore absorb a total of 42% of the total variability (Table 5.2.1.3.15).

The results show that the variables BRD, MRL, RA and RW contribute the most to define axis PC1, while the variables LA, LL and PW contribute to the definition of second axis PC2, while the variables PS and PB contribute to the definition of axis PC3 (Table 5.2.1.3.16).

The 3D PCA dispersion results based on the morphological variables studied show a high level of diversity within Brassica crops and wild relatives with the formation of seven groups, the first group A contains the accessions BH9 and CR5, the second group B contains a mixed group between the different morphotypes, and likewise for group C, these last three groups A, B and C are positively correlated with the three axes PC1, PC2 and PC3.

Group D contains accessions of different types which are very agglomerated which suggests their resemblance on the morphological level, this group is negatively correlated with the PC2 axis, and positive with the PC1 and PC3 axes. The same for group E but contains fewer accessions than the preceding one. Group F contains only the accession CC44, which differs from the other accessions. This group is positively correlated with

the two axes PC2 and PC3 and negatively with PC1. The last group G contains the last three accessions CR3, CR7 in CC7, this last group is positively correlated with the three axes PC1, PC2 and PC3 (Figure 5.2.1.3.15).

From these results, we notice a very great intra- and inter-specific diversity, with a great dispersion and the divergence of certain accessions. This is explained by the fact that these accessions have specific characteristics compared to other accessions which affects their levels of variability and especially their divergence between them.

Indeed, the variables LL, PW and LA contribute to the definition of group A, while the variable LRD contributes the most to the definition of group B, group C is defined mainly by the variables RW and RA, group D is defined mainly by the variables LW, SL and MRD, while the E group is defined mainly by the variables LA HH and PL. The group F is defined by the variable IA, while the last group G is defined mainly by the variables RRA and RDM. This explains this level of variability within Brassica crops and wild relative accessions studied (Figure 5.2.1.3.16).

The hierarchical classification based on these different morphological parameters for all accessions shows important inter and intra-specific variability within Brassica crops and wild relatives with the formation of 8 large groups. Each group contains a mixture of the different morphotypes where classification was based on morphological variability.

The first group A contains accessions that have a large leaf area (LA), while group B contains accessions with a high dry weight of roots (RDM) compared to other accessions, the group C contains the accessions with longest leaves (LL). While the group D contains the accessions which have well-developed roots (RA).

The group E contains accessions with a high weight of roots (RW) then an important leaves area (LA) compared to other accessions. While group G contains accessions with a high roots angle (RRA, LRA) compared to other accessions. while the last group H contains accessions with a less values of different parameters compared to other accessions (Figure 5.2.1.3.16).

Table 5.2.1.3.15. – Percentage of variance of the main components identified by factor analysis.

Components	% Variance	% Cumulative
PC 1	23,257	23,257
PC 2	9,733	32,990
PC 3	9,018	42,008
PC 4	7,401	49,410
PC 5	6,635	56,044
PC 6	6,131	62,175

Table 5.2.1.3.16. – Correlation coefficient of the individual parameters with the main components.

Parameters	Components		
	PC1	PC2	PC3

IA	-,083	,150	,119
PB	,062	,110	,762
PS	,208	-,173	,669
HH	,147	-,089	,617
NL	,208	,009	,046
SL	,173	-,025	,386
LA	,013	,767	,054
LL	-,119	,820	-,081
LW	,154	-,038	-,046
PL	-,158	,096	,232
PW	-,131	,727	-,047
RLA	,011	,079	,048
RRA	-,242	,208	-,151
BRD	,780	-,051	,099
MRD	,757	-,087	,114
MRL	,665	-,096	,140
LRD	-,056	,098	,055
RA	,901	-,080	,124
RW	,882	-,037	,079
RDM	-,342	,051	-,415

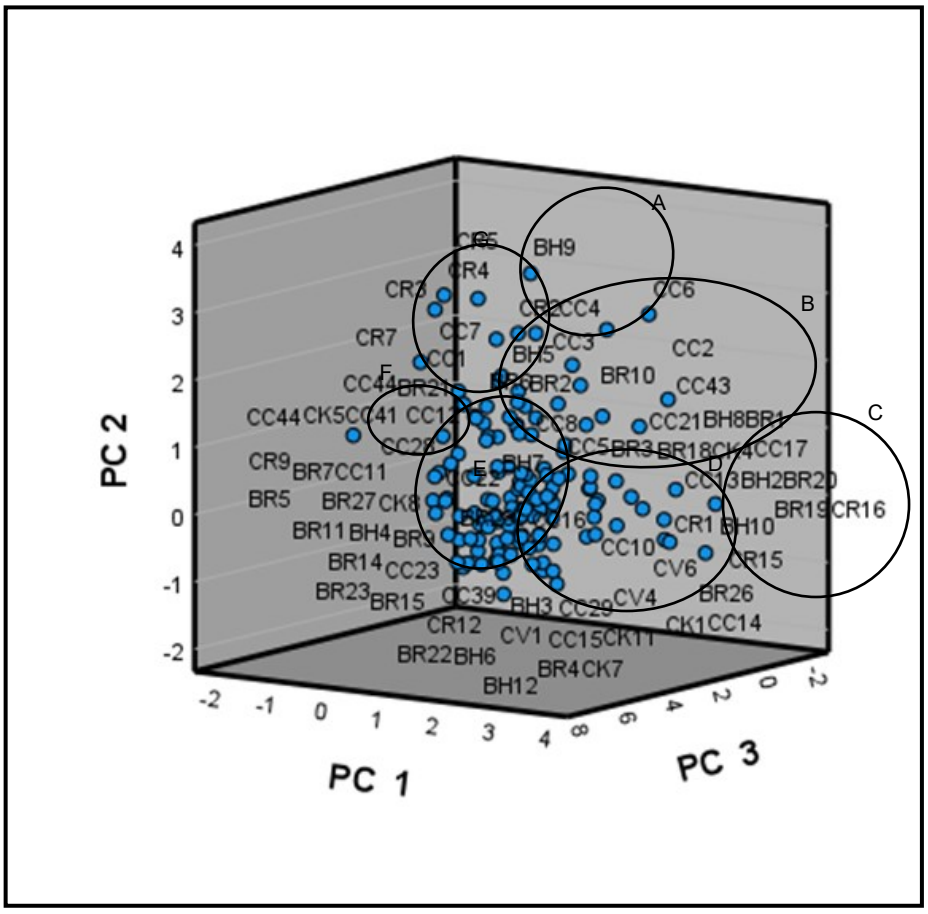


Figure 5.2.1.3.15. – Distribution of studied materials in the space described by the first three components among Brassica crop and wild relatives.

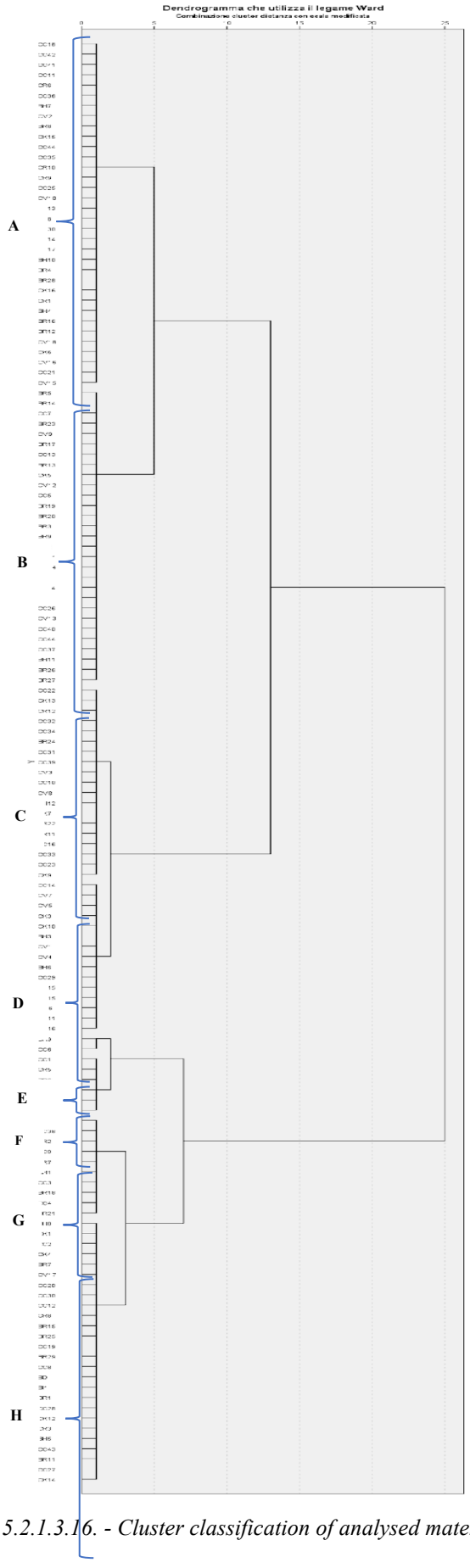


Figure 5.2.1.3.16. - Cluster classification of analysed materials

5.2.2. Effect of water stress on plant phenotyping of kale (*Brassica oleracea* var. *acephala*) and kohlrabi (*Brassica oleracea* var. *gongylodes*)

5.2.2.1. Introduction

The *Brassicaceae* family plays an important role in the horticultural profile of the countries bordering the Mediterranean basin. The great interest to the related crops belonging to this family is linked to healthy value of their produce, as they are rich in vitamins and bioactive compounds, such as glucosinolates and polyphenols (flavonoids and anthocyanins), which perform antioxidant and anti-inflammatory activities (Di Bella et al., 2021).

For this reason, are defined like superfood because they are characterised for these compounds which present activities against chronic-degenerative human's diseases (Branca et al., 2002). The domestication of crops seems to have occurred in the Mediterranean basin, where kale (*Brassica oleracea* var. *acephala*) represents the primary result of the process selection by man and that, subsequently, allowed the origin and the consequent diversification of cauliflower (*Brassica oleracea* var. *botrytis*) and broccoli (*Brassica oleracea* var. *italica*) and *Brassica oleracea* var. *gongylodes* (Ragusa, 2011).

This trial was splitted in two different experiments: A and B.

Trial A

In this trial some accessions of *Brassica oleracea* var. *acephala* were analysed. Brassica oleracea var. acephala differs from other crops of the same species by the poliennal biological cycle and for the long vegetative phase; the kale in fact emits the vegetative organs during from autumn to spring seasons and during the summer it produces the reproductive organs dispersing the seed.

The poliennal cycle today, however, represents an almost completely lost character for the other *B. oleracea* crops following their domestication process. The differentiation of the inflorescence occurs irregularly but does not hinder the emission process of new vegetative organs.

The leaves are characterised by a typical flavour and represent one of the ingredients of the Mediterranean diet. *Brassica oleracea* var. *acephala* is often cultivated in a small group, often close to the home (Branca et al., 2008).

The plant has dimensions that vary in relation to the cultivars considered. They can show an erect, branched stem, up to two metres high, with dark green leaves, elongated with elliptical lamina, with a flat smooth (as in the old Sicilian kale) or curled (as in the case of curly kale) (Ordàs et al., 2008) (Figure 5.2.2.1.1.A.).

Figure 5.2.2.1.1.A. Brassica oleracea var. acephala.

There is a wide diversity among the types of European kales for different traits, in general, the descriptors more discriminating are the branching and the shape of the plants, as well as the leaves and petiole. The wealth of kale genetic resources, analysed in the study by Branca et al. (2013), can be the basis for improving the production of kale and can offer a good support for agro-industrial production systems.

The aim of this work was the study of the influence of irrigation regime on productive and qualitative traits of kale under organic farming system, to select accessions resistant to the typical abiotic stresses of the Mediterranean environment.

5.2.2.2.A. Material and method

The trial was carried out in Santa Croce Camerina (36°51'13.3'' N 14° 29'32.0'' E, Contrada Randello, Ragusa) (Figure 5.2.2.2.1.A.) in a certified organic greenhouse. The aim of the research was evaluating six accessions of *Brassica oleracea* var. *acephala* subjected to two water regimes, 100% and 35% of crop evapotranspiration (ETc) (Consoli et al., 2014; Capra et al., 2008). Four accessions were provided by the University of Liverpool (Uni Liverpool) and conserved at the genebank of The University of Warwick-UK and two from the University of Catania (UNICT), stored at the genebank of the Department of Agriculture, Food and Environment (Di3A) (Table 5.2.2.2.1.A.).

Figure 5.2.2.2.1.A. Santa Croce Camerina field.

Table 5.2.2.2.1.A. *Brassica oleracea* var. *acephala* accessions.

Accession	Code	Origin
HRIGRU3597	UNILV1	Germplasm Bank of the Warwick-UK
HRIGRU8302	UNILV2	Germplasm Bank of the Warwick-UK
HRIGRU4887	UNILV3	Germplasm Bank of the Warwick-UK
HRIGRU7546	UNILV4	Germplasm Bank of the Warwick-UK
UNICT3381 BH50	UNICT1	Germplasm Bank of the University of Catania
UNICT4538 BH10	UNICT2	Germplasm Bank of the University of Catania

The seeds of each accession were placed in a cellular tray arranged in a cold greenhouse in light natural conditions (from 4.6 to 9.2 MJ m^{-2} d^{1}) in Catania (37°31'10" N 15°04'18" E; 105 m a.s.l.) on using organic farming practices.

The containers were filled with Brill soil (Geotec, Italy) and were irrigated according to ordinary techniques. The transplant was performed from the 27th of December 2018 in Santa Croce Camerina, using single rows, with 1.0 m between the rows and 0.5 m between the plants along the rows, at crop density of 2 plants/m^2.

The experimental design was a random block with three replicates of 10 plants each. The total volumes used, during the trial, was 20.45 m^3 of water for 35% ETc , 51.65 m^3 for 100% ETc. The harvest of the biomass (roots and leaves) was performed on 10th of April. The treatments performed were against snails (Ferramol), aphids (Pyganic 2.5 ml /l) and *Pieris brassicae* (Bacillus 1.5 g/l) and were effectuated granular fertilizations based on micro and macro-elements.

Bio-morphometric traits

The height (cm), collar and shaft diameter (cm), leaf number (n), fresh biomass leaves, stem, and roots (g). were evaluated. The samples were frozen at -80°C, lyophilised, ground to a fine powder, and utilised for further analysis and fresh leaf were storages for enzymatic analysis.

Total phenols content (TPC)

The TPC was performed utilized by Folin- Ciocalteu methods in according to Picchi et al. (2012). Extracts were prepared from 30 mg of lyophilised powder with a 1:1 mixture of EtOH and 0.02 N HCl and treated as above. An aliquot of 0.2 ml of sample was diluted with 2 ml distilled H_2O and 0.5 ml of Folin–Ciocalteu reagent and left at room temperature for 5 min. The solution was then treated with 1 ml of 20% Na_2CO_3 and incubated in the dark for 1 h. The absorbance of the blue coloured solution was measured at 730 nm. The total phenols were estimated by comparison with the standard curve obtained with gallic acid. The results are expressed as gallic acid equivalents (GAE) (mg GAE kg d.m.).

Enzymatic characterization

The enzymatic activities of plant material were analysed, developing the best analysis protocol. The samples were collected, transported to the laboratories of the Horticulture and Floriculture section, and subjected to subsequent analyses: catalase (CAT), ascorbate

peroxidase (APX), superoxide dismutase (SOD) activities, malondialdehyde (MDA) and proline (PRO) content.

Catalase (CAT), Ascorbate Peroxidase (APX)

For Catalase (CAT) and Ascorbate Peroxidase (APX), fresh tissues (500 mg) were ground to a fine powder with liquid nitrogen. Each sample was homogenized in 3 mL of 0.2 M potassium phosphate buffer (pH 7.8) with 0.1 Mm EDTA. Then samples were centrifuged at 15000 *x* g for 20 min at 4°C. The supernatant was removed, and the pellet suspended in other 2 mL of the same buffer and another centrifugation for 15 min at 15000 *x* g was done.

The total supernatants were stored on ice until the analysis. CAT activity was analysed using Aebi and Lester (1984) method. The assay sample was prepared combining 2 mL of extract (diluted in 50 mM potassium phosphate buffer, pH 7.0) and 1 mL of 10 mM H_2O_2. The H_2O_2 decomposition was determined as an absorbance decrease, in a UV/Vis spectrophotometer, at 240 nm, and expressed in terms of Units per protein content (U/g protein). For the APX assay the samples preparation was the same as described above.

The APX activity was done with the Colorimetric Assay Kit (Elabscience®, Catalog No: E-BC-K353), and according to Nakano and Asada (1981) with some modification: 50 mM potassium phosphate buffer (pH 7.0), 0.5 mM ascorbic acid solution, 0.5 mM H_2O_2 solution and 10 µL of leaf extract formed 1 mL of assay mixture. H_2O_2 solution was added immediately before the start of the reaction, and the absorbance was recorded at 290 nm for 3 min. The results were expressed in terms of Units per protein content (U/g protein).

Superoxide dismutase (SOD)

Superoxide Dismutase (SOD) was determined by a Colorimetric Assay Kit (invitrogen by Thermo Fisher Scientific). The results were reported on U/mg of protein.

Lipid peroxidation (MDA)

Lipid peroxidation was defined by the malondialdehyde (MDA) content, according to Dhisda et al. (1981), with some modifications: 200 mg of fresh leaves were homogenized with 4 mL of 0.1% trichloroacetic acid (TCA) and centrifuged at 10000 *x* g for 15 min. The supernatant was removed and 1 mL of it was mix with 2 mL of 20% TCA and 2 mL of 0.5% thiobarbituric acid (TBA). Sample were put at 95°C for 30 min in an extractor hood and then put on ice. The absorbance value was recorded at 532 nm and 600 nm, respectively. MDA concentration was calculated with its extinction coefficient (155 mM^{-1} cm^{-1}) and the results were expressed in terms of nmol/g of fresh weight.

Proline (PRO)

Proline content was quantified following a modified Myara et al. (1982) method: 100 mg of fresh sample was treated with liquid nitrogen. A 3% sulfosalicidic acid solution (5 µL/mg fresh weight was added, then the samples were centrifuged for 5 min at room temperature with the maximum speed. In another clean tube a mixture of 3%

sulfosalicidic acid (100 µL), glacial acetic acid (200 µL), acidic ninhydrin (200 µL) was prepared. Then 100 µL of supernatant was added and put it at 96°C for 60 min. When the incubation time was finished, the reaction was stopped on ice. The extraction was done adding 1 mL of toluene in the sample, 20 sec of vortex, and 5 min of incubation. The toluene phase was put in a clean tube and the absorbance was measured at 520 nm. The results were expressed as µg/g of fresh weight.

Protein content

Total protein content will be evaluated by the Pierce™ Coomassie Plus (Bradford) (Thermo Scientific™): 0.3 mL of sample was mix with 1.5 mL of Coomassie reagent and the absorbance was read at 595 nm.

Data analysis

Data were submitted to the Bartlett's test for the homogeneity of variance and then analyzed using the analysis of variance (ANOVA) using CoStat release 6.311 (CoHort Software, Monterey, CA, USA). Means were statistically separated based on Student–Newmann–Kewls test, when the 'F' test of ANOVA for treatment was significant at least at the 0.05 probability level. Significance was accepted at $P \leq 0.05$ level (Snedecor, G.W. and Cochran, 1989).

5.2.2.3.A. Result and discussion

Results of the analysis of variance for all studied variables are reported in Table 5.2.2.3.1. A. Except for stem height, weight, and TPC interaction I X A resulted not significative for all traits.

Table 5.2.2.3.1.A. Significance of main factors and their interactions resulting from ANOVA for the studied variables.

Significance			
Traits	**Irrigation Regime (I)**	**Accession (A)**	**I X A**
Stem height (cm)	***	***	**
Collar diameter (mm)	*	***	n. s.
Stem diameter (mm)	*	***	n. s.
Number of leaves (n)	**	*	n. s.
Stem weight (g/plant)	***	n. s.	**
Leaves weight (g/plant)	***	n. s.	n. s.
Roots weight (g/plant)	**	n. s.	n. s.
TPC (mgGAE kg^{-1} d.m.)	***	***	***

The bio-morphometric traits of the six accessions of kale subjected to the two irrigation regimes are reported in Table 5.2.2.3.2.A. On average of irrigation regimes, the stem height was influenced by the accession, with values ranging from 22 cm (mean value of UNILV3 and UNICT1, not statistically different) to 89.5 cm (UNICT2) in the mean of the treatments. Averaged for accessions, at 100% of ETc the stem height resulted 2.8-fold higher than that recorded at 35% of ETc. The height of the stems reflected their weight, that varied from 93 (UNILV3) to 715 (UNILV4) followed by UNICT2 with 489 g/plant (Table 5.2.2.3.2.A.).

The collar diameter was affected both by the irrigation regime, fluctuating from 18.4 cm to 26.4 cm respectively for 35% ETc and 100% ETc, and by the genotype, varying from 16.1 to 35.7 respectively for LIV2 and UNICT2 (Table 5.2.2.3.2.A.) In both the thesis, the accession UNICT2 showed the highest values. For the stem diameter were observed the same trend observed for the collar one and it varies significantly from 29.6 to 35.4 cm respectively for 35% ETc and 100% ETc.

Number of leaves were affected both by the irrigation regime, varying from 22.0 to 33.9 respectively for ETc 35 and ETc 100, and by the genotype, varying from 14.5 to 53.0 for LIV4 and LIV3 respectively. The stem weight was affected by the interaction only by the irrigation regime, changing from 50.5 to 1091.0for 35% ETc and 100% ETc respectively. The plants produced 22.0 leaves when stressed, and 33.9 when well irrigated. The leaves weight and roots weight showed the same tendency observed for the stem weight (Table 5.2.2.3.2.A.).

Plants in sub-optimal conditions showed greater root production with an average incidence of 9% on the overall biomass vs 7% recorded at 100% of the ETc. The accession UNILV2 gave the best performance at 35% ETc, increasing its production only by 12%, while UNILV3 and the accessions belonging to UNICT increased their production over 40% passing from 35% to 100% ETc.

The leaves weight, that represents the edible part of the plant, resulted in well irrigated thesis higher than 1000 g/plant (mean of accessions), while it was 3 times reduced when 35% ETc was applied. The accessions UNICT1 gave the best performance in both the two studied irrigation regimes, with 654 (35% ETc) and 1994 g/plant (100% ETc). Plants in sub-optimal conditions showed greater roots production with an average incidence of 9% on the overall biomass vs 7% recorded at 100% ETc.

Table 5.2.2.3.2.A. Bio-morphometric traits of the six accessions of kale subjected to different irrigation regimes.

	Stem height (cm)		Collar diameter (mm)		Stem diameter (mm)		Leaf number (n)		Stem weight (g/plant)		Leaves weight (g/plant)		Roots weight (g/plant)	
	35%ETc	**100%ETc**	**35%ETc**	**100%ETc**	**35%ETc**	**100%ETc**	**35%ETc**	**100%ETc**	**35%ETc**	**100%ETc**	**35%ETc**	**100%ETc**	**35%ETc**	**100%ETc**
UNILV1	28.8±7.4	83.8±6.7	16.8±1,6	23.3±2.9	22.8±2.3	27.9±0.3	20.0±0.0	24.5±2.1	95.0±9.9	507.0±1.9	239.0±4.2	966.0±3.8	56.0±9.8	155.1±5.8
UNILV2	26.5±12.0	75.0±1.4	16.1±1.7	18.8±0.8	21.8±3.4	27.2±3.5	35.5±7.8	40.5±2.3	106.0±7.6	292.0±1.8	281.1±5.5	1004.0±4.1	44.0±5.7	67.0±7.1
UNILV3	13.5±3.5	34.7±3.8	17.2±1.4	21.3±1.5	26.2±2.0	24.3±2.3	27.0±1.4	53.0±3.0	50.5±0.0	137.0±1.2	305.0±3.2	749.2±2.4	63.1±8.4	73.0±3.6
UNILV4	44.3±3.6	71.4±1.7	19.6±3.8	27.8±0.5	37.6±6.7	56.2±0.9	14.5±6.4	20.5±2.4	339.0±7.5	1091.0±6.2	474.3±7.6	1277.0±2.7	63.0±9.9	128.1±2.1
UNICT1	10.3±2.5	30.3±1.5	19.2±2.4	31.3±0.7	43.6±1.2	43.9±1.0	15.0±0.2	31.5±0.4	100.0±2.8	390.0±3.8	654.0±3.3	1994.2±1.8	75.2±6.5	286.0±1.4
UNICT2	33.3±2.5	145.6±0.1	21.3±6.4	35.7±0.1	25.3±1.4	32.5±0.1	20.0±0.0	33.5±1.6	148.0±6.5	830.0±1.7	335.0±8.2	1072.5±6.9	32.0±4.1	167.2±6.7
Means	26.1±12.6	73.5±4.1	18.4±2.0	26.4±6.4	29.6±8.9	35.4±12.3	22.0±8.0	33.9±11.7	139.8±10.2	541.2±4.6	381.4±10.4	1177.2±8.0	55.6±9.3	146.1±8.4
LSD(P<0.05) $_{Accessions}$	9.4	8.2	8.3	9.8	8.4	18.7	10.3	19.0	22.4	32.7	359.0	647.0	42.2	87.9
LSD(P<0.05) $_{Irrigation\ Regimes}$	12.7		3.3		5.2		7.9		2.0		190		4.3	

Total phenols content (TPC)

The TPC recorded in leaves of kale resulted higher in sub-optimal conditions. In general phenol content is genotype dependent and is influenced by biotic and abiotic stresses. Averaged for accessions it resulted 429.7 in sub-optimal conditions and 257.1 mgGAE kg^{-1} d.m. in well irrigated thesis. The accession UNICT2, on average of the irrigation regimes, gave the highest value 722.4 mgGAE kg^{-1} d.m. (Figure 5.2.2.3.1.A.).

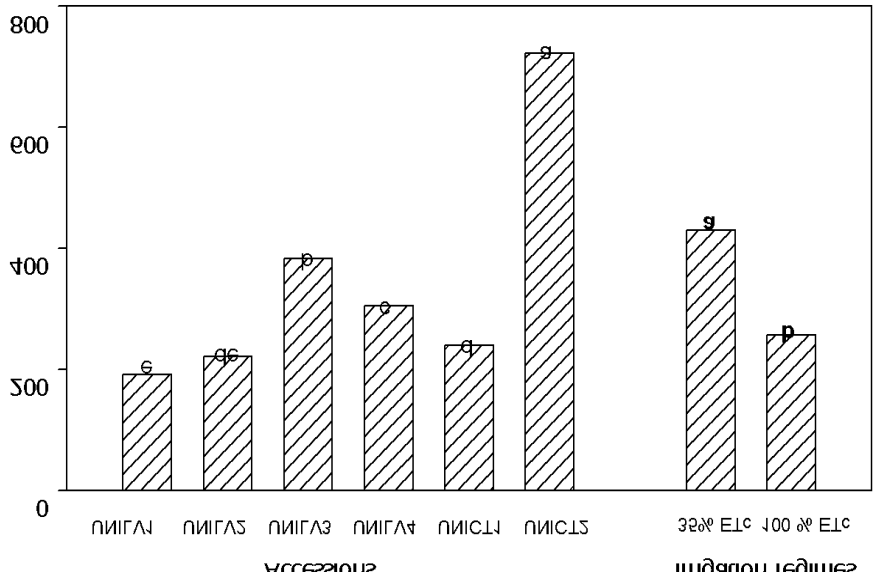


Figure 5.2.2.3.1.A. TPC (mgGAE kg^{-1} d.m.) on average of irrigation regimes and accessions. Different letters among accessions or between irrigation regimes indicate significative differences at P< 0.05.

Enzyme activities

UNILIV4 (5.88 U/g prot) treated with 35% ETc showed the highest values for Catalase (CAT) and Ascorbate Peroxidase (APX). UNICT1 and UNILIV2 are the accessions with the highest and the lowest CAT activity into the group treated with 100% ETc, with 3.21E-06 U/g prot and 1.02 U/g prot, respectively (Figure 5.2.2.3.2.A).

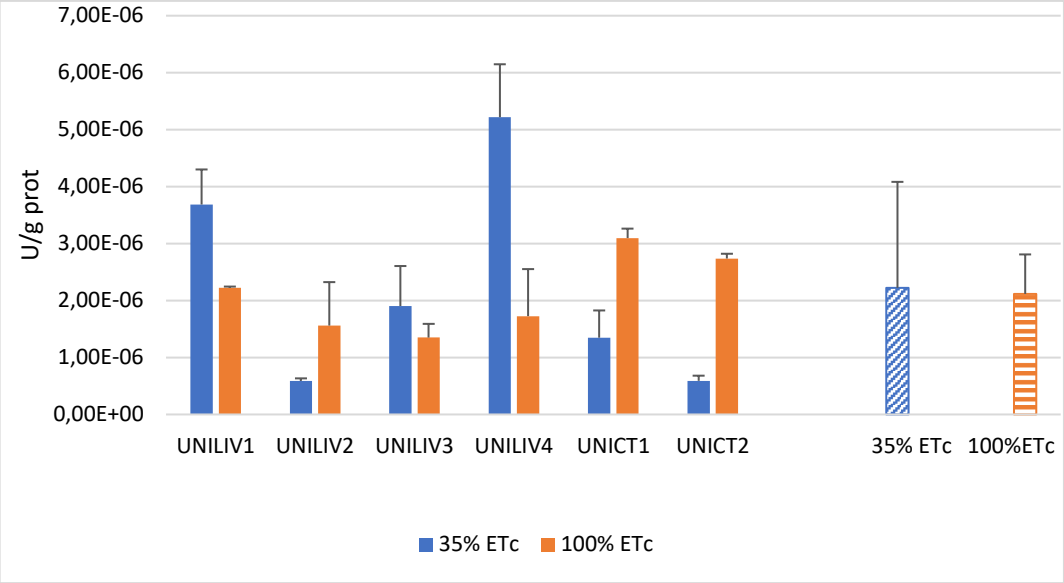


Figure 5.2.2.3.2.A. CAT activity in B. oleracea var. acephala with different irrigation condition.

For *B. oleracea* var. *acephala* accessions, UNILIV4 presents the highest APX activity in the 35% ETc treatment, instead UNILIV1, present the lowest APX activity. UNILIV1 and UNICT1 are the accessions that show the highest ant the lowest APX activity in samples treated with 100% ETc (9.19E-04 U/g prot and 1.56E-04 U/g prot) (Figure 5.2.2.3.3.A.).

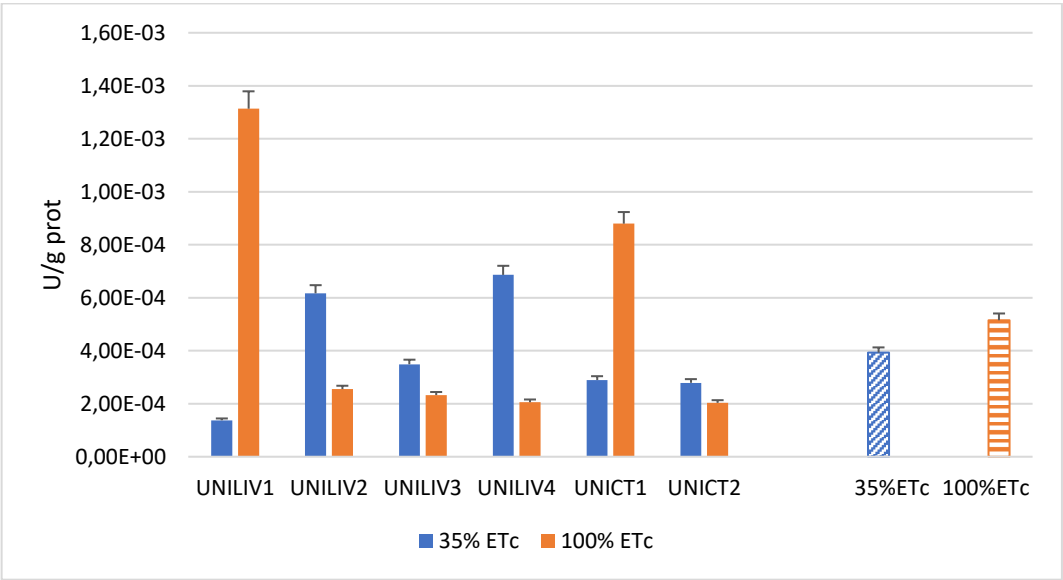


Figure 5.2.2.3.3.A.APX activity in B. oleracea var. acephala with different irrigation condition.

Superoxide dismutase (SOD)

UNILIV2 is the *B. oleracea* var. *acephala accession* with the highest enzymatic activity, for the sample treated with 35% ETc and 100% ETc, respectively (Figure 5.2.2.3.4. A.). The trend was the same.

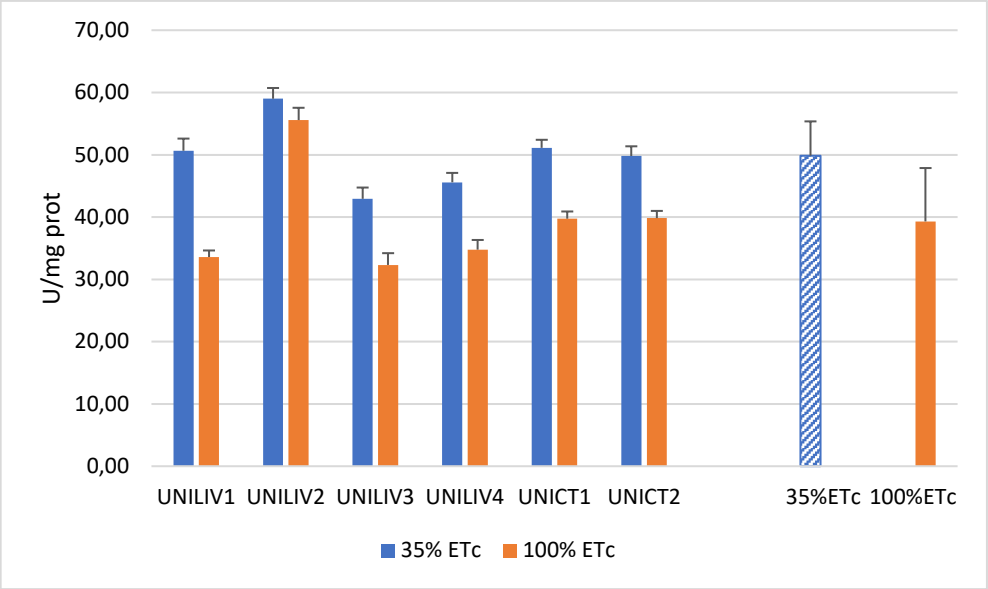


Figure 5.2.2.3.4.A.SOD activity in B. oleracea var. acephala with different irrigation condition.

Lipid peroxidation

The MDA content showed no significant differences in both treatments. *B. oleracea* var. *acephala* accessions it's similar regarding the values in the two different treatments, with an average of 8.11 nmol/g FW for samples treated with 35% ETc, and 5.98 nmol/g FW for samples treated with 100% ETc (Figure 5.2.2.3.5.A.).

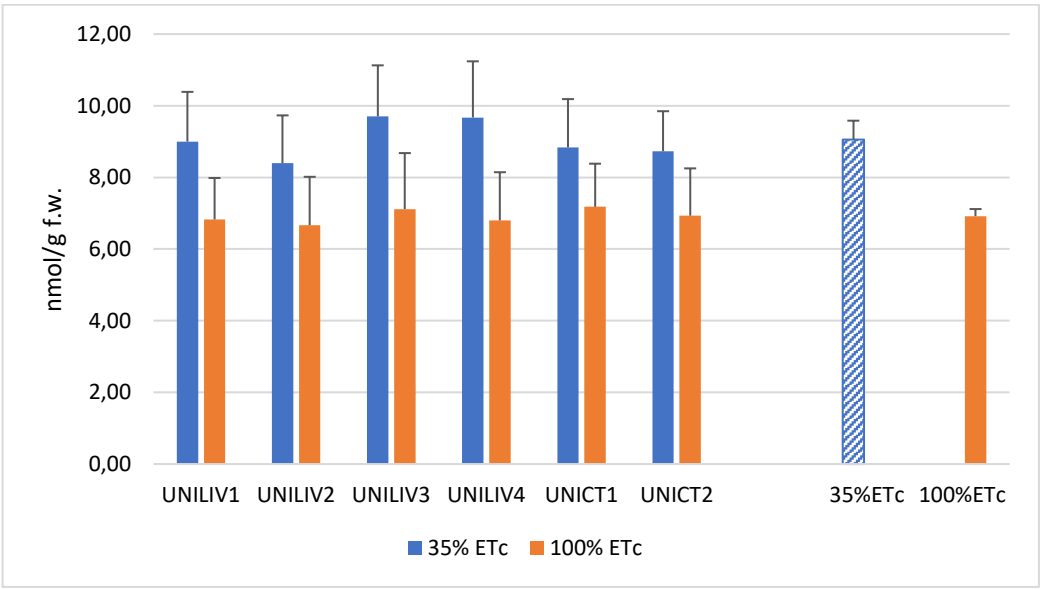


Figure 5.2.2.3.5.A.MDA content in B. oleracea var. acephala with different irrigation condition.

Proline (PRO)

UNICT2 (19.02 µg/g FW) and UNILIV4 (12.42 µg/g FW) revealed the greatest PRO content for 35% ETc and 100% ETc, respectively, unlike UNILIV3 (15.87 µg/g FW) and UNICT1 (11.49 µg/g FW), that have the lowest PRO content (Figure 5.2.2.3.6.A.).

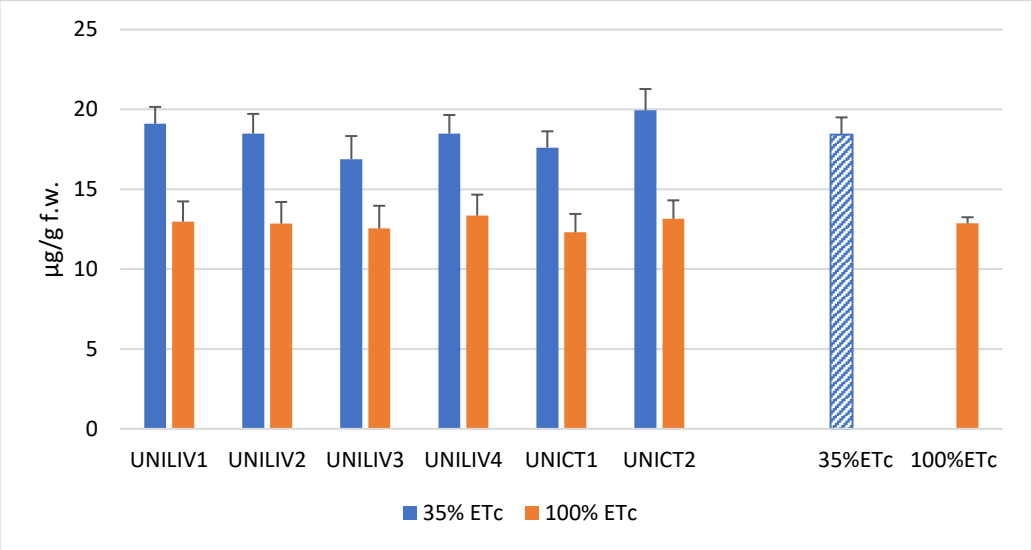


Figure 5.2.2.3.6.A.PRO content in B. oleracea var. acephala with different irrigation condition.

The aim of this work was to show that the morphometric traits and TPC content were influenced both by the drought stress, that characterised and by the genotype taken into consideration. The accessions tested in relation to the two-irrigation regime showed large differences for the studied traits. In general, the accessions provided by UNICT were characterised by high value of the plant fresh weight and TPC in comparison to the UNILIV ones. Nevertheless, the UNILIV accessions seem to be much were resistant to the water stress as they showed less reduction of the stem height and stem weight in sub-optimal conditions than the UNICT ones. The data gave useful information about the selection of accessions that gave good performance in terms of yield and quality even in sub-optimal conditions.

TRIAL B

The aim of this experiment was the bio-morphometric, physiological, and biochemical evaluation of *Brassica oleracea* var. *gongylodes* L. (kohlrabi). The kohlrabi is a biennial plant with bulbous stem, green or purple red with white flesh (Marcinkowska et al., 2021). Kohlrabi was first cultivated in north-western Europe and subsequently spread throughout Europe and around the world in the United States, Canada, Asia, and Africa. Kohlrabi is consumed all year round. *Brassica oleracea* var. *gongylodes* L. is an important vegetable cultivated and widely consumed for the round swollen stem that represents the edible part of the plant (Zhang et al., 2015). It presents a delicate and slightly sweet taste. Purple kohlrabi shows an abundant accumulation of anthocyanin.

many studies have described the health promotion properties associated with metabolites of kohlrabi, such as vitamin C, potassium, and low amounts of fat (Park et al., 2017). The stem part of this plant is commonly used as a cooked vegetable, but the raw grated stem forms a component of salads (Sassi et al., 2020).

5.2.2.2.B. Material and method

The objective of trial B was the evaluation of two genotypes of *Brassica oleracea* var. *gongylodes* under three water stress condition: 35% ETc (T3), 55% ETc (T2) and 100% ETc (T1) (Figure 5.2.2.2.1.B.) (Table 5.2.2.2.1.B.).

Figure 5.2.2.2.1. B. Brassica oleracea var. gongylodes.

Table 5.2.2.2.1.B. *Brassica oleracea* var. *gongylodes* genotypes evaluated.

Code	Species	Name	Genebank
VURV *(Libochovicka Bila Rana)*	*Brassica oleracea* L. var. *gongylodes*	Kohlrabi	VURV (CZE)
UNILIV *(Domino Gs)*	*Brassica oleracea* L. var. *gongylodes*	Kohlrabi	Warwick (UK)

The seeds of each accession were placed in a cellular tray arranged in a cold greenhouse as Trial A. The transplant was performed on the 27th of December 2018 in Santa Croce Camerina, using single rows, with 1.0 m between the rows and 0.5 m between the plants along the rows, at crop density of 2 plants/m^2. The experimental design was a random block with three replicates of 10 plants each. The treatments performed were against snails (Ferramol), aphids (Pyganic 2,5 ml /l) and *Pieris brassicae* (Bacillus 1,5 g / l) and were effectuated granular fertilizations based on micro and macro-elements.

Bio-morphometric characterization

The traits of fresh and dry biomass, leaf number, leaf area, petiole length, enlarged stem longitudinal, and transversal diameter were recorded.

Physiological characterization

SPAD index at the end of the trial was measured on 20 fully expanded leaves in 3 plants treatment (3 plants replication) by means of a portable chlorophyll metre SPAD-502 (Minolta Camera Co., Osaka, Japan). At the end of the trial, the gas exchange in three plants per treatment was measured with a CO_2/H_2O infrared gas analyser (LCi, ADC Bioscientific Ltd., Hoddesdon, UK). The reliefs were carried out in the morning (from 10:00 to 14:00). For each drought stress treatment, the net photosynthetic rate (AN), stomatal conductance (gs), transpiration rate (E), were determined. The chlorophyll *a* fluorescence (Fv/Fm) was recorded using a modulated chlorophyll fluorimeter OS1-FL (Opti-Sciences Corporation, Tyngsboro, MA, USA). The leaf was dark-adapted using cuvette clips for 15 min (Opti-Sciences Corporation, Tyngsboro, MA, USA). The chlorophyll a fluorescence was reported as the Fv/Fm ratio, where Fm = the maximum fluorescence and Fv = the variable fluorescence.

The relative water content (RWC) was evaluated on fully opened leaves. The RWC was expressed according to the formula: RWC% = (FW - DW/TW - DW) * 100.

Total phenols content (TPC)

The TPC was performed utilized by Folin- Ciocalteu methods in according to Picchi et al. (2012). Extracts were prepared from 30 mg of lyophilised powder with a 1:1 mixture of EtOH and 0.02 N HCl and treated as above. An aliquot of 0.2 ml of sample was diluted with 2 ml distilled H_2O and 0.5 ml of Folin–Ciocalteu reagent and left at room temperature for 5 min. The solution was then treated with 1 ml of 20% Na_2CO_3 and incubated in the dark for 1 h. The absorbance of the blue coloured solution was measured at 730 nm. The total phenols were estimated by comparison with the standard curve obtained with gallic acid. The results are expressed as gallic acid equivalents (GAE) (mgGAE kg d.m.)**.**

Glucosinolates (GLSs)

The extraction method of GLSs was based on the International Standard Method ISO 9167-1 (ISO 9167-1, 1992) and European Commission (European Comission, 1990), with several modifications (Aires and Carvalho, 2017).

The extracts with desulfo glucosinolates were injected in a HPLC-DADUV/ Vis equipped by a C18 (250 × 4.60 mm, 5 μm) column with a mobile phase of ultrapure water (solvent A) and 20 mLL−1 acetonitrile: water (v/v) (solvent B), with a flow rate of 1.5 $mLmin^{-1}$ and an injection volume of 10 μL, with binary gradients according to Aires and Carvalho (2017). The GSs peak identification and quantitative estimations were made using pure standard GSs as internal standard (IS) (benzyl GL at 1 mgmL−1). The chromatograms were recorded at 229nm and used to identify GSs by retention time (RT)

and UV spectra in comparison with commercial standards. All reagents used in analytical determinations were HPLC grade.

The quantification of GLS was based on the internal standard method, according to the fundamentals of HPLC and the guidelines of the International Conference on Harmonisation (ICH) (IHC, 2005). Each GL content was quantified as μmoles·100 g^{-1} dry weight (d.w.): CGLS sample = ($C$is × HPLC Area GLS sample × Rf × Df)/(HPLC area is × d.w.) × 100, where CGLS sample is the concentration of each GL in the sample; $C$is is the concentration in μmoles·mL^{-1} of internal standards added to sample and injected in the HPLC, Rf is the response factor of each GLS, and Df is the dilution factor. The Rf factors, for each GLS, detected at 229 nm, published in ISO 9617-1 standards were considered (ISO 9167-1, 1992).

Enzymatic characterization

The enzymatic activities of plant material were analysed, in according the trial A. The enzymatic characterizations carried out were catalase (CAT), ascorbate peroxidase (APX), superoxide dismutase (SOD), malondialdehyde (MDA) and proline (PRO) content. For all the enzymatic analysis, fresh and lyophilized samples were texted, to understand the best analysis method. Even fresh samples were considered.

Data analysis

Data were submitted to the Bartlett's test for the homogeneity of variance and then analysed using the analysis of variance (ANOVA). Means were statistically separated based on Student–Newmann–Kewls test, when the 'F' test of ANOVA for treatment was significant at least at the 0.05 probability level. Significance was accepted at $P \leq 0.05$ level (Snedecor, G.W. and Cochran, 1989). The program utilized for analysis is CoStat©.

5.2.2.3.B. Results and discussion

Bio-morphometric characterization

For bio-morphometric traits, data analysis showed that the most significant traits were fresh leaf biomass and stem diameter, with reference to genotype and treatment respectively. No significant differences were found for other characters (Table 5.2.2.3.1.B.).

Table 5.2.2.3.1.B. Bio-morphometric traits of the two accessions of kohlrabi subjected to different irrigation regimes.

Genotypes	Treatments	Leaf fresh biomass (g)	Enlarged stem fresh biomass (g)	Enlarged stem longitudinal diameter (mm)	Enlarged stem transversal diameter (mm)	% DM leaves	% DM enlarged stem	% DM root
VURV		186.8b	349.9	63.1b	91.1	11.7	8.6	18.8
UNILIV		414.7a	410.1	70.4a	96.4	11.7	8.9	18.2
	T1	279.6	498.4a	69.3	108.7a	10.8	7.6b	17.6

	T2	279.8	315.1b	67.8	93.8b	11.4	8.7a	17.4
	T3	220.2	360.2b	63.9	82.2c	11.9	9.1a	21.4
Significance								
Genotypes (G)		***	ns	*	Ns	ns	ns	ns
Treatments (T)		ns	**	ns	***	ns	**	ns
G x T		***	ns	**	Ns	ns	ns	ns

Physiological characterization

Drought stress is common to accelerate phenological phases and reduce normal growth and development periods (Ghobadi et al., 2006). Physiological changes are important considerations in plant adaptation mechanisms to resist this stress (Ebrahimiyan et al., 2012). No significant differences were observed for the SPAD index in both genotypes in all treatments (Figure 5.2.2.3.1.B.).

Significant differences for AN were observed in T3 treatments compared with the control plants in both genotypes. In particular, the plants irrigated at 35% of ETc showed a reduction of ~25% and 40% compared with control plants respectively for VURV and UNILIV (Figure 5.2.2.3.2.B.). The stomatal conductance showed indeed a reduction only in UNILIV with a reduction of ~70% (Figure 5.2.2.3.3.B.) compared with the T1 and T2. Similar trend was observed for the Evapotranspiration rate (data not shown). It has been shown that, in drought conditions, plants optimize carbon assimilation and minimize water losses, reducing stomatal conductance (Medrano et al., 2002). As observed by Puglielli et al. (2017), the trend of photosynthetic activity and stomatal conductance follows that of the substrate water content in which the plant is grown.

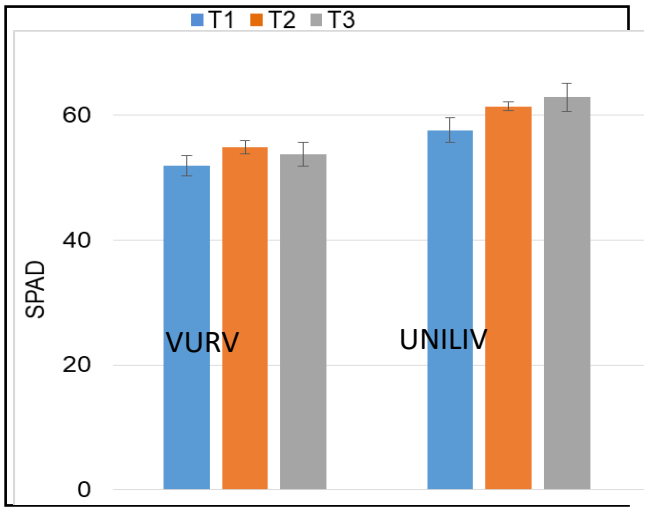


Figure 5.2.2.3.1.B. SPAD index in VURV and UNILIV genotypes exposed to drought stress treatments. Values are means ± standard errors (*n*=3).

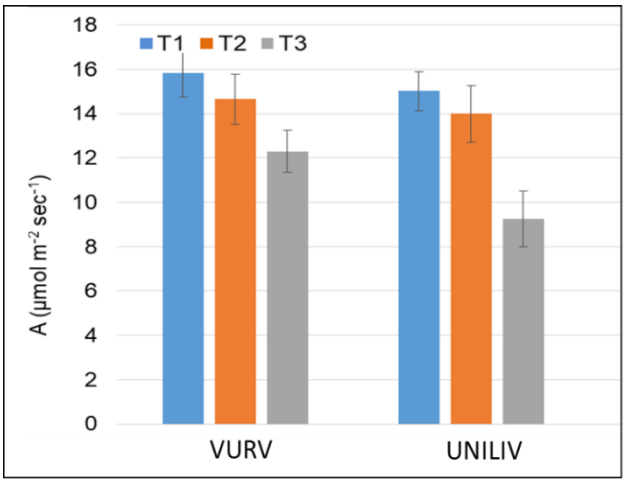


Figure 5.2.2.3.2.B. Net photosynthesis (AN) in VURV and UNILIV genotypes exposed to drought stress treatments. Values are means ± standard errors (*n*=3).

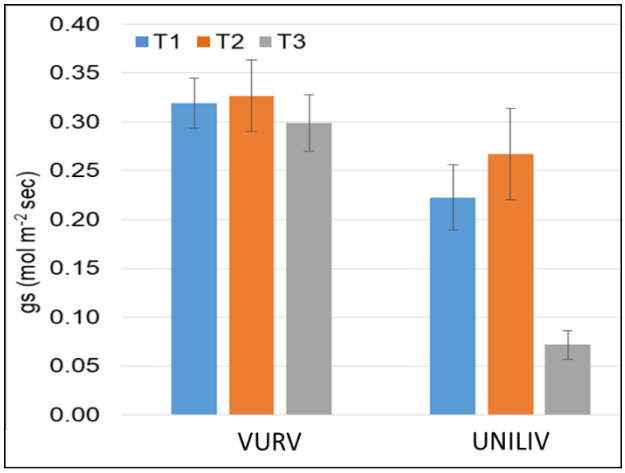


Figure 5.2.2.3.3.B. Stomatal conductance (gs) in VURV and UNILIV genotypes exposed to drought stress treatments. Values are means ± standard errors (*n*=3).

No significant differences were observed for the Fv/Fm index in both genotypes in all treatments (Figure 5.2.2.3.4.B.). Under drought stress, photoinhibition can increase because the plant's capacity to use the available light can be reduced (Osmond 1999). Our results showed that water stress did not influence photoinhibition, because the values of Fv/Fm were within the typical ranges of healthy plants (Maxwell, 2000). This demonstrates that the damage of the foliar tissues is not irreversible, that PSII was not permanently damaged (Farieri et al., 2016).

The leaf water potential and the Relative Water Content are indices used in numerous studies to evaluate the responses of species in conditions of drought stress (Álvarez et al., 2011).

In our study, the RWC under T2 drought stress did not show significant differences compared with control plants in both genotypes. In the condition of severe drought stress (33% ETc), a significant reduction of ~20% and ~ 25% was observed respectively for VURV and UNILIV (Figure 5.2.2.3.4.B.). The ability to maintain high the leaf water

level in conditions of water shortage is an important strategy adopted by genotypes tolerate the drought stress (Blum, 2005).

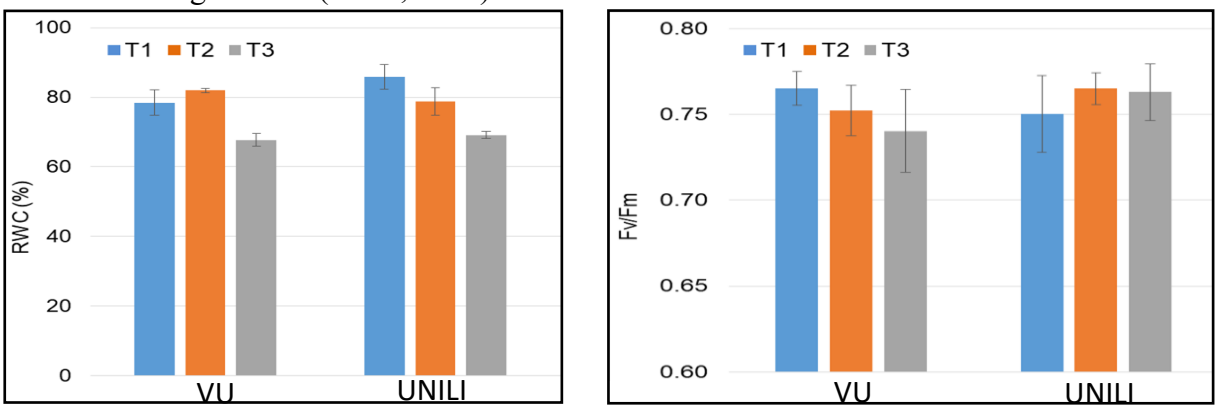


Figure 5.2.2.3.4.B. Relative water content (RWC) and Maximum quantum efficiency of the PSII (Fv/Fm) in VURV and UNILIV genotypes exposed to drought stress treatments. Values are means ± standard errors (n=6)

Total phenols content (TPC)

The genotype VURV showed a highest total phenols content under 55% ETc (T2), instead the highest total phenols amount in the genotype UNILIV was detected under 35% ETc (T3). No significative correspondence was observed between the two genotypes (Figure 5.2.2.3.5.B.).

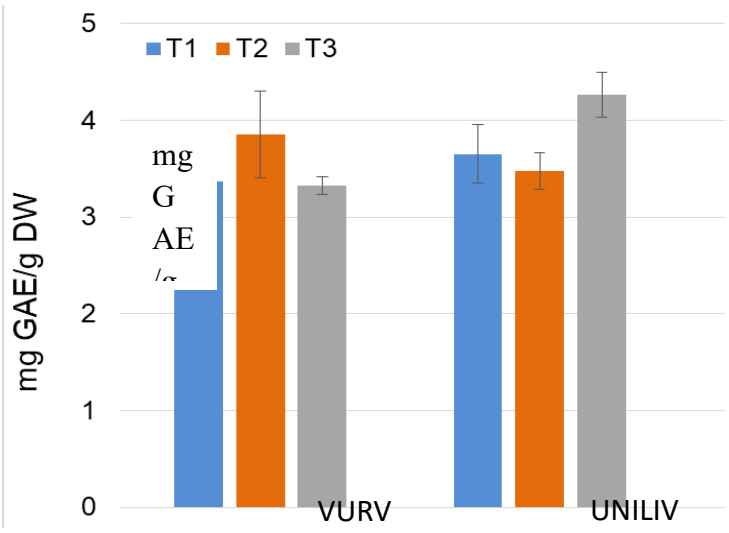


Figure 5.2.2.3.5. B. Total phenols amount (mg GAE/g d.w.$^{-1}$).

Glucosinolates profile

The genotype 'Libochovicka Bila Rana' revealed an average GLS total content of 385.62 µmol g^{-1} d.w. and 'Domino' 302.45 µmol g^{-1} d.w. The highest total GLS content was detect in the 'Libochovicka Bila Rana' genotype under severe water stress (T3, 35% ETc) and the lowest total content in 'Domino' control plants (Table 5.2.2.3.2.B..). The total GLS content increased in both genotypes with the severity of the water stress treatment.

Water stress has been related with increased glucosinolate accumulation in different *Brassica* species, although the intensity and duration of drought appears to be an important factor in the accumulation of each specific glucosinolate, as well as the developmental stage of the plant when the stress is applied (Martínez-Ballesta et al., 2013).

Table 5.2.2.3.2. B.. Glucosinolates profile of the genotypes 'Libochovicka Bila Rana' and 'Domino' in three irrigation conditions. Gib) Glucoiberin; (Prog) Progoitrin; (Graf) Glucoraphanin; (Gbrass)Glucobrassicin; (4-Metx)4-Methoxyglucobrassicin; (Ngb)Neoglucobrassicin.

Genotype	Irrigation treatment	Glucosinolates µmoles/100g dry weight						Total GLS
		Gib	**Prog**	**Graf**	**Gbrass**	**4-Metx**	**Ngb**	
VURV	(T1)	85.4±1.4	24.7±1.1	106.3±1.0	42.5±1.2	14.7±2.3	14.0±1.8	287.6±0.9
	(T2)	73.4±2.2	54.2±1.0	51.61±1.2	83.6±1.1	25.8±1.7	19.6±2.0	308.0±1.5
	(T3)	250.8±1.3	31.6±1.4	152.8±0.9	76.1±2.0	17.6±2.1	32.4±1.3	561.2±1.8
UNILIV	(T1)	102.8±1.5	30.1±2.2	81.8±1.3	18.2±1.9	8.1±1.8	15.3±1.7	256.8±1.7
	(T2)	96.5±1.7	35.4±1.7	111.2±1.1	46.7±1.6	13.2±1.2	16.8±1.5	319.8±2.0
	(T3)	92.2±1.9	39.5±1.6	59.9±1.5	93.4±1.5	27.7±2.0	18.4±1.0	330.9±1.5
Average		116.8±1.4	35.9±1.3	93.9±1.5	60.1±1.7	17.9±1.9	19.4±1.8	344.0±2.1

The glucosinolates Sinigrin, Sinalbin, Gluconapin, 4-Hydroxyglucobrassicin and Gluconasturtiin were not detect in either of the two genotypes.

In general, regardless the two genotypes and water stress condition, the glucosinolates Glucoiberin, Glucoraphanin and Glucobrassicin were those with the highest or the second highest content and the glucosinolates 4-Methoxyglucobrassicin and Neoglucobrassicin those with the lowest or the second lowest content.

For control plants, Glucoiberin was the main GLS for 'Domino' (102.81 µmol g^{-1} d.w) and Glucoraphanin for 'Libochovicka Bila Rana' (106.26 µmol g^{-1} d.w).

'Domino Gs' T2 (55% ETc) showed a high amount of Glucoraphanin (111.16 µmol g^{-1} d.w.) whereas 'Libochovicka Bila Rana' in this treatment showed higher content of Glucobrassicin (83.57 µmol g^{-1} d.w.).

Under the most severe water stress (35% ETc) was recorded the highest amount of a specific GLS, in this case 250.79 µmol g^{-1} d.w. of Glucoiberin in Libochovicka Bila Rana'. In this stress 'Domino' plants revealed higher amounts of Glucobrassicin (93.44 µmol g^{-1} d.w.) and Glucoiberin (92.22 µmol g^{-1} d.w.).

Catalase (CAT), Ascorbate Peroxidase (APX)

VURV was the *B. oleracea* var. *gongylodes* accession that show the highest catalase activity in both the 2 samples groups, with 3.11E-06 U/g prot, 3.25E-06 U/g prot and 1.84E-06 U/g prot, for 35% ETc, 55% ETc and 100% ETc, respectively (Figure 5.2.2.3.6.B.).

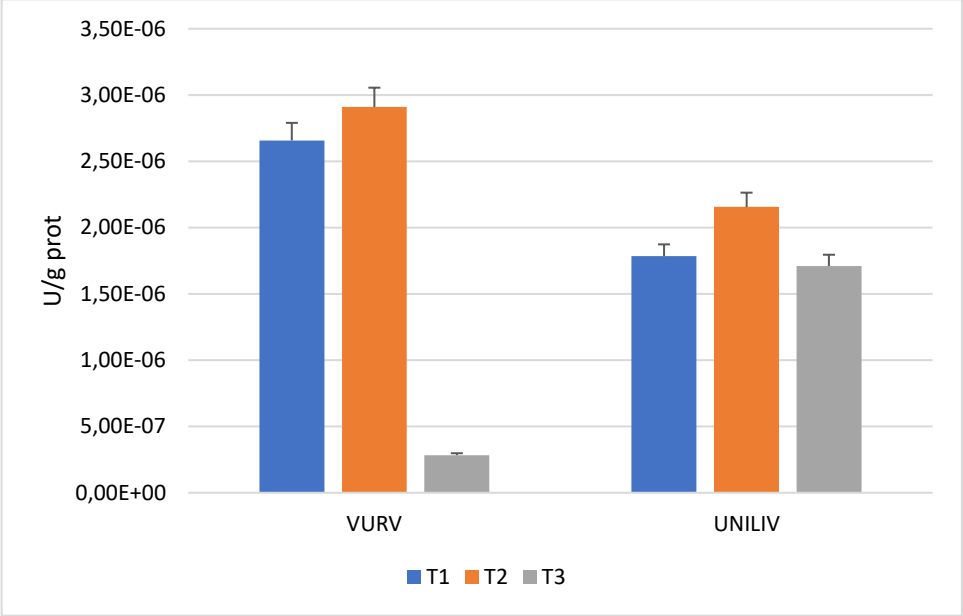


Figure 5.2.2.3.6.B. CAT activity in *B. oleracea* var. *gongylodes* with different irrigation condition.

UNILIV showed the highest APX activity for the other 2 treatments groups, 55% and 100%, with 2.65E-03 U/g prot and 3.03E-03 U/g prot) (Figure 5.2.2.3.7.B.).

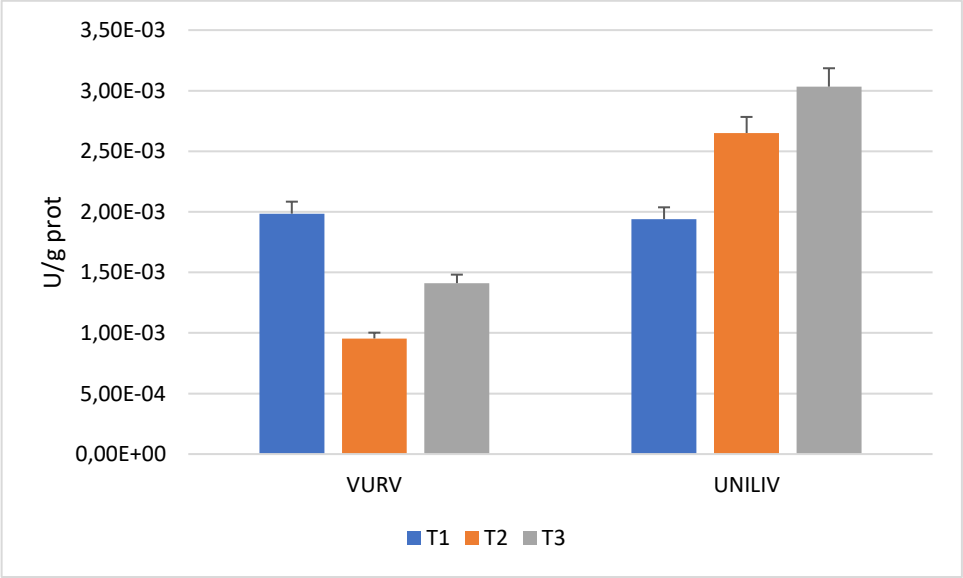


Figure 5.2.2.3.7.B, APX activity in B. oleracea var. gongylodes with different irrigation condition.

Superoxide dismutase (SOD)

The trend is similar, in both cases, with a higher enzymatic activity in the group of samples treated with 35% ETC, which progressively decreases in the groups treated with 55% ETc and 100% ETc (Figure 5.2.2.3.8.B.).

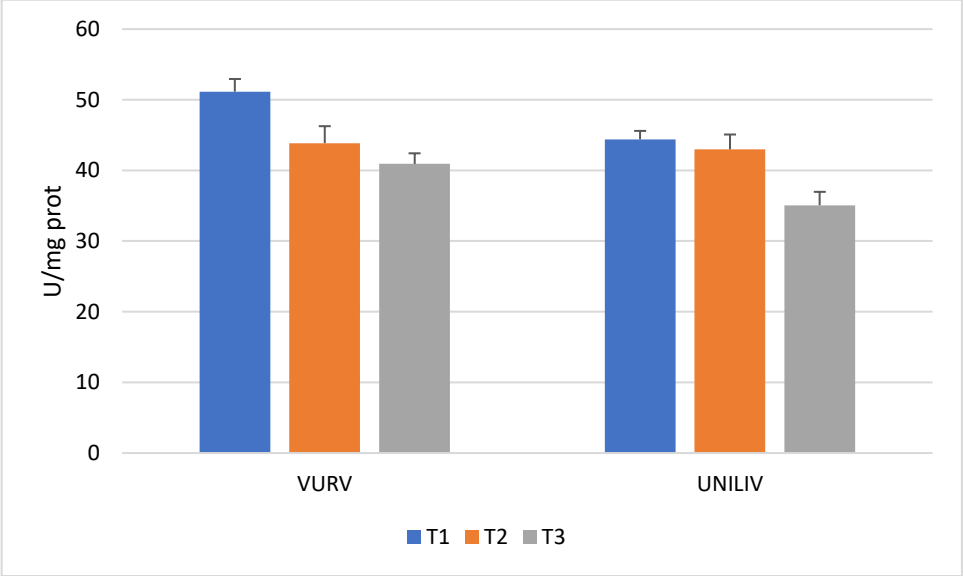


Figure 5.2.2.3.8.B. SOD activity in *B. oleracea* var. *gongylodes* with different irrigation condition.

Malondialdehyde (MDA)

UNILIV accession is the one that contains the greatest MDA content, with 9.02 nmol/g FW, 7.89 nmol/g FWand 7.14 nmol/g FWfor group treated with 35% ETc, 55% ETc and 100% ETc, respectively. VURV accession contains a similar amount of MDA into the 35% ETc sample (9.00 nmol/g FW), but a lower MDA content for the other two groups (6.19 nmol/g FWfor 55% ETc and 5.68 nmol/g FWfor 100% ETc) (Figure 5.2.2.3.9.B.).

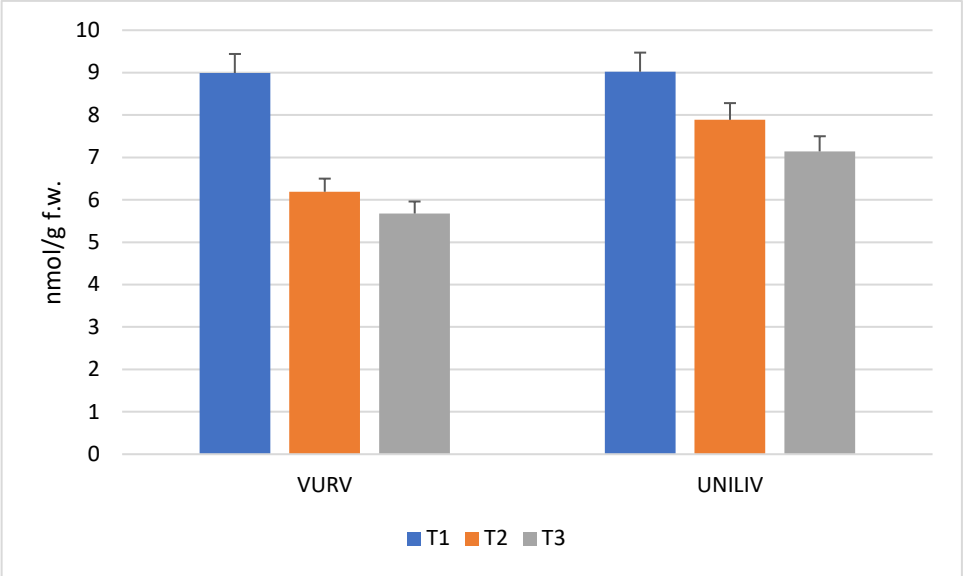


Figure 5.2.2.3.9.B. MDA in *B. oleracea* var. *gongylodes* with different irrigation condition.

Proline content (PRO)

For the PRO content, samples treated with 35% ETc have the greatest values, followed by 55% ETc and 100% ETc. UNILIV is the accession with the highest PRO content for samples treated with 35% and 100% (17.51 µg/g FW and 13.4 µg/g FW, respectively) (Figure 5.2.2.3.10.B.).

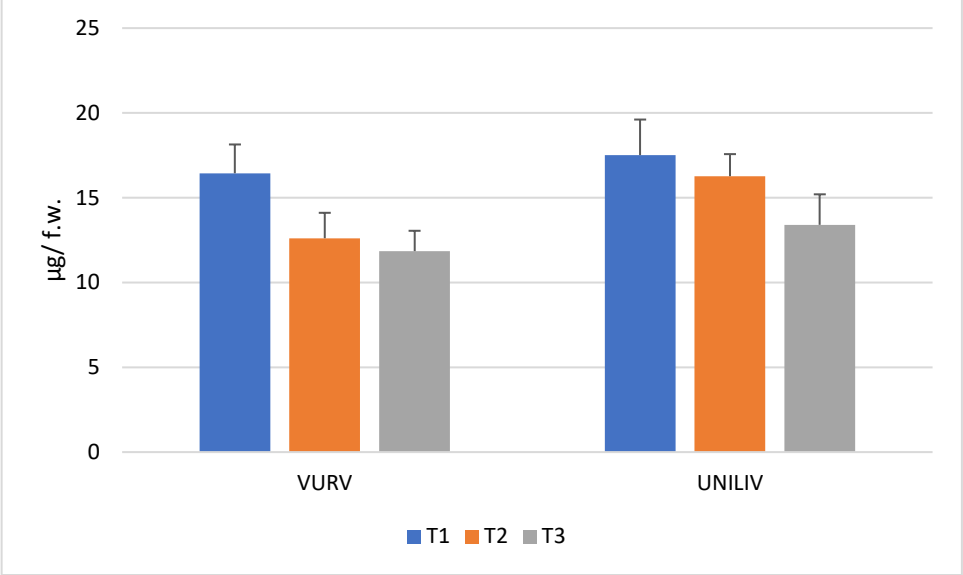


Figure 5.2.2.3.10.B. PRO content in *B. oleracea* var. *gongylodes* with different irrigation condition.

Drought stress is one of the main problems affecting agriculture especially in countries characterized by water scarcity, such as the Mediterranean area

The solution to this problem can be achieved through the identification of genotypes tolerant to drought stress (Libochovicka Bila Rana – VURV). The trial carried out showed how the genetic pathway appears very effective. The monitoring of physiological and biochemical parameters appears to be a tool both for the evaluation of genotypes and for the management of irrigation.

The total GLS content in both genotypes, 'Libochovicka Bila Rana' from Check Republic and 'Domino' from England, increased with the degree of water stress severity. Glucoiberin, Glucoraphanin and Glucobrassicin were the GLS detected with higher contentes whilst 4-Methoxyglucobrassicin and Neoglucobrassicin those with lower content.

The effect of water stress on the accumulation and profile of glucosinolates in B. acephala and B.gongulodes was studied in a time course experiment. There was a quantitative variation in both content and profile of glucosinolates in the treated samples as compared to the untreated control seedlings. Under this stress, there was a significant difference in total glucosinolate content.

Aliphatic glucosinolates constitute the major fraction of glucosinolate pool. When the pools of aliphatic and indole glucosinolates were analyzed independently, it was also found that although both aliphatic and indole glucosinolates were induced by these conditions, indole glucosinolates had a more profound effect, contributing majorly toward increased levels of total glucosinolates. Interestingly, aliphatic glucosinolates were induced maximally by Glu and wounding whereas all the tested abiotic stress significantly enhanced the accumulation of indole glucosinolates.

Our results corroborated the hypothesis that glucosinolates are important components of plant defense against biotic stress in B. ol varities. Similar results were reported previously from other crops in which increased accumulation of total glucosinolates (Mailer and Cornish. 1987). product by Brassicaceae plants, is considered as being told by environmental factors similar as soil, climate and cultivation conditions including fertilization, crop time, and factory organ (Martínez-Ballesta and al., 2013).

In general, the diversity of GLS biographies is advanced in *Brassica oleracea* as opposed to *Brassica rapa*.

The Brassicaceae plant tissues include one or further major GLS substantially composed of aliphatic GLS. In general, Brassicaceae vegetables GLS contain an alkyl side chain with 3 – 5 imitations (Ishida and al., 2014). From these bones, glucoiberin is present substantially in *Brassica oleracea* vegetables (cabbage, broccoli, and cauliflower) while, gluconapin and progoitrin are ubiquitous in numerous

Brassica vegetables similar as *Brassica rapa* (Chinese cabbage, mustard spinach, and turnip), *Brassica oleracea*, *Brassica juncea* (mustard herbage), and *Brassica napus* (rapeseed vegetable) (Ishida and al., 2014). Glucoerucin is substantially plant in cultivated *Eruca sativa* and wild rockets (*Diplotaxissp, Diplotaxis tenuifolia,*) rockets.

Despite several reports on a positive relationship between GLS production and abiotic stress, it is still unknown which are the mechanisms of resistance to drought and salinity conditions.

Determining a chemodiversity profile associated with phenotypes adapted to extreme environmental conditions, such as drought and salinity, could be a good strategy for prospecting GLS compounds and contents and quantity for coping with abiotic stresses.

Extensive studies in Brassicaceae family showed a positive correlation between salt stress and GLS content, [e.g. in broccoli (Ló;pez-Berenguer et al., 2009), canola (Khalifa, 2012), radish sprouts (Yuan et al., 2010), pakchoi (Keling and Zhujun, 2010)]. An increase in the signature of GLS content has also been reported for Brassicaceae taxa under drought stress, namey in *Brassica napus* (Champolivier and Merrien, 1997), Brassica oleracea (Radovich et al., 2005), Brassica rapa (Zhang et al., 2008), Brassica juncea (Tong et al., 2014), and Brassica carinata (Ngwene et al., 2017).

5.2.3. Individuation Brassica oleracea complex species (n=9) genotypes for resistance to water stress

5.2.3.1 Introduction

The awareness that the climate changes underway are affecting vegetable productions, which are necessarily supported by irrigated crops, and the correspondent significant damages recorded in recent decades confirm the priority of identifying genotypes resistant to water stress, in the pre-breeding screening phase, able to increase the water use efficiency (WUE) of the crops. This awareness is quite widespread in Mediterranean countries which undergo a gradual process of desertification which weakens not only natural ecosystems but also and above all agricultural ones. For vegetables, this awareness is quite accentuated given the importance that the availability of water represents for vegetable crops.

Sicily is particularly exposed to the ongoing climate changes which, in addition to affecting the crops due to the modest availability of water during the summer months, subject them to peaks of meteoric precipitation intensity during the autumn and winter months. In the arid summer months usually, we have registered during the last decade progressive increases in temperature which, associated with the reduced availability of irrigation water, put a strain on the growth and development of plants and on the performance of several vegetable crops.

As regards the *Brassica oleracea* crops, and therefore the different morpho-types, those afferent to Brassica oleracea that refer to the var. *acephala* (kale), var. *botrytis* (cauliflower), var. *capitata* (cabbage), var. *italica* (broccoli), var. *gongylodes* (kohlrabi), while as regards the *Brassica* wild relatives (n=9) populations of six have been taken in consideration *B. balearica*, *B. bourgeana*, *B. desnottesi, B. drepanensis, B. hilarionis, B. incana* (4 accessions), *B. macrocarpa* (3 accessions), *B. montana, B. rupestris* (3 accessions), *B. souliei, B. tirrenica, B. tournefort, B. villosa* (2 accessions).

. These Brassica wild populations are represented by very rustic perennials plants that can colonize hostile and inaccessible environments such as the rocky slopes of hills, and of mountains, but which can also be widespread at the base of rocks and in more fertile soils. The diffusion in the most fertile soils is limited by the presence of herbivorous animals that are greedy for it, and it is for this reason that these populations

are often taken in the rocky slopes inaccessible to the animals. The spread of these populations in marginal environments both for soil fertility and availability of water and for extreme thermal conditions, which during the summer months determine thermal values above 40 ° C for a few weeks, determines the interest in them in recent years. for the identification of potential sources of resistance against biotic and abiotic stresses.

Water stress negatively affects the crops afferent to B. oleracea both as regards the production of vegetative organs (cabbage, kale, kohlrabi) and reproductive organs (broccoli and cauliflower). For the former, certainly the leaves are the most sensitive organs due to the transpiration process which causes the loss of water from the plant to the atmosphere and consequently the cellular turgor of the tissues of the leaves, shoots, and stem. For crops from reproductive organs, water stress interacts both on the growth of the first plant, on the size of the inflorescence and on the quality of the latter.

\Based on these considerations, the third line of research was activated as part of the activities of my doctoral thesis which had the aim of analysing a set of accessions of the *Brassica* core collection of the BRESOV project to identify genotypes resistant to water. stress and to understand the genetic basis of these resistances through the study of the transcriptomes provided by the RNA of the resistant genotypes compared with the resistant ones.

5.2.3.2 Materials and Methods

Plant material

During the second year of my PhD this trial started. Four genotypes were selected from different accession of brassica oleracea and brassica wild (Table 5.2.3.1) for their drought resistance after preliminary drought resistance screening above the accession reported.

Table 5.2.3.1 List of accession, in green row the 4 selected genotypes.

ACCESSION CODE	COMMON NAME
BB 2	B. balearica
BBO 4	B. bourgeana
BD 4	B. drapanensis
BDE1	B. desnottesi
BH 1	B. oleracea var. acephala
BH 10	B. oleracea var. acephala
BH 100	B. oleracea var. acephala
BH 14	B. oleracea var. acephala

BH 50	B. oleracea var. acephala
BHI 42	B. hilarionis
BI 1	B. insular
BM 28	B. macrocarpa
BM 30	B. macrocarpa
BM 6	B. macrocarpa
BN 10	B. nigra
BR 100	B. oleracea var. italica
BR 127	B. oleracea var. italica
BR 15	B. oleracea var. italica
BR 206	B. oleracea var. italica
BR 211	B. oleracea var. italica
BR 325	B. oleracea var. italica
BR 354	B. oleracea var. italica
BR 358	B. oleracea var. italica
BR 359	B. oleracea var. italica
BR 360	B. oleracea var. italica
BR 364	B. oleracea var. italica
BR 365	B. oleracea var. italica
BR 367	B. oleracea var. italica
BR 368	B. oleracea var. italica
BR 369	B. oleracea var. italica
BR 37	B. oleracea var. italica
BR 370	B. oleracea var. italica
BR 41	B. oleracea var. italica
BR 41	B. oleracea var. italica
BR 80	B. oleracea var. italica
BSO 2	B. souliei
BT 5	B. tournefort
BTR 1	B. tirrenica
BU 10	B. rupestris
BU 19	B. rupestris
BU 3	B. rupestris
BV 6	B. villosa
BV 7	B. villosa
BX18	B. montana
BY 7	B. incana
BY15	B. incana
BY6	B. incana
CA 8	B. oleracea var. sabauda
CC42	B. oleracea var. capitata
CR 34	B. oleracea var. gongylodes
CR 50	B. oleracea var. gongylodes
CR 52	B. oleracea var. gongylodes
CRI09H2200006	B. oleracea var.gongylodes

CRI09H2200023	B. oleracea var. gongylodes
CV 238	B. oleracea var. botrytis
CV 239	B. oleracea var. botrytis
CV 24	B. oleracea var. botrytis
CV 240	B. oleracea var. botrytis
CV 241	B. oleracea var. botrytis
CV 242	B. oleracea var. botrytis
CV 243	B. oleracea var. botrytis
CV 244	B. oleracea var. botrytis
CV 245	B. oleracea var. botrytis
CV 246	B. oleracea var. botrytis
CV 247	B. oleracea var. botrytis
CV 249	B. oleracea var. botrytis
CV 25	B. oleracea var. botrytis
CV 250	B. oleracea var. botrytis
CV 26	B. oleracea var. botrytis
CV192	B. oleracea var. botrytis
CV194	B. oleracea var. botrytis
HRIGRU12936	B. oleracea var. gongylodes
HRIGRU12942	B. oleracea var. gongylodes
HRIGRU2405	B. oleracea var. italica
HRIGRU3475	B. oleracea var. gongylodes
HRIGRU3597	B. oleracea var. acephala ricci
HRIGRU4690	B. oleracea var. tronchuda
HRIGRU4716	B. oleracea var. italica
HRIGRU4887	B. oleracea var. acephala
HRIGRU5416	B. oleracea var. italica
HRIGRU5567	B. oleracea var. capitata
HRIGRU6211	B. oleracea var. gongylodes
HRIGRU6226	B. oleracea var. acephala
HRIGRU6229	B. oleracea var. acephala
HRIGRU6421	B. oleracea var. acephala
HRIGRU7432	B. oleracea var. italica
HRIGRU7546	B. oleracea var. acephala
HRIGRU7547	B. oleracea var. acephala
HRIGRU7552	B. oleracea var. alboglabra
HRIGRU8302	B. oleracea var. gemmifera
UNICT5040	B. oleracea var.italica x B. olearacea var, botrytis
UNICT5041	B. oleracea var.italica x B. olearacea var, botrytis
UNICT5042	B. oleracea var.italica x B. olearacea var, botrytis
UNICT5043	B. oleracea var.italica x B. olearacea var, botrytis
UNICT5044	B. oleracea var.italica x B. olearacea var, botrytis
UNICT5048	B. oleracea var.italica x B. olearacea var, botrytis
UNICT5049	B. oleracea var.italica x B. olearacea var, botrytis
UNICT5050	B. oleracea var.italica x B. olearacea var, botrytis
UNICT5051	B. oleracea var.italica x B. olearacea var, botrytis

UNICT5052	B. oleracea var.italica x B. olearacea var, botrytis
UNICT5056	B. oleracea var.italica x B. olearacea var, botrytis
UNICT5057	B. oleracea var. italica x B. olearacea var, botrytis
UNICT5060	B. oleracea var. autofecondata
UNICT5061	B. oleracea var. autofecondata
UNICT5062	B. oleracea var. autofecondata
UNICT5063	B. oleracea var. autofecondata
UNICT5065	B. oleracea var. autofecondata
UNICT5066	B. oleracea var. autofecondata
UNICT5067	B. oleracea var. autofecondata
UNICT5068	B. oleracea var. autofecondata
UNICT5069	B. oleracea var. autofecondata
UNICT5070	B. oleracea var. autofecondata
UNICT5071	B. oleracea var. autofecondata
UNICT5072	B. oleracea var. autofecondata
UNICT5073	B. oleracea var. autofecondata
UNICT5075	B. oleracea var. autofecondata
UNICT5076	B. oleracea var. autofecondata
UNICT5077	B. oleracea var. autofecondata

The plants were sowing in June 2020 in containers in an open green house in organic conditions. After one month the plants were transplanting in 5 L (20 cm diameter) pots on 14th of July, in an experimental field placed in Catania, Via Valdisavoia 3, (37° 31' 5.59" N 15° 4' 12.23" E).

The temperature was recorder during the trial for 5 days the average temperature was about 26 °C (Figure 5.2.3.1). The resistance plants selected were two accession of *Brassica macrocarpa* originated from Marettimo and Favignana islands such as resistant plants in addition to *settembrino* broccoli (*B. oleracea* var. *italica*). Concerning sensible plants, *maiolino* broccoli (*B. oleracea*. var. *italica*) were selected (Figure 1) (Table 2).

The growing substrate was composed of two parts of organic soil BRILL© and one part by volcanic coarse sand. The irrigation was performed exclusively in the control pots with 50 ml $days^{-1}$ $pots^{-1}$. Maximum. Minimum and average temperatures were registered and leaf SPAD data were registered plant by plant for evaluating its chlorophyll amount and then the effect of the water stress the physiological status of the plant (Table 3). Plantlets were also analysed detecting the leaves number (green, chlorotic, and necrotic ones) and a score was attributed for their drought resistance from the less tolerant to the best (0,1,2, respectively).

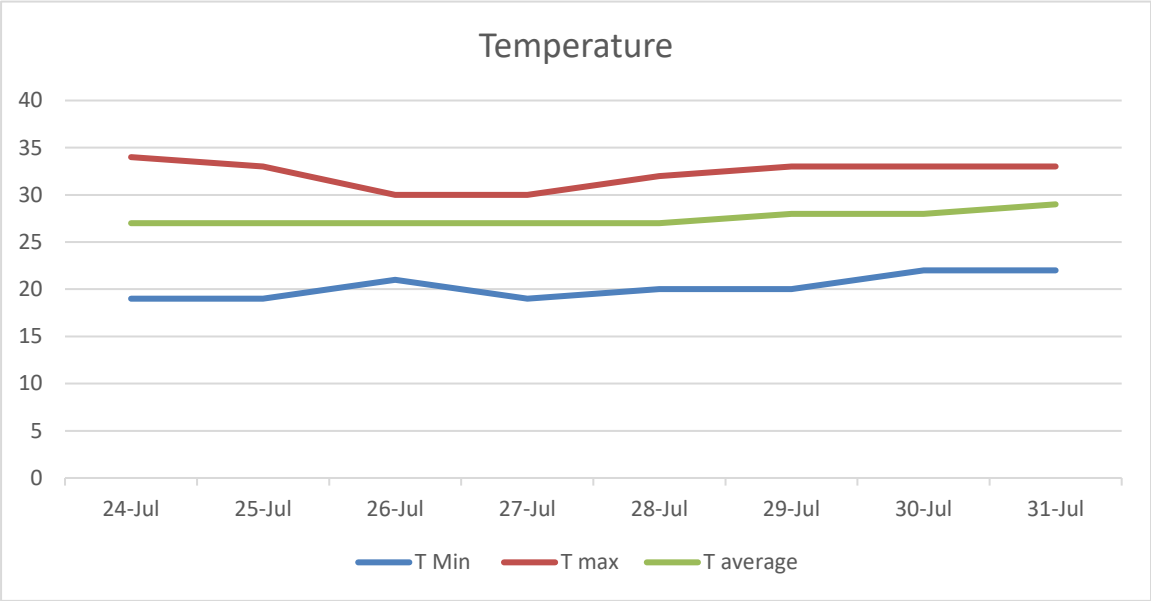


Figure 5.2.3.1. Daily maximum, minimum and average temperature (°C)

This study provides the use of four different varieties, in particular *Brassica macrocarpa* of Marettimo (FL), *Brassica macrocarpa* of Favignana (FK), *Brassica oleracea* var. italica lettering (BY) and *Brassica oleracea* var. italica maiolino (BW).

Figure: 5.2.3.2 *Brassica macrocarpa* di Marettimo (FL), *Brassica oleracea* var. italica settembrino (BY), Brassica *macrocarpa* di Favignana (FK), *Brassica oleracea* var. italica maiolino(BW)

Each variety includes six biological replicates: three controls (CK) and three stressed (DR) plants.The treatment provided the regular irrigation of the plants starting from the day of the transplant until July 24th, 2020, meanwhile the control plants were irrigated with 50 cl of water, instead of the stressed plants that were not irrigated at all.

Physiological characterization

SPAD index at the end of the trial was measured on 20 fully expanded leaves in 3 plants treatment (3 plants replication) by means of a portable chlorophyll meter SPAD-502 (Minolta Camera Co., Osaka, Japan). SCORE index to individuate the sensible (0) and the resistance genotype (2).

RNA extraction

The RNA was extracted using the Spectrum Plant Total RNA kit (Sigma-aldrich®, Saint Louis, MO, USA) and then its purity and concentration were evaluated by spectrophotometer at 260/280 nm wavelengths using Nano Drop ® ND 1000 (Nano Drop Technologies, Wilmington, DE, USA).

Malonildialdehyde (MDA) content

Malonildialdehyde (MDA) is an organic compound resulting from lipid peroxidation of polyunsaturated fatty acids, which are the main components of cellular membrane. The ROSs (reactive oxygen species) degrade polyunsaturated lipids to form malondialdehyde, which is considered an important marker of oxidative stress in cells. The MDA quantification protocol was derived from Hodges et al. (1999) and Landi (2017). Two hundred fifty microlitres of supernatant were transferred to a new 1.5 ml screw cap microcentrifuge tube with an equal volume of positive reaction solution [0.5% TBA in 20% (w/v)] trichloroacetic acid (TCA), and other 250 µl of supernatants were added to an equal volume of negative reaction solution [20% (w/v) TCA]. Blank tubes consisted of 250µl of 80% ethanol with 250µl of positive reaction solution or negative reaction solution. The tubes were incubated at 95°C for 30 min in a heat block (Eppendorf Thermomixer Compact5,350 Mixer). The reaction tubes were cooled at room temperature (RT) and centrifuged at 3,000g for 10 min at 4°C and the supernatant was recovered. One hundred fifty microlitres of each sample or blank were transferred to a clear 96-well microplate and the absorbance of each well was measured at 440, 532 and 600 nm. MDA1 equivalents were calculated in (nmol/ml) as $(A - B)/157.000) \times 10^6$, where $A = [(A532+_{TBA}) - A600+_{TBA}) - (A532-_{TBA} - A600 -_{TBA})]$ and $B = [(A440+_{TBA} - A600+_{TBA}) \times 0.0571]$. Each absorbance read was repeated three times.

Amount of Hydrogen Peroxide (H_2O_2)

Hydrogen Peroxide is commonly produced in plants during normal physiological processes and in response to stressful situations. Many studies show particular attention to determination of the amount of H_2O_2, which is a reactive oxygen species (ROS), and therefore it is involved in oxidative processes.

The amount of hydrogen peroxide in the 24 accessions of Brassicaceae was determined according to the method described in (Velikova et al. 2020)

Transcriptome analysis

On July 28th, 2020, fully expanded, leaves of each genotype were collected and immediately frozen with liquid nitrogen. RNA isolation was carried out by using the Spectrum Plant Total RNA Extraction kit (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions. RNA degradation and contamination were monitored by electrophoresis with 1% agarose gel. RNA purity and concentration were assayed using the Nano Drop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Before to be sequenced, the RNA samples were subjected to quality parameter evaluation. RNA integrity was assessed using the Agilent Bioanalyzer 2100 system (Agilent Technologies, Santa Clara, CA, USA).

Library preparation for transcriptome sequencing

One µg of RNA was used as input material for library preparations. Sequencing libraries were generated using NEB Next ® Ultra ™ RNA Library Prep Kit for Illumina® (New England Biolabs, Ipswich, MA, USA) following manufacturer's recommendations. Briefly, mRNA was purified from total RNA using poly-T oligo-attached magnetic beads. Fragmentation was carried out using divalent cations under elevated temperature in NEB Next First Strand Synthesis Reaction Buffer (5X). First strand cDNA was synthesized using random hexamer primer and M-MulV Reverse Transcriptase (RNase H) as synthesizing enzyme. Second strand cDNA synthesis was subsequently performed using RNase H to insert breaks into the RNA molecule and DNA Polymerase I as synthesizing enzyme. Remaining overhangs were converted into blunt ends via exonuclease/polymerase activities. After adenylation of 3′ ends of DNA fragments, NEBNext Adaptor with hairpin loop structure were ligated to prepare for hybridization. To select cDNA fragments of preferentially 150~200 bp in length, the library fragments were purified with AMPure XP system (Beckman Coulter, Beverly, MA, USA). Then 3 µl USER Enzyme by NEB were used with size-selected, adaptor-ligated cDNA at 37 °C for 15 min followed by 5 min at 95 °C before PCR. Then PCR was performed with Phusion High-Fidelity DNA polymerase, Universal PCR primers and Index (X) Primer. Finally, PCR products were purified (AMPure XP system) and library quality was assessed on the Agilent Bioanalyzer 2100 system.

Clustering and next generation RNA sequencing

Cluster generation and sequencing were performed by Novogene (UK) company Limited (25 Cambridge Park, Milton Road, Cambridge, CB4 OFW, United Kingdom). The clustering of the index-coded samples was performed on a cBot Cluster Generation System using a PE Cluster kit cBot-HS (Illumina). After cluster generation, the library preparations were sequenced on Illumina HiSeq2000 platform to generate pair-end reads. Raw data (raw reads) in fastq format were firstly processed through in-house perl scripts. In this step, clean data were obtained by removing reads containing adapters, reads containing poly-N and low-quality reads. At the same time, Q20, Q30, GC-content and sequence duplication level of the clean data were calculated. All the downstream analyses

were based on clean data with high quality.

De novo assembly and gene functional annotation

De novo transcriptome assembly was made up by Trinity software (2.6.6 version) with min_Kmer_Cov = 3 and min_glue = 4. Hierarchical Clustering was carried out by Corset (4.6 version) to remove redundancy (parameter -m 10), so that the longest transcript of each cluster has been selected as Unigene. The assembly assessment and gene prediction have been performed by Benchmarking Universal Single-Copy Orthologous (BUSCO software, 3.0.2 version), whereas the gene functional annotation was obtained by exploiting seven different databases: National Centre for Biotechnology Information (NCBI), non-redundant protein sequences (Nr, Diamond software, 0.8.22 version, e-value threshold 1e-5), NCBI non-redundant nucleotide sequences (Nt, NCBI blast software, 2.9.0 version, e-value threshold 1e-5), Protein family (Pfam, hmmscan software, HMMER 3.1 version, e-value threshold 0.01), Cluster of Orthologous Groups of Proteins (KOG/COG, Diamond software, 0.8.22 version, e-value threshold 1e-5), Swiss-Prot (Diamond software, 0.8.22 version, e-value threshold 1e-5), Kyoto Encyclopedia of Genes and Genome (KEGG, Diamond and KAAS software, 0.8.22 version, e-value threshold 1e-5) and Gene Ontology (GO, blast2GO software, b2g4pipe_v2.5 version, e-value threshold 1e-6). To identify the transcription factor, iTAK (hmmerscan software) tool was used to infer the TF families (Pérez-Rodríguez et al. 2009; Jin et al. 2014).

Quantification of gene expression and differential expression analysis

Gene expression level was estimated by RSEM software (1.2.28 version) by mapping back each clean read onto assembled transcriptome and readcounts for each gene were then obtained from the mapping results. Furthermore, the readcounts of each gene have been used as input data for DESeq2 (1.26 version, padj ≤ 0.05), to obtain differentially expressed genes (DEGs). Comparisons were made to identify the set of differentially expressed genes between control (ck) and water stress (dr) treatments for each comparison (FL_ck vs FL_dr; FK_ck vs FK_dr; BY_ck vs BY_dr; BW_ck vs BW_dr). An adjusted p-value cutoff of 0.05 and a $\log_2$ fold change (Log_2FC) threshold of +/- 5 was adopted to filter the significantly up- and down-regulated genes. A correlation analysis was performed to demonstrate experiment repeatability and to reveal differences in gene expression among samples. Principal Component Analysis 3D plot and a heatmap were obtained by using R language, considering as input data the read counts of each sample, including biological replicates.

Real-Time validation of selected DEG candidates using qRT-PCR

Gene ontology and KEGG enrichment analysis

Based on differentially expressed genes (DEGs), the GO enrichment was accomplished by using blast2go (b2g4pipe_v2.5 version) software (e-value = 1e-6). Furthermore, to analyse the *brassica* transcriptome, all the unigenes were submitted to KEGG database for the systematic analysis of gene function. KOBAS software (v.3.0, corrected p-value ≤ 0.05) has been applied to test the statistical enrichment of differentially expressed genes

in KEGG pathway.

5.2.3.3. Results and discussion

Physiological characterization

The SPAD and SCORE show the most sensitive and resistant genotype. Four genotypes were selected in three replicates for the two theses: the control and the water stress in total were analyzed twenty-four plants. Two genotypes of wild species Brassica macrocarpa from Marettimo and one from Favignana from the islands Egadi and two genotypes of Brassica oleracea var. Italica one called Maiolino which were the resistance and one called Settembrino the sensible

Table 5.2.1.3; plantlets tested for their drought sensible and resistance before preliminary screening; green rows indicating drought resistant genotypes.

ACCESSION CODE	WATER STRESSED			IRRIGATED
	SPAD	ΔSPAD	SCORE	SPAD
BB1	22,7	80,78	1	28,1
BB2	27,4	86,63	1	31,63
BBO4	34,5	116,95	1	29,5
BD4	31,7	76,39	1	41,5
BDE1	31,2	76,28	1	40,9
BH 10	47,65	93,63	1	50,89
BH 100	33,1	80,73	1	41
BH 14	40,9	90,11	0	45,39
BH 1R	40,12	112,48	0	35,67
BH 50	40,2	106,32	1	37,81
BHI SP42	33,5	95,14	1	35,21
BI1	31,3	80,67	0	38,8
BM 28	28,3	81,56	2	34,7
BM 30	21,9	62,39	2	35,1
BM 6	27,7	71,76	0	38,6
BN 10	29,6	75,25	1	39,33333
BR 100	57,21	130,35	1	43,89
BR 127	43,72	124,38	0	35,15
BR 15	36,43	78,65	1	46,32
BR 206	43,10	163,94	0	26,29
BR 211	35,1	86,54	1	40,56
BR 325	41,38	111,39	0	37,15
BR 354	38,74	98,03	0	39,52
BR 359	42,3	102,42	1	41,3
BR 360	35,3	91,93	2	38,4
BR 364	29	72,86	1	39,8

BR 365	38,2	92,05	0	41,5
BR 367	33,9	99,41	1	34,1
BR 368	28,4	74,35	0	38,2
BR 369	27,9	72,28	0	38,6
BR 37	29,21	70,69	1	41,32
BR 370	34,7	90,84	1	38,2
BR 41	28,3	71,28	0	39,7
BR 41 S2	43,12	108,61	1	39,7
BR 80	34,19	93,62	0	36,52
BR358	38,16	65,68	1	58,1
BSO2	28,2	65,89	0	42,8
BT5	29,26	-	1	37,7
BU 10	39,7	114,74	1	34,6
BU 19	21,9	57,78	1	37,9
BU 3	30,26	73,79	1	41,01
BV 7	28,51	93,11	0	30,62
BV6 P1B	35,31	87,84	1	40,2
BX18	37,5	119,43	1	31,4
BY 7	39,1	134,18	0	29,14
BY15	41,52	132,95	0	31,23
BY6 P2	35,72	93,70	0	38,12
CA8	33,2	75,11	1	44,2
CC42	34,3	86,18	1	39,8
CR 34	32,2	86,33	1	37,3
CR 50		0,00	0	39,9
CR 52	34,2	92,18	1	37,1
CRI09H2200006	35,12	117,46	1	29,90
CRI09H2200023	32,22	100,37	0	32,1
CV 238	29,8	71,29	1	41,8
CV 239	32,3	73,74	1	43,8
CV 24	41,4	104,02	0	39,8
CV 240	37,1	82,63	1	44,9
CV 241	33,2	83,21	0	39,9
CV 242	31	66,38	1	46,7
CV 243	29,6	70,14	1	42,2
CV 244	31,4	78,89	0	39,8
CV 245	29,3	67,51	1	43,4
CV 247	33,1	90,68	1	36,5
CV 249	30,3	91,27	1	33,2
CV 250	35,1	105,72	0	33,2
CV 26	40,9	65,86	0	62,1
CV192	45,2	93,39	1	48,4
CV194	41,8	81,01	1	51,6

CV246	34,8	77,51	1	44,9
CV25S3P2	42,3	82,30	1	51,4
HRIGRU12936	34,34	87,83	0	39,1
HRIGRU12942	32,41	89,28	1	36,3
HRIGRU2405	35,57	130,29	0	27,3
HRIGRU3475	43,14	137,26	0	31,43
HRIGRU3597	36,57	77,64	1	47,1
HRIGRU4690	42,51	121,94	0	34,86
HRIGRU4716	41,21	131,87	0	31,25
HRIGRU4887	35,25	121,55	0	29.00
HRIGRU5416	28,1	70,21	0	40,02
HRIGRU5567	50,42	144,39	0	34,92
HRIGRU6211	32,31	100,37	1	32,19
HRIGRU6226	43,43	105,39	1	41,21
HRIGRU6229	28,44	78,74	1	36,12
HRIGRU6421	27,54	56,07	1	49,12
HRIGRU7432	32,11	85,99	0	37,34
HRIGRU7546	28,13	67,64	1	41,59
HRIGRU7547	31,31	89,46	0	35.00
HRIGRU7552	43,11	135,10	1	31,91
HRIGRU8302	31,61	69,79	0	45,29
UNICT5040	33,2	80,00	0	41,5
UNICT5041	34,3	77,60	1	44,2
UNICT5042	32,2	93,33	1	34,5
UNICT5043	45,2	140,81	1	32,1
UNICT5044	41,8	91,27	0	45,8
UNICT5048	39,3	89,12	0	44,1
UNICT5049	36,8	95,83	1	38,4
UNICT5050	34,3	76,56	1	44,8
UNICT5051	25,6	66,67	0	38,4
UNICT5052	36	73,62	1	48,9
UNICT5056	34,5	74,84	0	46,1
UNICT5057	27,2	66,18	1	41,1
UNICT5060	41,2	100,73	1	40,9
UNICT5061	36,4	93,81	1	38,8
UNICT5062	33,1	99,70	0	33,2
UNICT5063	35	84,54	1	41,4
UNICT5065	24,8	69,27	1	35,8
UNICT5066	29,1	81,28	0	35,8
UNICT5067	30,7	73,62	1	41,7
UNICT5068	22,2	58,27	1	38,1
UNICT5069	27,5	74,12	1	37,1
UNICT5070	32,1	84,25	0	38,1

UNICT5071	26,5	64,01	1	41,4
UNICT5072	30,4	66,67	1	45,6
UNICT5073	32	81,84	0	39,1
UNICT5075	31,8	93,81	1	33,9
UNICT5076	31,8	98,76	1	32,2
UNICT5077	33,7	93,87	0	35,9

Figure 5.2.3.5 Leaf resistant and sensible

MDA analysis

Two histograms were developed for each variety to represent the results. The first one compares the MDA values between biological replicates. On the X-axis three control replicates (ck) and three stressed replicates (dr) are reported; while the Y-axis represents the MDA values expressed in ng/mg of fresh tissue. The second histogram was obtained from the mean of the values of biological replicates for both treatments. Only The second histogram is shown below.

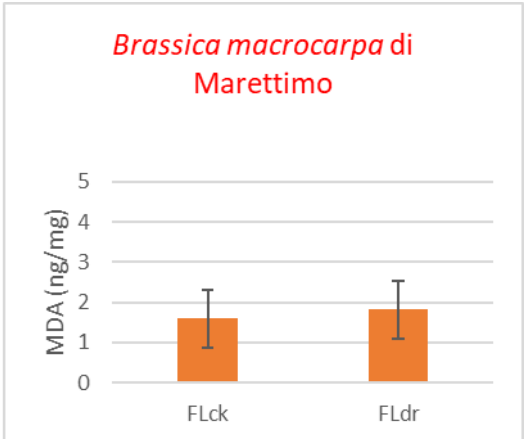


Figure 5.2.3.6. *Brassica macrocarpa* of Marettimo

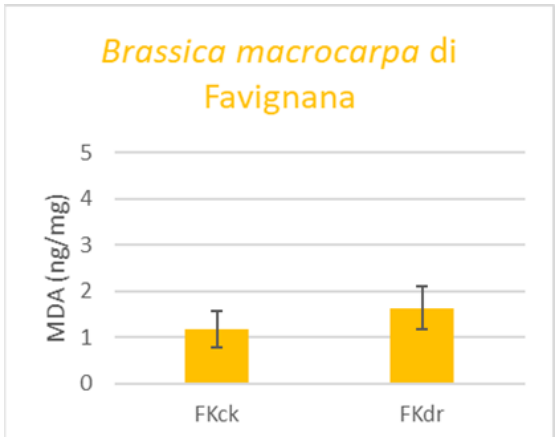


Figure 5.2.3.7. *Brassica macrocarpa* of Favignana

The stressed biological replicates (dr) show higher values than control replicates (ck). However, this difference is not statistically significant.".

The average of stressed biological replicates (dr) reveals higher amounts of MDA than the mean of the control replicates (ck). However, the difference between the control and the stressed plants is not statistically significant.

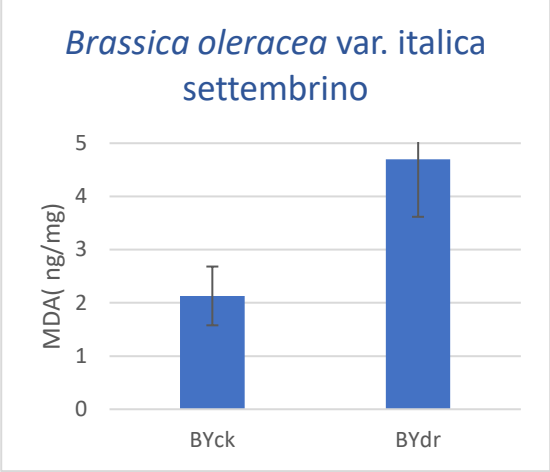


Figure 5.2.3.8 *"Brassica o.*var. italica settembrino"

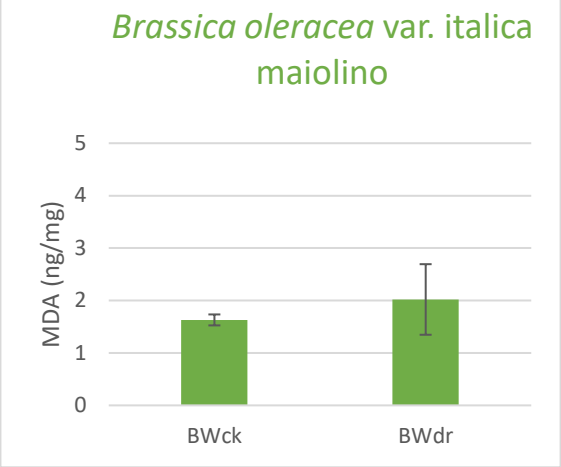


Figure5.2.3.9"*Brassica o.* var. *italica* maiolino''

Stressed biological replicates (dr) have significantly higher values than their control replicates (ck). In fact, the statistical analysis ANOVA confirms the difference between treatments ($p < 0,005$).

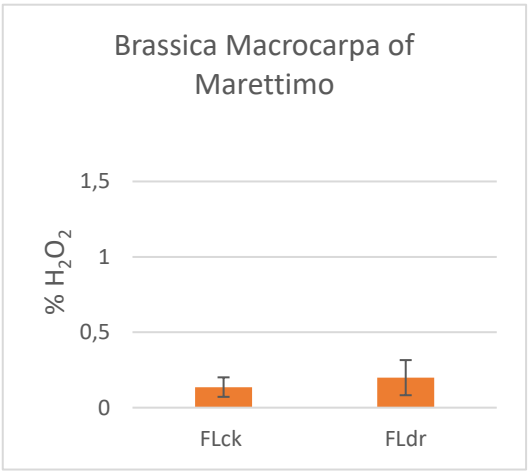
The average values of stressed biological replicates (dr) have higher amounts of MDA than the controls (ck). However, this difference is not statistically significant.

These results showed important differences about the response of the four varieties to the treatment.

Brassica macrocarpa of Marettimo and *Brassica macrocarpa* of Favignana, though phenotypical and MDA analysis, seem to be more tolerant than the two *Brassica oleracea* landraces. Therefore, the results obtained by the MDA analysis confirm what was observed at phenotypic level. Differently, *Brassica oleracea* var. italica settembrino and *Brassica oleracea* var. italica maiolino, show considerable differences in the phenotype and in the MDA results. In fact, the stressed biological replicates of the *Brassica oleracea* var. italica settembrino presents more phenotypic suffering and higher levels of MDA than the respective control replicates than of the Brassica *oleracea* var. italica maiolino one, resulting in higher sensitivity to the water stress.

Amount of Hydrogen Peroxide (H_2O_2)

For representing the results, two histograms were developed for each variety. In the first histogram the values of H_2O_2 between biological replicates were compared: on the X-axis the three control (ck) and stressed (dr) replicates are reported respectively, while on the Y-axis the percentages of H_2O_2 are reported. The second histogram was obtained from the mean values of biological replicates for both treatments. Only the second histogram is shown below.



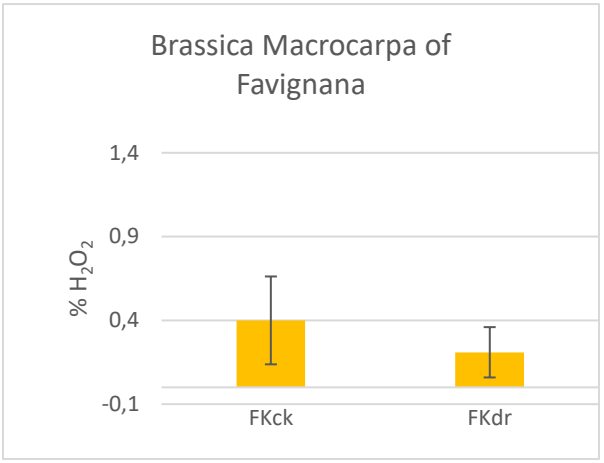
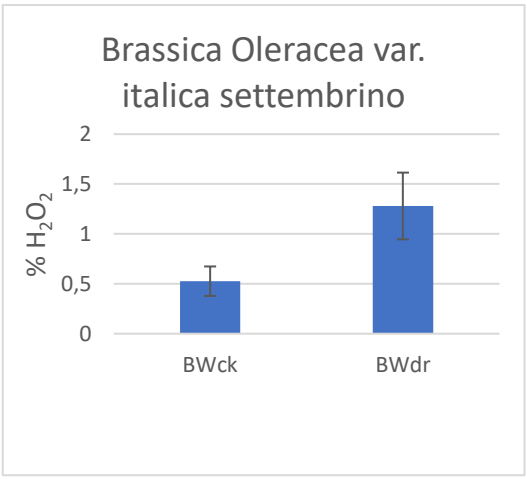


Figure 5.2.3.10. *Brassica macrocarpa* of Marettimo Figure 5.2.3.11 *Brassica macrocarpa* of Favignana".

The mean of the values of stressed biological replicates (dr) shows percentages of H_2O_2 slightly higher than the control replicates (ck). However, this difference is not statistically significant.

The mean values of stressed biological replicates (dr) reveal lower amounts of H_2O_2 than the control replicates (ck). Although hydrogen peroxide is considered an indicator of plant stress, *Brassica macrocarpa* of Favignana shows abnormal % of H_2O_2, probably caused by the particularly resistant genotype.



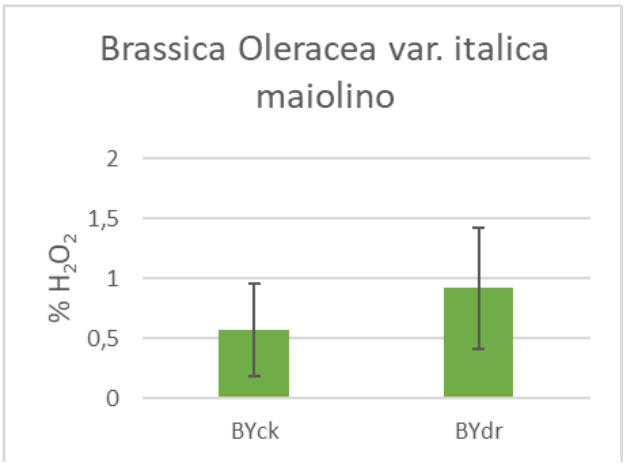


Figure 5.2.3.11 *Brassica O.*var. *italica* Settembrino" Figure 5.2.3.12"*Brassica O.* var. *italica* maiolino"

The stressed biological replicates (dr) have % of H_2O_2 significantly higher than control replicates (ck). In fact, the ANOVA statistical analysis confirms the difference between accession ($p < 0,005$).

The stressed biological replicates (dr) have higher amounts of H_2O_2 than control replicates (ck). However, this difference is not statistically significant.

Considering the results obtained, it is possible to confirm that the varieties *Brassica*

macrocarpa of Marettimo and *Brassica macrocarpa* of Favignana seem to be more tolerant to water stress than the two landraces *Brassica oleracea* var. italica settembrino and maiolino; the latter one seems in any case more resistant to water stress than the former.
Whereas, both *Brassica oleracea* var. italica settembrino and maiolino landraces are more sensitive to water stress, with considerable differences between control and stressed plants. These differences are clearly visible phenotypically and from the amount of H_2O_2. According to the previous analysis on the amount of MDA, *Brassica oleracea* var. italica settembrino seems to be the variety more sensitive to stress. These values are confirmed from the statistical analysis ANOVA that has a significant value (< 0,005).

Transcriptomic results

In the present study, we have performed a comprehensive identification of the transcriptional responses of the four *B. oleracea* complex species (n=9) genotypes in leaves by RNA-Seq approach. A flowchart of the pipeline for the *Brassica* leaf transcriptome sequencing and *de novo* assembly is reported in Figure 9. Before sequencing, RNA integrity was checked and the average RNA integrity number (RIN) was quite good, thus indicating that all the samples had adequate quality to be further processed and sequenced (data not shown). After sequencing, raw reads were filtered to remove reads containing adapters or reads of low quality. *De novo* assembly of clean reads resulted in 269,191 transcripts and 74,587 unigenes with N50 length of 1713 bp and 1654 bp, respectively, in line with previously reported N50 values (Evangelistella et al. 2017; Fu et al. 2016; Sicilia et al. 2019; Sicilia et al. 2020). To evaluate the assembly consistency, the filtered unique reads were mapped back into the reconstructed transcriptome using the alignment software bowtie2. Consequently, these results indicated that the sequencing quality was reliable to perform downstream analysis.

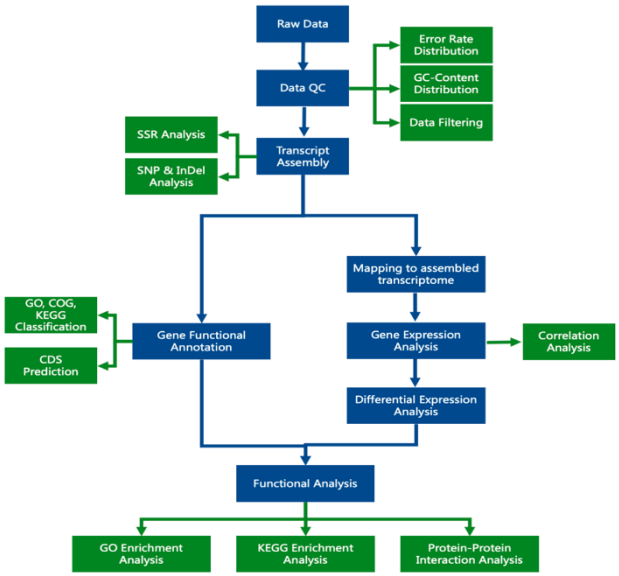


Figure5.2.3.13 The analysis workflow for species without a reference genome.

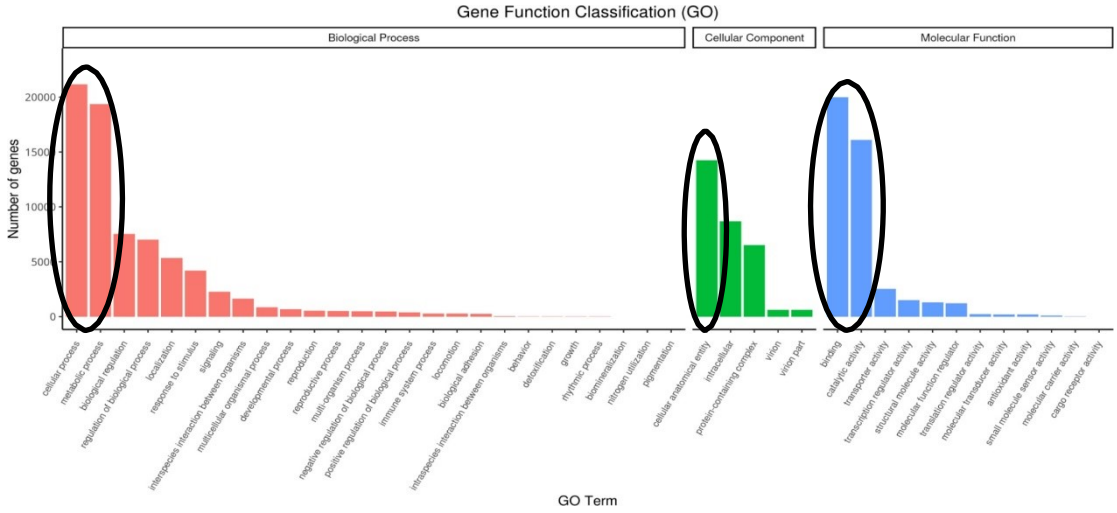
All assembled unigenes were blasted into public databases, including National Center for Biotechnology Information (NCBI), Protein family (Pfam), Clusters of Orthologous Groups of proteins (KOG/COG), SwissProt, Ortholog database (KO) and Gene Ontology (GO) (Table 23). A total of 88,139 unigenes were annotated in at least one searched database; the frequency of unigenes annotated in at least one database was 99.02 %. Among them, 61,047 (81.84 %) and 72,720 (97.49 %) assembled unigenes showed identity with sequences in the Nr and Nt database, respectively. The distribution of assembled unigenes homologous to sequences in KO, Swiss-Prot, Pfam, GO and KEGG databases were 27.94, 57.61, 48.01, 48.01 and 14.79%, respectively (Table5.2.1.4)

Table 5.2.1.4

Statistical Items	Number of Unigenes	Percentage (%)
Annotated in NR	61047	81.84
Annotated in NT	72720	97.49
Annotated in KO	20843	27.94
Annotated in SwissProt	42972	57.61
Annotated in PFAM	35816	48.01
Annotated in GO	35813	48.01
Annotated in KOG	11033	14.79
Annotated in all Databases	6819	9.14
Annotated in at least one Database	73857	99.02
Total Unigenes	74587	100

GO Classification

Gene Ontology (GO) terms was carried out to identify biological processes involved in water stress response. Considering the whole GO enrichment dataset emerged that the "cellular process", "metabolic process", "biological regulation" and "regulation of biological process" were the three most enriched GO terms found in biological process category. For the cellular component (CG) category the result showed that "cellular anatomical entity"," intracellular "," protein containing process" were the most enriched terms. As regards the Molecular Function (MF) category we observed that binding and



catalytic activity were found (Figure5.2.3.14)

Figure5.2.3.14. GO Classification.

KOG Classification

To predict any possible functions, all identified unigenes (74,587) were aligned too the KOG database to assign their corresponding KOG category (Figure x). Among the KOG categories, those clusters encoding for "Posttranslational modification, protein turnover, chaperones", "Translation ribosomal structure and biogenesis", "General function prediction only" represented the largest group of categories (Figure5.2.3.15)

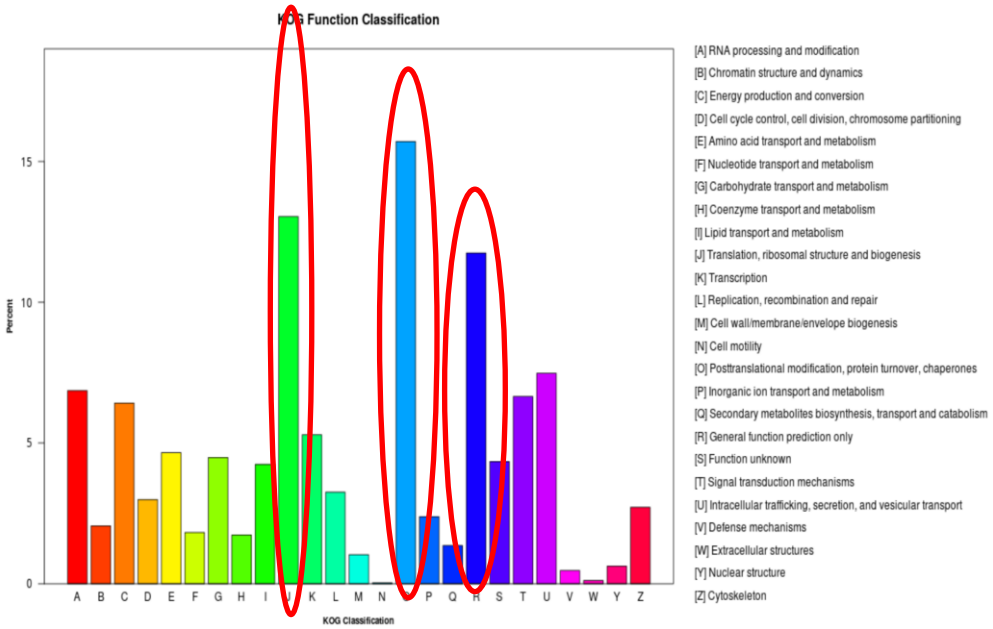


Figure5.2.3.15 KOG Classification.

The most representative genes are involved in Translation, Ribosomal structure and biogenesis, Posttranslational modification, Protein turnover, Chaperones and General function prediction only.

KEGG Classification

The sets of DEGs originated from the above-described three comparisons (FL_ck vs FL_dr; FK_ck vs FK_dr; BY_ck vs BY_dr; BW_ck vs BW_dr) were mapped onto KEGG enrichment pathways. The main KEGG pathway terms were plotted in the Figure 5.2.3.16. Considering the transcriptomic data, we observed that the most represented KEGG pathways were: "Signal transduction", "Translation", "Carbohydrate metabolism", "Transporter and

catabolism", "Energy metabolism", "Lipid metabolism", "Endocrine system" and "Cell growth and death" (Figure 5.2.3.16).

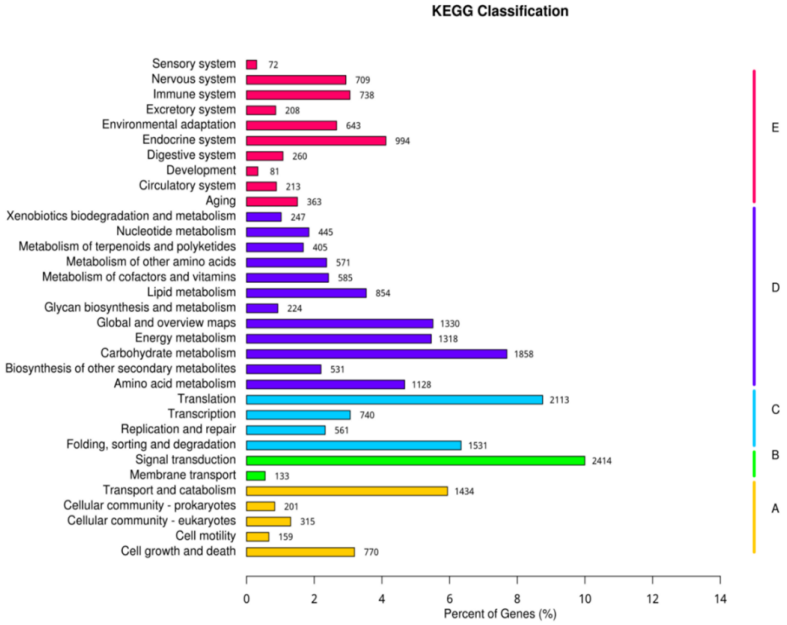


Figure5.2.3.16 KEGG Classification.

In the Y-axis the names of KEGG pathways and in the X-axis is the number of the genes annotated in the pathway and the ratio between the number in this pathway and the total number of annotated genes. The KEGG metabolic pathways gene involved in are divided into 5 branches: A: Cellular Processes, B, Environmental Information Processing, C: Genetic Information Processing, D: Metabolism, E: Organismal Systems.

Moreover, the most enriched pathways for each comparison are displayed (Figure 5.2.3.16 A, B, C, D, E, F, G,). Considering the FL (*B. macrocarpa* Marettimo) genotype, we observed that "transmembrane transporter activity", "transmembrane transport", "small molecule metabolic process" and "oxidoreductase activity" were the over-represented enriched metabolic pathways

Instead, the FK (*B. macrocarpa* Favignana) genotype showed that the" small molecule metabolic process", "oxidoreductase activity", "kinase activity" and "cellular protein modification process" were the most abundant metabolic pathways. By analysing the BY (*B. oleracea* var. *italica* Maiolino) genotype DEGs encoding for "transmembrane transporter activity", "transmembrane transport", "oxidoreductase activity" and "kinase activity" resulted over-regulate. In case of BW (*B. oleracea* var. *italica* Settembrino) genotype "oxidoreductase activity", "kinase activity", "cellular protein modification process" and "carbohydrate metabolic process" were the most enriched metabolic pathways

Considering the FL (*B. macrocarpa* Marettimo) genotype "plant hormone signal transduction", "carbon metabolism" and "biosynthesis of amino acids' " were the most enrichment terms. As regards the FK (*B. macrocarpa* Favignana) genotype, the most genes are involved in "plant hormone signal transduction", "peroxisome", "glyoxylate

and dicarboxylate metabolism", "carbon metabolism".

Considering the BY (*B. oleracea* var. *italica* Maiolino) genotype we observed that genes involved in "starch and sucrose metabolism" and "plant hormone signal transduction" were over-regulated. Finally, the BW (*B. oleracea* var. *italica* Settembrino) genotype reported that the most enriched metabolic pathways were "starch and sucrose metabolism", "plant hormone signal transduction", "pentose and glucuronate interconversions".

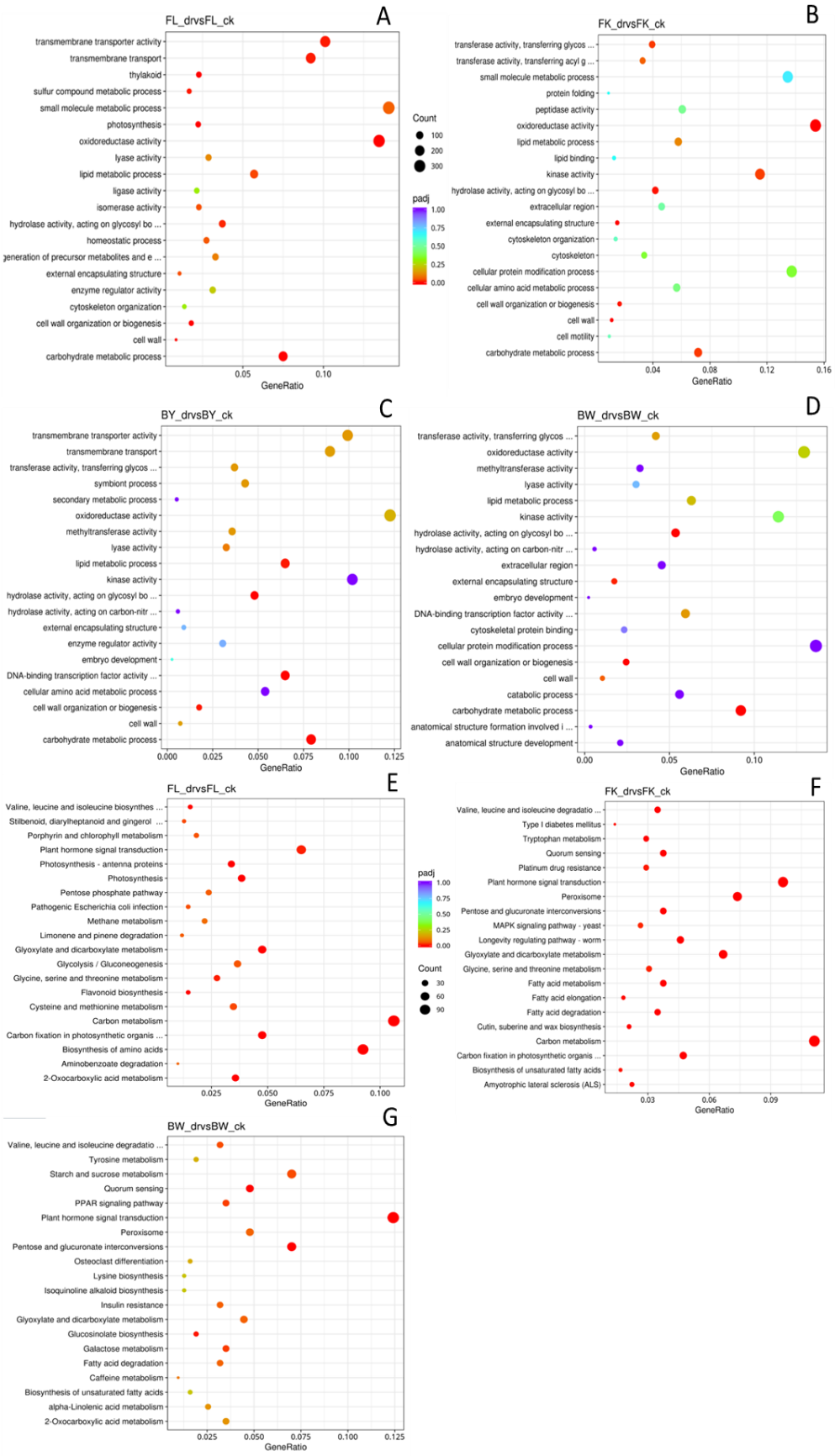


Figure 5.2.3.17 KEGG enriched pathways

Differential Expression Analysis

The characterization of *Brassica* transcriptional response to water stress was carried out by the identification of the unigenes whose expression level changed upon drought stress condition. According to the experimental design, a total of 4446 (1829 up- and 2617 down-regulated), 3184 (1555 up- and 1629 down-regulated) , 2890 (1323 up- and 1567 down-regulated) and 1591 (672 up- and 919 down-regulated) differentially expressed genes (DEGs) were identified in FL_dr vs FL_ck, FK_dr vs FK_ck, BY_dr vs BY_ck and BW_dr vs BW_ck, respectively.

Considering the FL sample data set, the Venn diagram analysis showed a total of 5071 genes are exclusively regulated under drought condition, while a total of 3707 genes are specifically regulated in control condition (Figure 5.2.3.17 A).

Regarding the FK sample data set, a total of 5535 genes were found upon drought condition, whereas a total of 4498 genes were observed in control condition (Figure 5.2.3.17 B). For the BY samples data showed a set of 4780 genus for the water stress and 4705 for the control (Figure 5.2.3.17 C). Instead, the accessions BW presented 32435 genes are in common, suggesting that their specific regulation is not depending by drought stress (Figure 5.2.3.17 D)

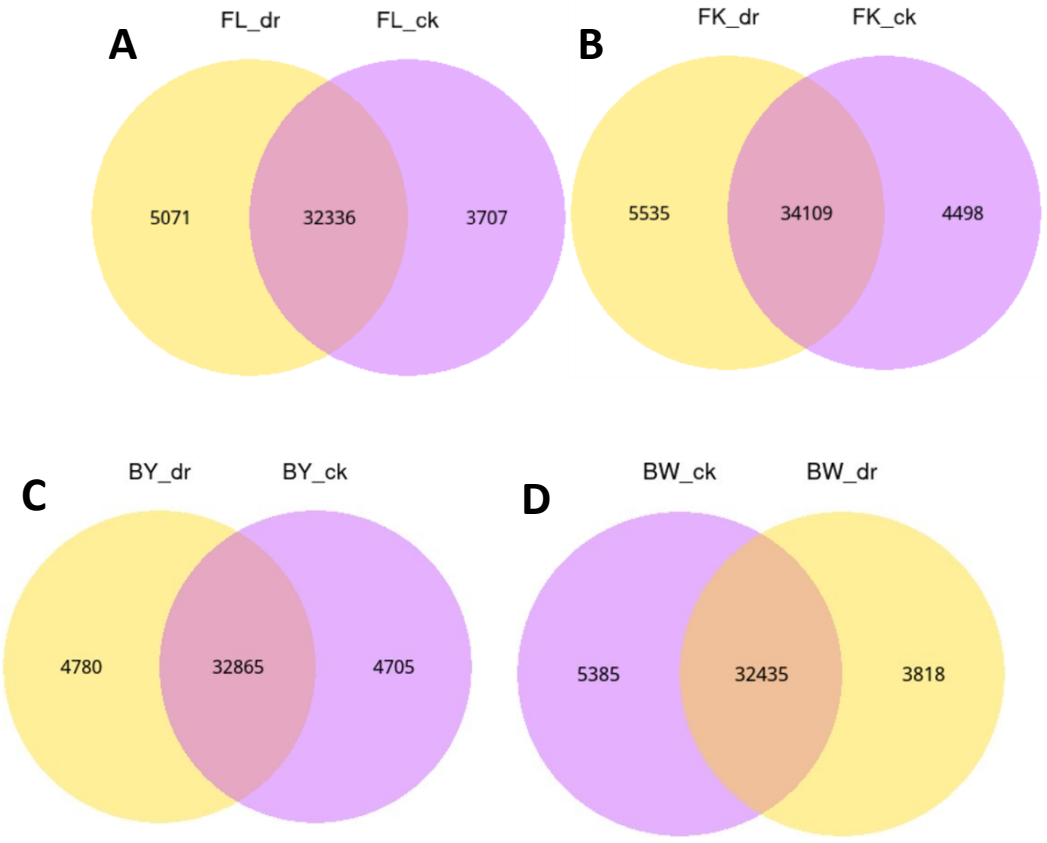


Figure 5.2.3.17 Venn Diagram of Expression Genes.

Moreover, samples either belonging to control or treated samples has been clustered as to find genes with similar expression patterns under various experimental conditions. (Figure 5.2.3.18)
In hierarchical clustering, areas of different colours denote different groups (clusters) of genes, and genes within each cluster may have similar functions or take part in the same biological process (Figure 5.2.3.19)

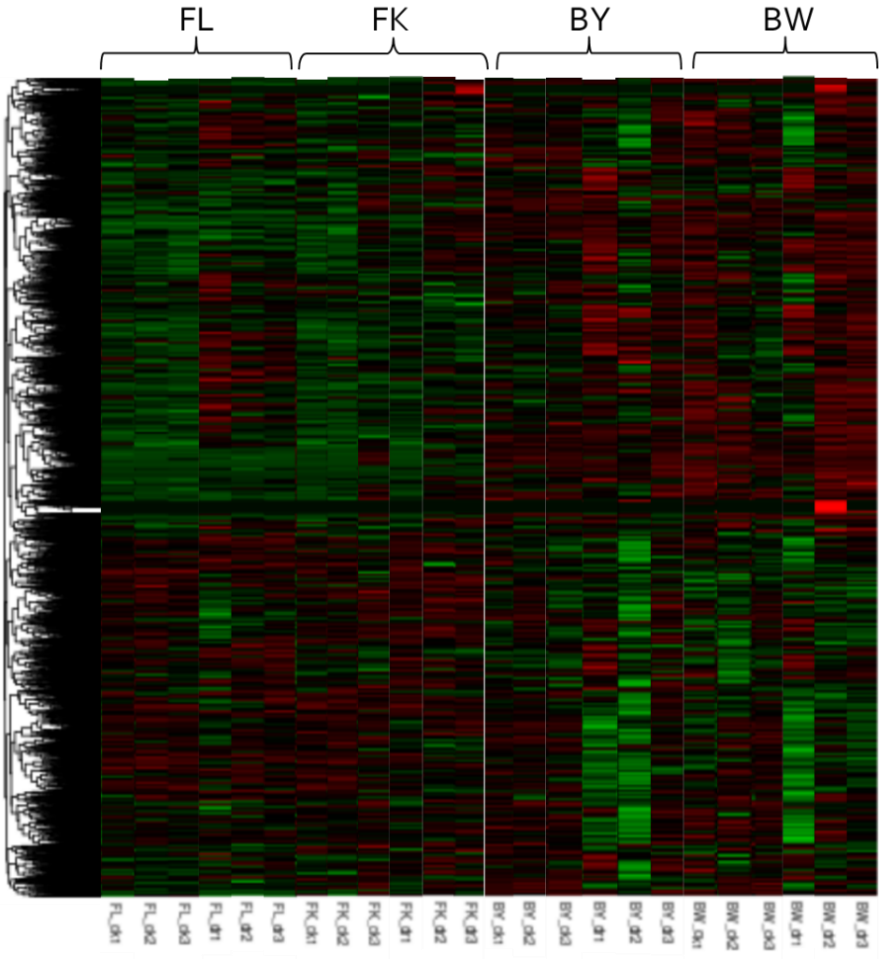


Figure 5.2.3.18 Cluster Analysis

In conclusion according with the previous results of MDA and H_2O_2, even the transcriptomic analysis confirms that the two landraces of *Brassica oleracea* var. *italica* are much more sensible to water stress than the two populations of *Brassica macrocarpa* compared.
Transcriptomic analysis in the *Brassica oleracea* var. italica settembrino revealed in total 2890 differentially expressed genes (including up- and down regulated genes). Brassica *oleracea var.* italica settembrino contains many DEGs involved in various biological functions like transmembrane transporter activity, transmembrane transport, oxidoreductase activity and kinase activity.

In conclusion, from the results obtained, we can say that *Brassica oleracea var.* italica settembrino is the most sensitive landrace to water stress whereas the *B. macrocarpa* population of Marettimo is the most resistant.

5.2.4. Innovative products of interest for nutraceutical traits

5.2.4.1. Introduction

The valuable characteristics of the *B. oleracea* crops suggested us to identify potential innovative new products by using some neglected vegetable landraces to be placed on the market. The demand for specialty products is quite strong all-around Europe while their productions remain often rather occasional and related to home/suburban gardens and farms where these crops are grown in not specialised fields.

Among the research initiatives aimed at vegetable product innovation, attention has been paid since some decades to the sprouts of various vegetable species, or to the production of young seedlings, also named microgreens, obtained from the germination of seeds, provided with or without a radicle. These productions are traditional in several Asiatic countries and own peoples for several centuries have known and utilised them for the high nutritional properties and digestibility of these products, compared to the corresponding seeds, which are often characterised by high nutraceutical value conferred by several antioxidant compounds, and not only them as we will describe in the frame of this PhD thesis.

Recently, sprouts production has been subjects of particular attention in various European and North American countries following the experimental evidence which certifies in addition to the antioxidant properties also the anticancer ones. This is the case, for example, of broccoli sprouts which have high concentrations of total polyphenols, vitamins, and glucosinolates compounds compared to the corresponding traditionally fresh product, as such as the broccoli hypertrophic inflorescence, namely head/corymb. The use of sprouts for human diets has the advantage of allowing the intake of lower daily quantities of product and a greater quantity of nutraceutical compounds.
Recently, *Brassica oleracea* sprouts are increasingly requested and consumed, characterised by higher content of glucosinolates (GLSs) compared to the classic mature product (up to 100 times higher). (Di Bella et al. 2021)

Our attention was paid to the Sicilian landrace Broccolo nero represents a neglected crop grown on the slopes of Mont. Etna, so called for the high content of anthocyanins which provide its characteristic reddish dark colour of the leaf midrib and of the main and the secondary stems.

Today, in fact, the attention of the modern consumer is increasingly turning to looking for foods with high nutraceutical value with the aim of contrasting, also through the adoption of a correct and healthy diet. The positive effects determined by the consumption of fruits and vegetables are due to the large number of potentially protective compounds contained into horticultural products and into the derived foods, affecting different biochemical pathways (Aggarwal and Shishodia, 2006). From the numerous studies in literature, the phytochemicals produced by the plant show beneficial effects on human health. Such compounds, including polyphenols, carotenoids and glucosinolates, represent non-nutritious compounds of the plant capable of acting by different ways and

levels; in fact, they prevent oxidative stress, stimulate the immune system, and reduce the risk of cancers as they inhibit cell mutations and the proliferation of cancer cells (Kim and Park, 2009; Kestwal et al., 2011). In this context, among the plant molecular compounds that have relevance on human health, in recent years scientific research has been approaching several studies on microRNAs (miRNAs).

We also evaluated the presence of some miRNAs described in the literature for brassicas, during the growth stages of the plant, from the sprout to the adult ones, to underline their possible use for nutraceutical and pharmacological purposes could be of interest for food industries.

Based on these considerations, this line of research was activated to broaden the knowledge on the Sicilian sprouting broccoli landrace Broccolo nero, which has shown high amounts of antioxidant compounds, in several plant organs and products, in previous evaluation trials. We paid attention to the amount and the profile of the GLSs compounds, and to the presence of some plant miRNAs, selected through an in-silico study, in the exosome-like preparation extracted from the juice of *Broccolo nero* sprouts for better understand the epigenetic and molecular mechanisms underlying cellular communication between plants and animals.

We evaluate the amount and the profile of GLSs and of some plant miRNAs, selected through an in-silico study, in exosome-like preparation extracted from the juice, obtained both by sprouts, microgreens, baby-leaves and leaves of adult plants of Broccolo nero for evaluating their possible use for food, nutraceuticals and pharmacology purposes by the use of suitable vegetable preparations.

5.2.4.2. Materials and methods

Plant Material and Experiment Conditions

We compared the nutraceutical profile of different stages of growth and development of the plant of the Sicilian landrace of sprouting broccoli (*Brassica oleracea* L. var. *italica* Plenck) 'Broccolo Nero' (BN) of the Di3A active collection of the University of Catania lists in the vegetable germplasm repository of the Department of Agriculture, Food and Environment (Di3A) at the University of Catania (repository Di3A Università di Catania UNICT 4939) (Figure 5.2.4.2.1.; Table 5.2.4.2.1.).

We established one growing cycle which started during the spring season in April 2020. The Seeds were sown in cellular trays using organic substrate (Terri® Bio) placed in an unheated greenhouse in Catania (37°31'10'' N 15°04'18''E) under natural light of 9.2 MJ m^{-2} d^{-1}). The mean temperature registered was 22.6°C ± 11.4°C from April to June.

The plants were collected at three different stages of their growth: the sprouts were collected at the cotyledon disclosure without coats and roots; the microgreens were collected at the appearance of the first true leaf; the baby leaves were collected at the 3-4th true leaf growth phase, and the leaves from adult plant just after inflorescence appearance.

Figure 5.2.4.2.1. Broccolo nero

Table 5.2.4.2.1. Accessions

Genotype	**Code**	**Stage**
Broccolo nero	UNICT 4939 BR 365	Sprouts
Broccolo nero	UNICT 4939 BR 365	Microgreens
Broccolo nero	UNICT 4939 BR 365	Baby-leaves
Broccolo nero	UNICT 4939 BR 365	Leaves from adult plants

Plant bio-morphometric characteristics

The plants were characterised for their main morphometric parameters, as such as the weight of 10 individuals, hypocotyl length, and cotyledon width and length for the sprouts, the width and length of the first leaf for the microgreens, as well as the number, length, and width of the true leaves, were recorded for the baby-leaves. The leaves from the adult plants were collected after six months from their transplanting (seven months after sowing) (Figure 5.2.4.2.2.).

The young seedlings were harvested in the three growth stages such as: i) *sprouts* (seedlings with spread cotyledons without seed coating, harvested after 7 days); ii) *microgreens* (seedlings with the first true leaf, harvested after 15 days); iii) *baby-leaves* (seedlings with 3-4 true leaves collected after 29 days). Young leaves of adult plants (LAP) in the growing phase were collected from plants transplanted six months before, in fourth pots of 10 liters filled with the same substrate utilised for the sowing.

The LAPs were collected in June to compare their biochemical and genetic profiles for looking for the presence of miRNAs in comparison to the three novel foods. The collected samples were subjected to the washing, drying, and weighing phases. After six months after the transplanting the plants were characterised for the main morphological descriptors UPOV and IBGPR.

Figure 5.2.4.2.2. Baby-leaves bio-morphometric characterization.

Juice extraction

Part of the samples of *sprouts, microgreens, baby-leaves* and of LAPs of Broccolo nero were weighed, placed inside a cold extractor (Hurom slow juicer) to separate the juice fibrous part (Figure 5.2.4.2.3.). The juice and fiber were stored inside falcon of 50 ml and their contents was weighed and refrigerated at -80 °C. After 5 days, the preserved falcons were placed inside the Heto PowerDry LL 3000 Freeze Dryer for 90 hours at -55 °C to obtain the freeze-dried samples.

When the freeze-dried juice was obtained, a part was used for detecting the amount and the profile of the GLSs compounds and the other set of the sample was used for the characterization of genetic components, by extraction and analysis of miRNAs. Plant samples were frozen at -80 C° and they were freeze-dried and ground to obtain a fine powder. Then, they were stored in glass jars at -20 C° for glucosinolate and miRNA analysis.

Figure 5.2.4.2.3. Extractor (Hurom slow juicer).

Glucosinolate Analysis

The extraction of glucosinolates from the plant samples was carried out following the Bennet extraction protocol (Bennet et al., 2017). The freeze-dried samples of the BN sprouts, microgreens, baby-leaves and LAPs were used for the extraction of glucosinolates. For each freeze-dried sample, we utilised 100 mg of powder, which was dissolved in 1,5 mL 70% MeOH (Sigma–Aldrich Chemie GmbH, Steinheim, Germany) at 70°C for 30 min, mixed by vortex every 5 min to facilitate the extraction, and then centrifuged (10000 rpm, 15 min, 4°C). Supernatant was collected, and the methanol was completely removed using a rotary evaporator under vacuum at 37°C. The dry material obtained was dissolved again in 1 mL of ultrapure water and filtered through a 0.22 µm PVDF syringe filter.

For the identification and quantification of glucosinolates, we used a HPLC-PDA-ESI-MSn system (Agilent 1200 HPLC-DAD, Barcelona, Spain) coupled to Bruker MS Ion Trap (Bremen, Germany). Chromatographic conditions and identification and quantification techniques was done in according to (Moreno et al 2010)

MiRNAs analysis

Regarding the analysis of miRNAs, their presence into the products of Broccolo nero landrace was researched based on a silico study and on facts described in literature; in particular, the research focused on miRNAs that appear to have an antiproliferative effect

in cancer cells or a protective activity against certain pathologies (Terzo et al., 2019; Terzo et al., 2020).

The miRNAs that were the object of this work were the miR-414, the miR-156 and the miR-398, chosen as mentioned, for the possible health value that they can offer to human health. Through some *in vitro* studies carried out in collaboration with the Biometec Department of the University of Catania, miR-414 has been shown to have properties that inhibit the proliferation of melanoma cells; miR-156 has been studied for its atheroprotective activity (Hou D. et al., 2018) and finally miR-398 has been studied because it may target a gene implicated in hypercholesterolemia.

To verify that the leaf extracts of the four development stages of Broccolo nero contained the miRNAs of interest, they were analysed by Real Time RT-PCR, using specific step loop primers for each miRNA (Table 5.2.4.2.2.). For miRNA extraction, liquid nitrogen was added to the frozen juice samples to avoid RNA degradation and to facilitate its grinding; a refrigerated mortar was used to crush and homogenise the sample. The extraction of miRNA from 100 mg of pulverised product was carried out using the mirPrimer→ microRNA isolation kit (Sigma-Aldrich), according to the protocol indicated by the manufacturer. The assay and evaluation of the quality of the micro-RNA samples obtained were performed by reading the NanoDrop 1000 spectrophotometer at the wavelength of 260 nm, 280 nm and 230 nm.

Subsequently, 300 ng of each sample were retro-transcribed using a reaction mixture consisting of: 2.5 ⌈l of dNTPs mix (2.5 mM), 1 ⌈l of M-MLV RT (50 U/⌈l), 2 ⌈l of DTT (100 mM) and 4 ⌈l of RT Buffer 5X. 1⌈l of stem-loop-specific primers (1 ⌈M) and H_2O – DEPC were then added to the reaction up to the final volume of 20 ⌈l; the chemistry used for the Real Time analysis involved the use of syber green as intercalating agent (Table 5.2.4.2.3.).

The reaction was carried out using the following thermal profile: 65 °C for 5 min, 16 °C for 30 min, 37 °C for 60 min and 90 °C for 5 min. Once the cDNA was obtained, Real Time PCR was performed using the 7300 Real Time PCR System thermal cycler. The amplification reaction of each sample was prepared using quantities reported below, in a final volume of 25 ⌈l. The amplification protocol includes for the following temperatures for the following cycles: 1 cycle at 95 °C for 10 min, 40 cycles at 95 °C for 15 sec, 52 °C for 30 sec and 72 °C for 30 sec. The acquisition of fluorescence was obtained during the extension phase. At the end of PCR cycles, the Melting curve was performed to verify the specificity of the amplification signal on the products obtained.

Table 5.2.4.2.2. *Primers used for amplification miRNA in Real-Time RT-PCR.*

Primer FW miR414	StemLoop miR414
GTGCAGGGTCCGAGGT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGTAAGA
Primer FW miR156	StemLoop miR156
GTATACTGACAGAAGAGAGTG	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGTGCTC
Primer FW miR398	StemLoop miR398
GTATACTGTGTTCTCAGGTCA	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAAGGGG
Primer RW Universale	
GTGCAGGGTCCGAGGT	

Table 5.2.4.2.3. Component of the amplification reaction.

Real Time PCR	
Specific retro-transcription product for each miRNA	10 ⌈l
Specific primers (foward e reverse) for the miRNAs of interest (10 ⌈M)	1 ⌈l
PCR Master Mix 2X containing *Syber green*	12,5 ⌈l
H_2O DNasi ed RNasi *free*	1,5 ⌈l

Cancer Cell lines utilised

The cell line A2058 (malignant melanoma cells) (ATCC®) was used CRL11147D-™). A2058 cells were cultured using RPMI 1640 growth medium (Sigma Aldrich), a medium enriched with: 10% fetal serum (FBS) (Sigma Aldrich), 1% of L-glutamine (50 U / mL) (Sigma Aldrich) and 1% streptomycin / penicillin (50 U / mL) (Sigma Aldrich). The cell line was maintained in culture (Thermo Scientific, model HERAcell150i) in standard atmosphere conditions, at a temperature of 37 ° C and 5% of CO_2.

Isolation and identification of plant nanovesicles

For the isolation of the exosome-like nanovesicles, the juice obtained from the squeezing of Broccolo nero sprouts, microgreens, baby-leaves and LAPSs. The juice, obtained with a Slow Juicer (HURON), was centrifuged following the procedure described by Thèry C. and collaborators (2002), modified in terms of time and speed of centrifugation. Specifically, 50 ml of juice was centrifuged at 3000 rpm for 10 min. The recovered supernatant was then further centrifuged at 10000 x g for 1 h and subsequently at 20,000 x g for 3 h. At this point, the recovered liquid phase was subjected to ultracentrifugation at 110000 x g for 90 min; finally, the exosome pellet was resuspended in PBS and filtered using filters 0.45 µm. For further verification of the isolated exosomes-like, NANOSIGHT, one was used to determine the size distribution profile of nanoparticles in liquid suspension.

In silico study of miRNA

Recent scientific evidence has confirmed the presence of miRNA from exosomes plants, introduced through the diet, into the serum and plasma of humans and other mammals (Chen X. et al., 2008). Based on these studies, the characterization of the molecular content of the exosomes-like previously isolated from *Brassica oleracea*, it initially used one in silico study, consulting some databases (http://rhesus.amu.edu.pl/mirnest/ and http://plantgrn.noble.org/psRNATarget/), to identify those plant miRNAs that target genes that in humans are involved in differentiation processes, apoptosis, and cell proliferation.

Molecular analysis of gene expression

Based on the results obtained from the in-silico study, two miRNAs were identified plants (miR-414 and miR-1533) that target the Spindlin 1 (SPIN1) genes and Neuropilin 2 (NRP2). The expression of the genes SPIN1, NRP2 and Cycline D, involved in the proliferative processes, were evaluated by semi-quantitative RT-PCR. Specifically, total RNA was extracted using PureLink RNA Mini Kit (Ambion), according to the protocol indicated by the manufacturer. The assay and quality assessment of the RNA samples were performed by reading on NanoDrop 1000 spectrophotometer at wavelengths of 260 nm, 280 nm e 230 nm.

The total RNA (0.5 μg) was then back-transcribed into cDNA using M-MLV reverse transcriptase (Invitrogen), according to the protocol indicated by the manufacturer. The primers used for the subsequent amplifications are reported in detail in Table 5.2.4.2.4.:

Table 5.2.4.2.4. Primer sequence utilized.

Gene	**Primer sequence**
PGK1-FW	TTAAAGGGAAGCGGGTCGTT
PGK1-RW	CAGGCATGGGCACACCAT
SPIN1-FW	CTGATTCGAGATGTCGC
SPIN1-RW	GAACATCGTAGGCTGCAGGA
NRP2-FW	CACTGGAGAACTGCATGGAA
NRP2-RW	CCGAGTAGGAACAGGTGCAG
Cyclin D-FW	CCGAGAAGCTGTGCATCTAC
Cyclin D-RW	GGCGGTAGTAGGACAGAAG

The PGK1 gene was used as a housekeeping gene to normalize expression levels in the samples. The reverse transcription reaction was prepared using the protocol reported in the Table 5.2.4.2.5.:

Table 5.2.4.2.5. Component for trascriptomics reaction.

RT-PCR	
MoMuLV reverse transcriptase buffer 5x	4 µl
Random primers	3µl
dNTPs (2,5 mM)	2,5 µl
DTT (0,1 M)	2 µl
MoMuLV reverse transcriptase (100 U/µl)	1 µl
RNA (100 ng/µl)	5 µl
H_2O – DEPC	3 µl

For the Polymerase chain reaction (PCR) we proceeded to the amplification of 2µl of cDNA in a reaction mix containing: 1X buffer, dNTPs (0.2 mM), primer (0.4 µM), DNA pol (1U). Amplification was performed using the following thermal profile: 1 cycle at 94 ° C for 3 min; 30 cycles at 95 ° C for 1 min, 64 ° C for 1 min and 72 ° C for 1 min; final extension at 72 ° C for 5 min. The PCR products were then separated by electrophoretic run-on agarose gel at 1% in TBE, in the presence of the Syber safe intercalator.

MiRNA extraction and amplification in Real Time RT PCR

To verify that the exosome-like preparation, obtained from the juice of the Broccolo nero sprouts, contained the miRNAs identified through bioinformatics proceeded to analyses its content by Real Time RT-PCR, using primers specific step-loops for miR-414 and miR-1533. The extraction of the total RNA from the exosome-like preparation was performed using the Kit miRNeasy from Qiagen, according to the protocol indicated by the manufacturer, obtaining the total RNA enriched with small RNAs such as miRNAs. At this point, 50 ng of total RNA in one was retro-transcribed for each sample reaction mixture consisting of: 2 µl of dNTPs mix (2.5 mM), 1 µl of M-MLV RT (50 U / µl), 2 µl of DTT and 4 µl of RT Buffer 5X. 1µl of primers were added to the reaction microRNA-specific (1 µM) and H2O - DEPC until a final volume of 20 µl is obtained (Table 5.2.4.2.6.). The reaction was incubated 5 min on ice and the following profile was used thermal: 30 min at 16 ° C, 1 h at 42 ° C and 5 min at 85 ° C. Once the cDNA was obtained from the RT-PCR, Real-Time PCR was performed using the use of the 7300 Real Time PCR System thermal cycler. The amplification reaction for each sample was prepared using the quantities reported in table 3, in a final volume of 25 µl.

In detail, the amplification protocol used includes 1 cycle at 95 ° C for 10 min, followed by 35 cycles at 95 ° C for 15 sec, 60 ° C for 30 sec and 72 ° C for 30 sec. The acquisition of fluorescence, in particular, was obtained during the phase of extension. At the end of the PCR cycles, the Melting curve was also performed to verify the specificity of the amplification signal on the obtained products.

Table 5.2.4.2.6. Component for amplification reaction.

Real Time PCR	
Specific retro-transcription product for each miRNA (c-DNA)	5 µl
Specific Primers (10 µM)	1 µl
PCR Master Mix 2X with *Syber green*	12,5 µl
H_2O DNasi and RNasi *free*	6,5 µl

Cloning in the pCRII-Topo vector

The amplification obtained from RT-PCR using the stem-loop primers for miR-414, and once purified it was cloned into a plasmid vector used later for transformation of competent bacterial cells, grown in an appropriate culture medium. Cloning was performed using the TOPO®TA-Cloning (Invitrogen) kit, which exploits the TA cloning mechanism (Table 5.2.4.2.7.). The reaction is set up using the quantities reported in table 4, according to the indicator protocol from the manufacturer.

Table 5.2.4.2.7. Kit TOPO®TA-Cloning (Invitrogen).

Kit TOPO®TA-Cloning (Invitrogen)	
cDNA	2 µl
Salt Solution	1 µl
TOPO® Vector	1 µl

Plasmid vectors, linked to the fragments of interest, were used to transform TOP10 chemically competent Escherichia coli cells (Invitrogen) by shock, a step that allows the thermal transfer of the plasmid inside the cell. The cells, following the addition of SOC medium, were incubated under shaking a 37 ° C for about 1 hour. Subsequently, the cell suspension was spread on Luria agar plates Bertani medium containing ampicillin (100 mg / L), in order to select the transformed clones and the plates were incubated at 37 ° C overnight. Once grown, the colonies were recovered from the plates and grown in LB for 24 hours. To verify the presence of the insert of interest, the plasmid DNA was extracted

and digested enzymatically with EcoRI. Finally, the positive samples were sequenced to verify the sequence inserted was that of the miR-414.

Transfection and cell contact

The custom mirVana mimic miRNA (Thermo Fisher) with sequence identical to miR-414. Synthetic miR-414 mimic, a double-stranded RNA molecule, is expected to bind specific to the SPIN-1 gene down by regulating it. The effects of miRNA mimic were compared with those of a control sequence (mirVana miRna mimic negative control, Thermo Fisher), not homologous to any human sequence and the mirVana mimic miRNA mir-1 positive control, to verify the transfection occurred. The LIPOFECTAMINE RNAiMAX reagent (Life Technologies). Specifically, lipofectamine is a transfection reagent (Invitrogen), used for increasing the transfection efficiency of RNA (including mRNA) or plasmid DNA, in vitro cell cultures. Furthermore, lipofectamine is composed of lipid subunits which can form liposomes, in an aqueous environment, capable of trapping the material of transfection allowing them to happen electrostatic repulsion of the membrane cell and enter cells. The cell line A2058 was plated in 12-well multiwell 24 h before transfection, in medium with antibiotics, in order to have a confluence of 60-80% to the time of treatment with imimi miRNAs. The various components for the transfection were prepared in optimum medium: lipofectamine (4 µl per well) plus miR-414 mimic (40 nM), mimic negative and positive control (40nm). The reagent was then added dropwise to the corresponding wells and plates were incubated at 37 ° C and 5% CO_2. Instead, Trypan Blue (TB), a dye capable of crossing cell membranes. The assay was performed 24 h and 48 h after transfection and, following the addition of TB, the cells were subsequently counted in Burker's cell count chamber.

miRNA extraction from Broccolo nero sprouts and Real Time RT PCR amplification

To underestimate the presence of miR-414 and some plant miRNAs described in the literature (miR-156a and miR-398a) in the root, stem and leaf of Brassica seedlings oleracea, they were sectioned. The different parts were, therefore, soak separately in liquid nitrogen to prevent RNA degradation e subsequently ground with the aid of a mortar, previously refrigerated.

The extraction of total RNA from the ground product (100 mg) was performed using the mirPremier® microRNA isolation kit (Sigma-Aldrich), according to the indicator protocol from the manufacturer. The assay and quality assessment of the obtained RNA samples were performed by reading on the NanoDrop 1000 spectrophotometer at a wavelength of 260 nm, 280 nm and 230nm. At this point, each sample (5 µl) was back-transcribed using a mixture of reaction consisting of: 2.5 µl of dNTP mix (2.5 mM), 1 µl of M-MLV RT (50 U / µl), 2 µl of DTT and 4 µl of RT Buffer 5X. 1µl of stem-loop primers were added to the reaction specific (1 µM) for the above miRNAs and H2O -

DEPC until a final volume of 20 µl was obtained. The reaction was carried out using the following thermal profile: 65 ° C for 5 min, 16 ° C for 30 min, 37 ° C for 60 min and 90 ° C for 5 min. Once the cDNA was obtained, Real-Time PCR was performed using the 7300 Real Time PCR System thermal cycler. The amplification reaction for each sample was prepared using i quantities shown in table 5, in a final volume of 25 µl.

In detail, the amplification protocol used includes 1 cycle at 95 ° C for 10 min, followed by 35 cycles at 95 ° C for 15 sec, 52 ° C for 30 sec and 72 ° C for 30 sec. The acquisition of fluorescence was obtained during the phase of extension. At the end of the PCR cycles, the Melting curve was also performed to verify the specificity of the amplification signal on the obtained products.

MiRNA extraction from adult plants belonging to the genus Brassica and Real Time RT PCR amplification

To evaluate the presence of miR-156a and miR-398a in the stem, leaves and sketch floral of the adult plants of *Brassica oleracea* var. *italica* and Black broccoli, we proceeded their separation and sectioning. The different parts were, then, separately immersed in liquid nitrogen to prevent RNA degradation and subsequently ground with the aid of a mortar, previously refrigerated (Figure 5.2.4.2.4.).

Figure 5.2.4.2.4. Sprouts roots, stems, and leaves.

The extraction of total RNA from the ground product (100 mg) was performed using the mirPremier® microRNA isolation kit (Sigma-Aldrich), according to the indicator protocol from the manufacturer. The assay and quality assessment of the obtained RNA samples were performed by reading on the NanoDrop 1000 spectrophotometer at a wavelength of 260 nm, 280 nm and 230nm. At this point, each sample (5 µl) was retro-transcribed using a mixture of reaction consisting of: 2.5 µl of dNTP mix (2.5 mM), 1 µl of M-MLV RT (50 U / µl), 2 µl of DTT and 4 µl of RT Buffer 5X. 1µl of stem-loop primers were added to the reaction specific (1 µM) for the above miRNAs and H2O - DEPC until a final volume of 20 µl was obtained. The reaction was carried out using the following thermal profile: 65 ° C for 5 min, 16 ° C for 30 min, 37 ° C for 60 min and 90 ° C for 5 min. Once the cDNA was obtained, Real-Time PCR was performed using the 7300 Real Time PCR System thermal cycler. The amplification reaction for each sample was prepared using quantities shown in table 6, in a final volume of 25 µl.

In detail, the amplification protocol used includes 1 cycle at 95 ° C for 10 min, followed by 35 cycles at 95 ° C for 15 sec, 52 ° C for 30 sec and 72 ° C for 30 sec. The acquisition of fluorescence was obtained during the phase of extension. At the end of the PCR cycles, the Melting curve was also performed to verify the specificity of the amplification signal on the obtained products.

5.2.4.3. Results and Discussion

Plant bio-morphometric characteristics

With reference to the weight of 10 individuals, sprouts, microgreens, and baby-leaves, they fluctuated respectively from 1.2 g to 3.5 g finally reaching 9.5 g (Table 5.2.4.2.1.). With the progress of the growth of the plant has shown increases of weight equal to three and to eight folds and about 100%, for microgreens and baby-leaves respectively (Table 5.2.4.2.1.). With reference to the longitudinal dimension of the hypocotyl, its elongation was witnessed which varied from 43.5 mm to 57.3 mm respectively for the sprouts and microgreen stages of the plant (Table 5.2.4.2.1.). As far as the dimensions of the cotyledon are concerned, there was a grouping of both the longitudinal and transverse dimensions which varied respectively from 12.1 mm to 34.3 mm and from 16.5 mm to 25.8 mm (Table 5.2.4.2.1.).

With reference to the number of true leaves due to the characteristics, it was detected only for microgreens and baby-leaves, as the little sprouts, as already mentioned, are characterised only by the presence of well-opened cotyledons. (Table 5.2.4.2.1.). The number of true leaves is equal to 1 for microgreens as the presence of the first true leaf distinguishes this growth phase of the plant, while it was equal to 4.5 for the baby leaves of Broccolo nero (Table 5.2.4.2.1.). The size of the larger true leaf showed significant increases in terms of longitudinal dimension for the baby-leaf phase. The longitudinal dimension of the leaf varied from 38.6 mm to 125.7 mm while the transversal dimension between 21.5 mm and 48.3 mm, respectively for microgreens and baby-leaves (Table 5.2.4.2.1.). The longitudinal dimension of the stem was measured, as already mentioned, only for the baby-leaves and was equal to 109.7 mm (Table 5.2.4.2.1.).

With reference to the young leaves of adult plants of Broccolo nero, they showed longitudinal and transverse dimensions of the petiole equal to 1.7 cm to 0.9 cm respectively (Table 5.2.4.2.1.). With reference to the leaf blade, the longitudinal dimension is equal to 22.2 cm while the transverse one is equal to 13.5 cm, while the area of the leaf blade is equal to 103.6 cm^2 (Table 5.2.4.2.1.).

The colour of the leaf is dark green due to the intense presence of anthocyanins clearly highlighted in the main and secondary rib which had an intense red colour (Table 5.2.4.2.2.).

Table 5.2.4.2.1. Bio-morphometric parameters of Broccolo nero in the different growing stages.

Characteristics	Sprouts	Microgreens	Baby-leaves
Weight of 10 individuals (g)	1.2±0.4	3.5±0.3	9.5±1.2
Hypocotyl length (mm)	45.3±0.2	57.3±0.4	-
Cotyledon length (mm)	12.1±0.3	34.3±0.6	-
Cotyledon width (mm)	16.5±1.3	25.8±0.3	-
Stem length (mm)	-	-	109.7±6.0
Number of true leaf (n)	-	1.0±0.0	4.5±0.5
Leaf length (mm)	-	38.6±2.5	125.7±1.2
Leaf width (mm)	-	21.5±0.3	48.3±1.5

Table 5.2.4.2.2. Characteristics of the plant after six months from transplanting (UPOV and IBPGR descriptors).

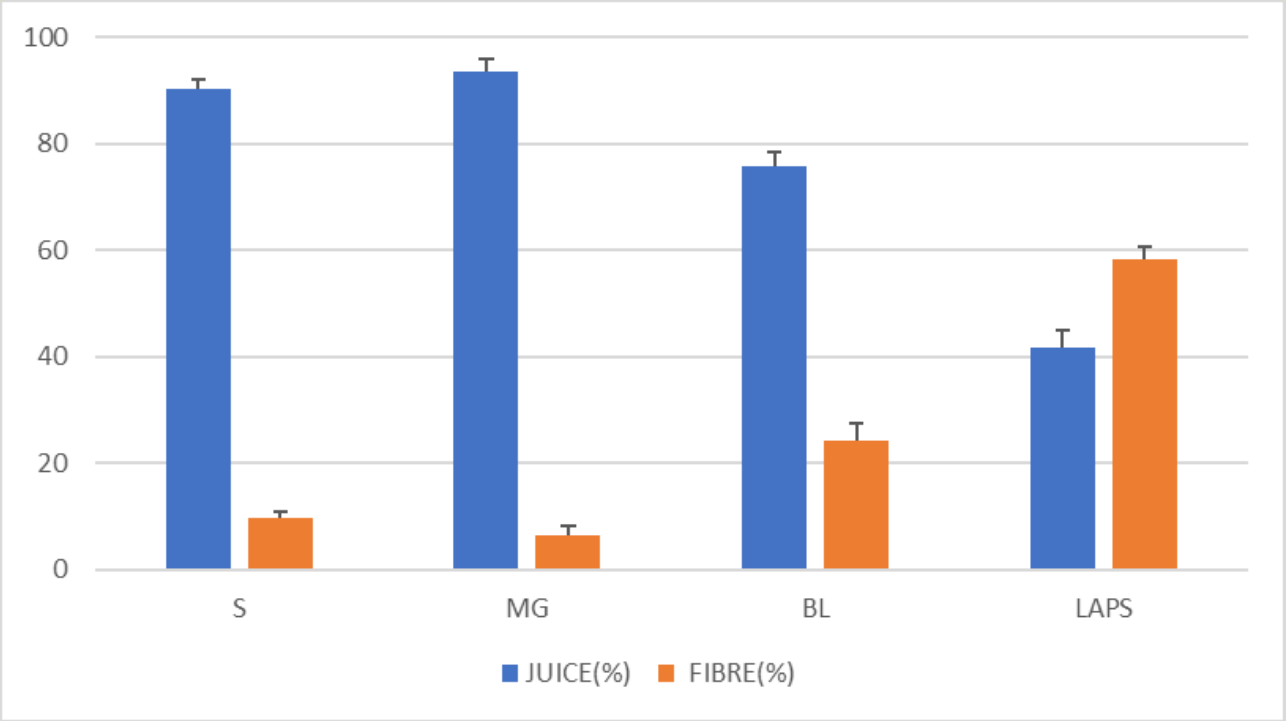
CODE	IBPGR o UPOV	DESCRIPTION	RESULTS
LEAF			
AF		Leaf area (cm²)	103.55±1.5
FGLU	4.2.12	Leaf longitudinal dimension (cm)	22.15±2.3
FGLA	4.2.13	Leaf trasversal dimension (cm)	13.5±1.4
FGCO	4.2.24	Leaf colour (1=yellow green; 2=light green; 3=green; 4=dark green; 5=purple green; 6=purple; 7=other)	5
PETIOLE			
PELU	UPOV_17	Petiole longitudinal dimension (cm)	1.7±0.3
PELA	UPOV_18	Petiole trasversal dimension (cm)	0.9±0.1

Evaluation of the juice yield

In the second phase of the work, the yield of the juice in the various stages of the plant growth was studied and we quantified the fibre obtained during the extraction.

The juice yield appeared inversely proportional to the growth stage of the plant and therefore to the days of its life (Table 5.2.4.2.1.).

Figure 5.2.4.2.1. Percentage of juice and fibre after centrifugation of Broccolo nero sprouts (S), microgreens (MG), baby-leaves (BL) and leaf of adult plants (LAPs).



The juice yield, in fact, varied from 93.5 % to 41.8%, passing from the buds to the young leaf of an adult plant (Figure 5.2.4.2.1)

The quantity juice obtained varied in relation to the Broccolo nero products evaluated. For the sprouts the juice extracted was the 90.3% of the weight of the sprouts utilised, the 93.5% for the microgreens, the 75.7% for the baby-leaves and the 41.8% for the LAPs (Fig.5.2.4.2. 1..).

On the other hand, the fibres obtained after extraction were 9.7%, they were obtained sprouts was reduced by about 6.5% in the case of microgreens, by about 24.3 % in the case of baby-leaves and even by 2.6% for the leaves of the adult plant taken into consideration (Figure 5.2.4.2.1).

On the other hand, the fibre yield appeared gradually higher with the growth of the plant, passing from 2.8% to 24.9% respectively for the buds and the growing leaf of an adult plant (Figure 5.2.4.2.1).

Evaluation of the Glucosinolates profile

The total amount of glucosinolates was higher for the juice obtained from the sprouts, the for the microgreens, baby-leaves, and LAPs ones for which it gradually decreases varying from 14.0 μM $g^{-1d.m.}$ at 4.9 μM $g^{-1d.m.}$ respectively for the sprouts and the LAPs (Figure 5.2.4.2.2.).

We have observed that the glucoiberin content does not follow a regular trend, in fact it is lower in the case of sprouts and of the LAPs, respectively 0.5 and 1.0 μM $g^{-1d.m.}$, than for microgreens and baby-leaves for which we registered values equal to 2.3 and 2.8 μM $g^{-1d.m.}$ respectively (Figure 5.2.4.2.2.). With reference to the glucoraphanin content, its value decreases during the growth of the plant decreasing from 4.8 to 1.5 μM $g^{-1d.m.}$ respectively for the sprouts and the young leaf of the adult plant (Figure 5.2.4.2.2.).

The trend of the content of 4-hydroxy-glucobrassicin in the various growth stages of the plant is also irregular; for the sprouts, it is equal to 2.5 μM $g^{-1d.m.}$, for microgreens there is a slight increase to 2.9 μM $g^{-1d.m.}$, and then reach the value equal to 0.0 and to 1.2 μM / g s.s. for the baby-leaves and LAPs respectively (Figure 5.2.4.2.2.).

As concerns with the glucobrassicin content,its content decrease during the growth of the plant from the sprout phase to the LAPs, and the corresponding values varied from 1.7 μM $g^{-1d.m.}$ to 0.9 μM $g^{-1d.m.}$ respectively (Figure 5.2.4.2.2.).

With reference to the glucoerucin content, there was a regular decrease in the various stages of the growth and development of the plant, from the sprout stage to the LAPs one with values equal to 1.2 μM $g^{-1d.m.}$ and 0.3 μM $g^{-1d.m.}$ respectively (Figure 5.2.4.2.2.).

With regards to the content of 4-methoxy-glucobrassicin and neo-glucobrassicin, significant values were highlighted only for the sprout stage, 1.7 μM $g^{-1d.m.}$, while in the other growth stages of the plant, such as microgreens, baby-leaves and young leaves of adult plant, the content of the above compounds is equal to 0.0 μM $g^{-1d.m.}$ (Figure 5.2.4.2.2.).

Finally, the total content of glucosinolates showed a decreasing and regular trend in the different phases of growth of the plant, passing from 14.0 μM $g^{-1d.m.}$ at 4.9 μM $g^{-1d.m.}$ respectively for the sprot and the young leaves of the adult plant (Figure 5.2.4.2.2.).

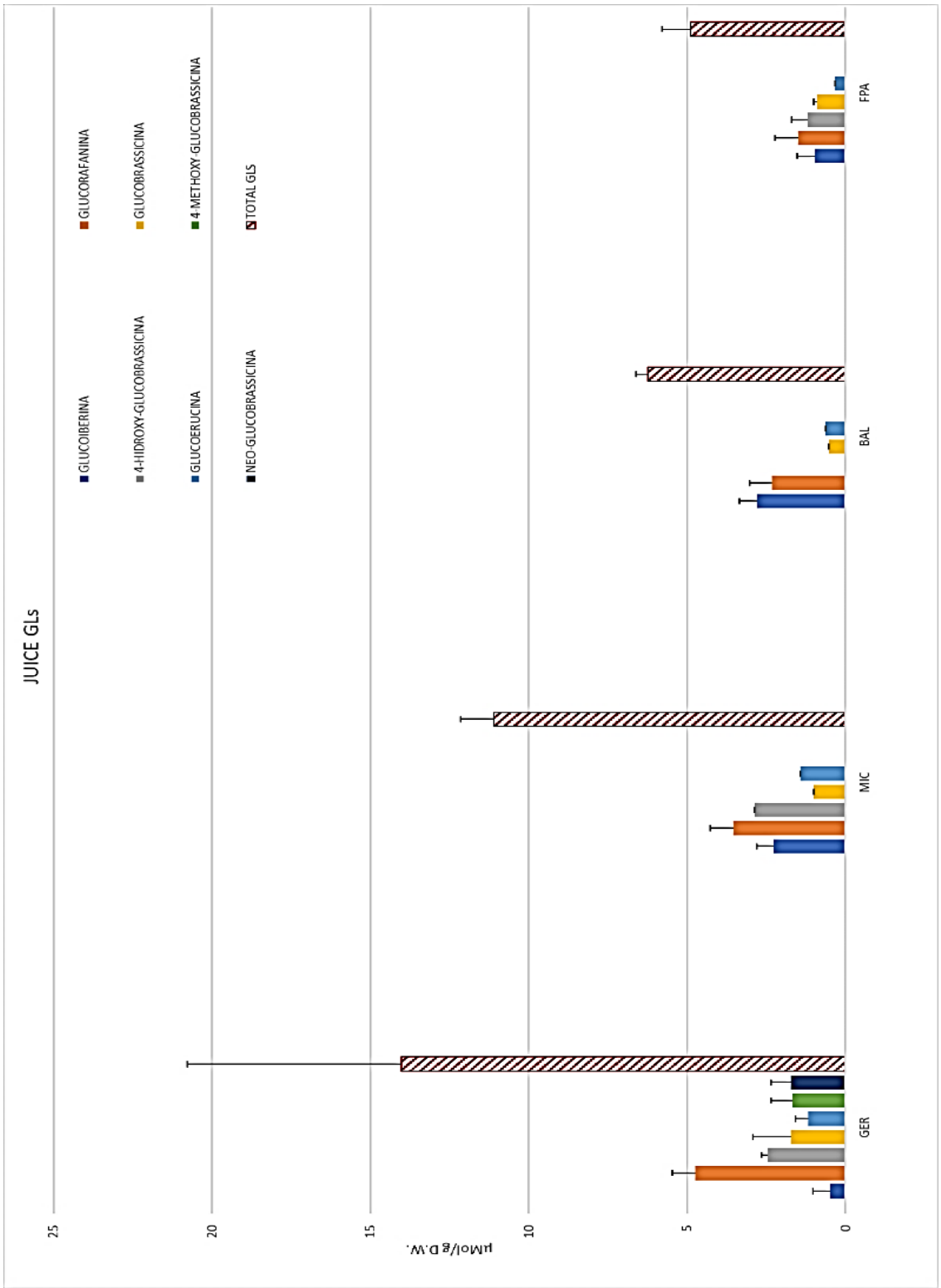
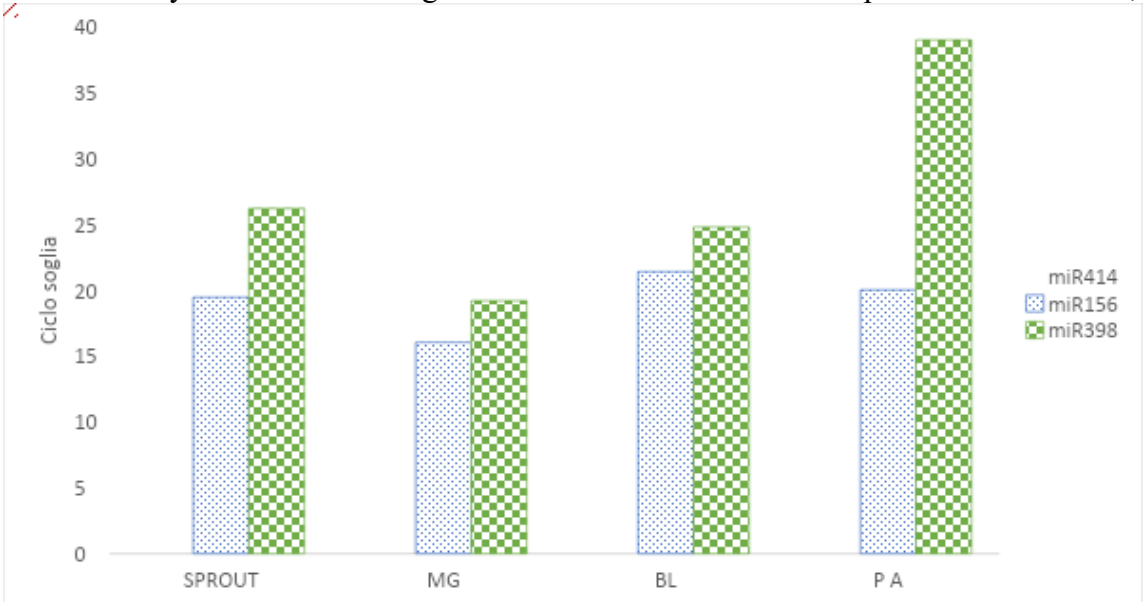


Figure 5.2.4.2.2. Glucosinolates profile of Broccolo nero sprouts (S), microgreens (MG), baby-leaves (BL) and leaf of adult plants (LAPs).

Evaluation of plant miRNAs

The analysis carried out using Real Time RT-PCR revealed the presence of miR-156,



miR-398 and miR-414 in the freeze-dried juice samples for all the plant stages studied.

An amplification curve was obtained for each sample and the relative Ct (Threshold Cycle) was inversely proportional to the quantity of initial cDNA template contained in the sample under examination.

With regards to miR-156, the lowest Ct value (i.e. the highest expression of the target, equal to 16) was observed at the microgreen growth stage while the highest Ct, equal to about 21.4, was obtained. at the baby-leaves stage of the plant; in the case of the other two stages of growth of the plant, the Ct values were equal to 19.5 and 20 for the sprout and young leaf of adult plant, respectively (Figure 5.2.4.2.3.).

Referring to miR-398 also in this case, the growth stage showed the lowest level of Ct is the microgreen one with a value equal to 19.2, while for the other growth stages, such as sprout, baby-leaves and young leaves of adult plant, the Ct levels are equal to 26.2, 24.8 and 39 respectively (Figure 5.2.4.2.3.).

Figure 5.2.4.2.3. Presence of the miRNAs of interest in Broccolo nero sprouts (S), microgreens (MG), baby-leaves (BL) and leaf of adult plants (LAPs).

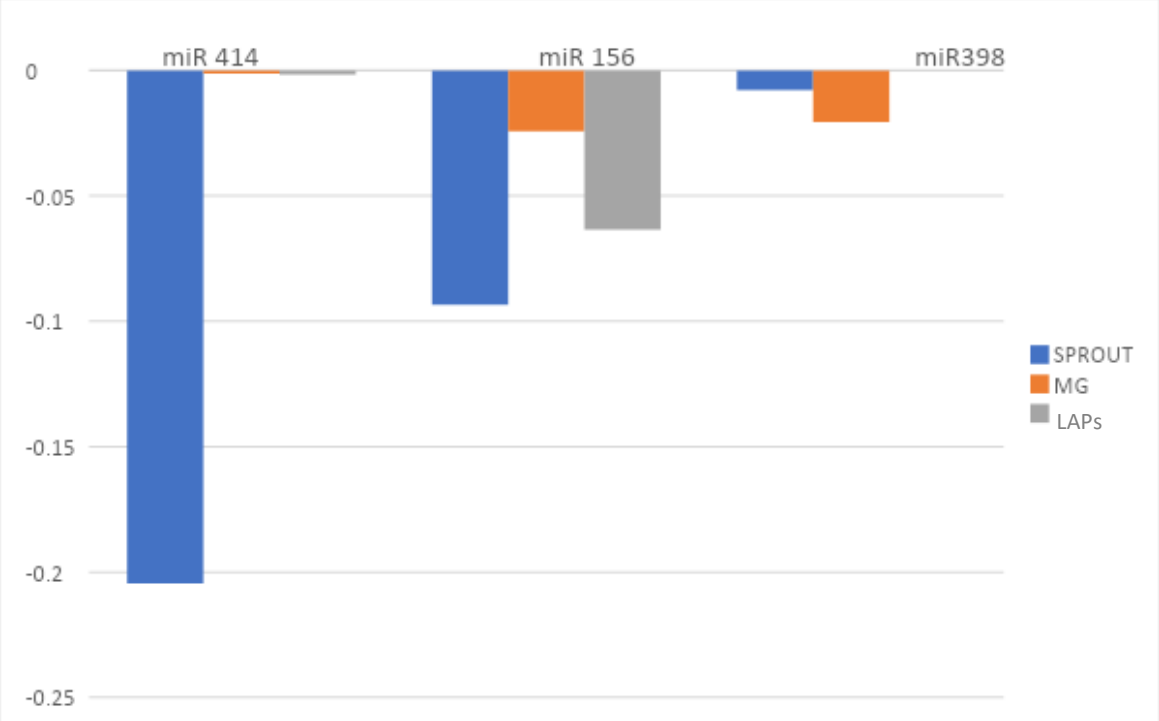
Finally, as can be seen in the figure, miR-414 showed the highest Ct values in all stages than the previous miRNAs, thus proving, among the miRNAs studied, the one with the lowest expression levels. With reference to the four phases of growth, the growth stage that has the lowest level of Ct is the microgreen one, with a value equal to 25.1, while high values were observed for the young leaf of adult plant and baby-leaves, whose Cts were 34.4 and 34.7 respectively; significant the presence of miR-414 at the sprout stage, which showed a Ct equal to 27.4 (Figure 5.2.4.2.4.)

The Real Time RT PCR analysis showed that the miRNA most present in all stages of plant growth is the miR-156 one and the stage of growth in which it is most expressed

for the microgreen stage; for the other stages the levels tend to decrease, even if, in any case, they remain quite significant. (Figure 5.2.4.2.4.).

Of all the microRNAs found, miR-414 is the least abundant in all stages of plant growth. The second graph shows the reduction in miRNA expression levels in the various stages of growth compared to the microgreen for which a value of 1 was considered. The presence of all the miRNAs in the different growth stages decreases compared to the microgreen and the values are lower than 1 with a range of 2 ^ ΔCt between about 0.01 and 0.2, as is possible to observe from the analysis of the threshold cycles (Figure 5.2.4.2.4.).

Figure 5.2.4.2.4. Expression of miRNAs in Broccolo nero sprouts (S), microgreens (MG), baby-leaves (BL) and leaf of adult plants (LAPs). the different phases of growth of Broccolo nero.



Analysis of the in-silico study

Two plant miRNAs, miR-414 (TCATCATCATCATCATCTTAC) and the miR-1533 (ATAATAATAATAATAATGATA), were identified through the in-silico study which target two human oncogenes, SPIN1 and NRP2 respectively.

The SPIN1 gene encodes a protein involved in the proliferation of cancer cells, through the activation of the Wnt signalling pathway and is up-regulated in melanoma by lnRNA, which binds to miR-425 and miR-489, activating the PI3K-AKT cascade. It has also been shown that the increase in SPIN1 expression is correlated with the increased migration capacity and invasiveness of the tumour (Xiangjun C. et al., 2017).

The NRP2 gene, on the other hand, encodes a transmembrane protein belonging to the family neuropilins, which binds to the SEMA3C protein (Ig domain, semaphorin 3C)

and the SEMA3F protein (Ig domino, semaphorin 3F) and interacts with the Vascular Endothelial Growth (VEGF). This protein is involved in development cardio, in the development of the axon and in the genesis of tumours (Rossi M. et al., 2014)

Gene expression analysis of SPIN1 and NRP2

Densitometric analysis of RT-PCR products, courses on agarose gel (Figure 5.2.4.2.5.), has highlighted the downregulation of both NRP2 and SPIN1 (Figure 5.2.4.2.6.). That result supported the hypothesis that the two genes are respectively targets of miR-1533 and miR-414.

Real Time PCR showed the presence of only iR-414 in the exosome extract, of which the amplification curve is reported, absent, however, for miR-1533

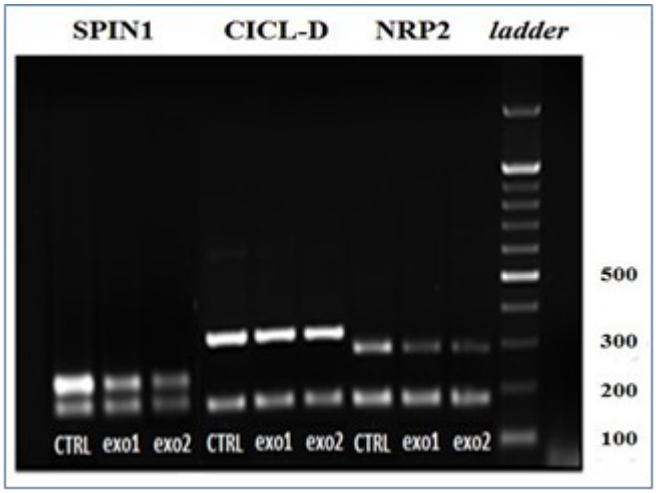


Figure 5.2.4.2.5. SPIN1, CICL-D e NRP2.

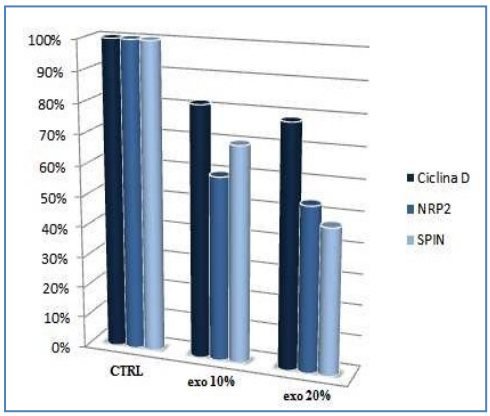


Figure 5.2.4.2.6. Gene expression.

Plasmid DNA sequencing

Plasmid DNA sequencing showed that the inserted sequence matches to miR-414, as can be seen from the following electropherogram (Figure 5.2.4.2.7.). Such a result confirms the presence of miR-414 in *Brassica oleracea*-like exosomes.

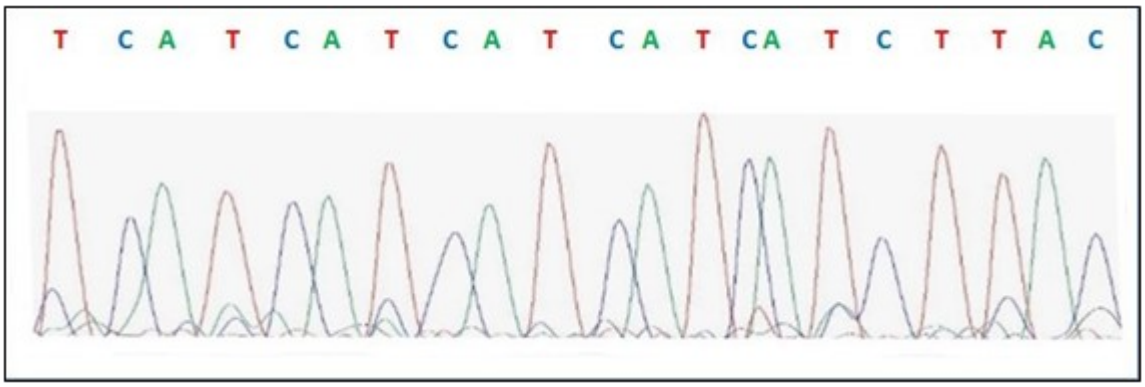


Figure 5.2.4.2.7. Plasmid DNA sequencing

Transfection and cell contact results

Transfection of the A2058 cell line with the miR-414 (40 nM) mimic showed, compared to the control, a reduction in the number of cells of 25% after 24 h and 50% after 48 hours (Figure 5.2.4.2.8.).

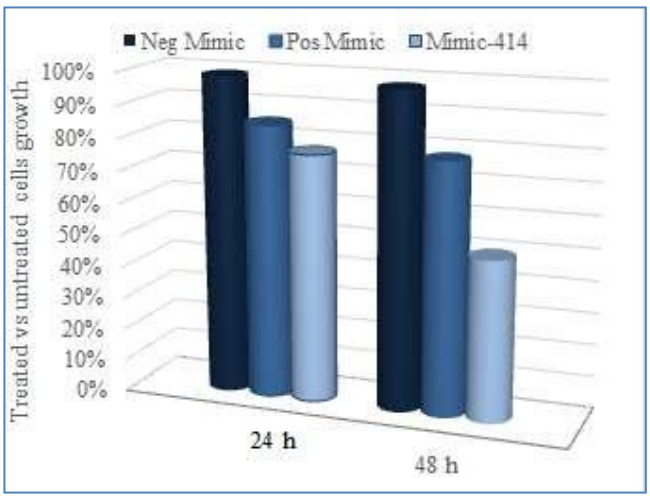


Figure 5.2.4.2.8. Cell contact results

MiRNA analysis in Brassica oleracea by Real Time RT PCR

Real Time RT PCR analysis revealed the presence of miR-156a miR-398 and miR-414 in the root, in the stem and in the leaf of the small buds of Brassica oleracea, of which the amplification curves are reported. Specifically, for each miRNA was found to have a greater expression at the level of the leaf compared to the stem and at the root,

MiRNA analysis in adult plants of the Brassica genus in Real Time PCR RT

Real Time RT PCR analysis revealed the presence of miR-156a in the stem and in the leaf of Brassica oleracea var. italica, of which it is possible to appreciate the curves of amplification. Specifically, a greater expression was found at the level of leaf with respect to the stem. In Black broccoli, however, none was found for amplification.

Furthermore, again by Real Time RT PCR, the presence of miR-398a was highlighted in the stem and leaf of Brassica oleracea var. italics. Again, the expression was found to be greater at the level of the leaf. As for the black broccoli, however, the expression was highlighted only at the level of the floral sketch. Based on the results acquired, the innovative vegetable products proposed, such as sprouts, microgreens and baby-leaves, provide a good source of antioxidants and of useful MiRNAs of great interest both for food and pharmaceutical industries.These new products are of great interest for diversifying the actual items of the ready to eat/ready to cook vegetable products by exploiting the great diversity of landraces widespread in our Country. The Sicilian sprouting broccoli landrace Broccolo nero has been characterised and evaluated for several years also in comparison to other traditional landraces emerging from the other ones for its great antioxidant profile and for the useful MiRNAs individuated. Of great interest is also the antioxidant profile and the MiRNAs found in the leaves which represent the waste portion of the plant could be useful for extract juice to use as integrator or nutraceutical.The scientific evidence and epidemiological studies of the last decades have strengthened the awareness that a correct lifestyle and the adoption of a healthy one nutrition constitute an inseparable combination of nutrition and health, covering a role of primary importance in maintaining the health of the individual. (Georgoulis et al., 2014; Schwingshackl et al., 2017; Wade et al., 2017). In this context, the Brassicaceae represent a botanical family of plants used for food, with a high glucosinolate (GLSs) content which determine the formation of unstable and biologically active intermediates, the isothiocyanates (ITCs), among which the best known is the sulforaphane (Wu et al., 2019). From some studies conducted both in vivo and in vitro, in fact, it is emerged the antioxidant and anticarcinogenic activity of the ITCs (Gasper et al., 2005), with a key role also in the regulation of inflammatory processes (Fimognari et al., 2012). Furthermore, experimental studies indicate that ITCs exert a protective action as well on the cells of the nervous system, counteracting the onset of neurodegenerative diseases e neuronal cell death (Kraft et al., 2004). However, some studies have shown the effects of Brassica extract also on cholesterol metabolism (Park et al., 1997; Kim et al., 2008).It was observed that exogenous miRNAs of plant origin, introduced in the body from the diet, are absorbed by the cells of the gastrointestinal tract mammals and, through the bloodstream, where they reach different tissues and organs, regulate cellular gene expression (Zhang et al., 2012).First, an in silico study was conducted to characterise the molecular content exosome-like, in order to identify those miRNAs that target the genes that in humans are involved in the processes of differentiation, apoptosis and proliferation cellular. Two plant miRNAs have been identified, miR-414 and miR-1533, and identified their target genes, respectively SPIN1 and NRP2, described in the literature as up-regulated in melanoma. Densitometric analysis of the RT-PCR products showed a down regulation of both SPIN1 and NRP2, confirming the initial hypothesis that the two genes are respectively miR-414 and miR-1533 targets. Furthermore, using specific stem-loop primers in Real Time RT PCR and sequencing, it was possible to confirm the presence of miR-414 in the extract of type exosomes. In addition, functional investigations conducted

using a miRNA mimic synthetic with sequence identical to miR-414, showed a reduction of cell proliferation, also found by counting cells with the use of Trypan Blue dye. These results confirm the initial hypotheses. A further goal of this work was to underestimate the presence of miR-414, of miR-156a and miR-398a directly in the root, stem, and leaf of Brassica oleracea sprouts, microgreens, baby-leaves, and in the waste parts of the adult Broccolo nero plants, to underestimate their possible use in field of nutraceuticals and pharmacology, opening new frontiers for medicine Specifically, the Real Time RT PCR analysis highlighted the presence of miR-156 in the root, stem, and leaf of Brassica oleracea new products and in the waste portion of the adult plant; in the black broccoli, however, it was found the presence of this miRNA, highlighting the difference between the tree grown stage of the plants. Experimental evidence suggests that such miRNAs require plant, introduced into the body through the diet, would be carried by exosome nanovesicles, resulting in being able to overcome the gastrointestinal barrier and accumulate at the level of multiple tissues and different fluids in humans and animal models (Liang et al., 2014; Mlotshwa et al., 2015; Snow et al., 2013). This suggests a potential cross-kingdom action of miRNAs and nanovesicles plant-derived exosomes-like, in the regulation of the expression of genes involved in pathogenetic processes of mammals and of man.Interesting studies suggest a potential cross-kingdom action of miRNA or exosome-like nanovesicles derived from plants, introduced through diet, in the regulation of the expression of genes involved in pathogenic processes in mammals and in humans. Zhang et al. published in 2012 the results of their research highlighting the regulation of cross-kingdom gene expression through miRNA. The study highlights the presence of plant-derived exogenous miRNA in human and mammalian plasma, introduced through food and probably carried through exosomes. The authors provide evidence that miRNA 168, a vegetable exogenous derived from rice (Orzya sativa), is stably present in the human serum of subjects that feed on it; demonstrating, moreover, that the same miRNA targets the miRNA of the LDLRAP1 gene, coding a low-density lipoprotein receptor adaptor protein that regulates the removal of LDL-cholesterol molecules from the blood circulation, resulting in regulation of its plasma levels. Another study conducted by Hou D. et al. 2018, showed that reduced levels of mir-156a of plant origin, most expressed in the early growth phase of some vegetables such as spinach, Cabbage, and lettuce, found in the serum of people with cardiovascular diseases and seem to correlate negatively with the presence of the disease. Through functional in vitro studies, they have shown that ectopic administration of mir-156a in endothelial cells reduces the adhesion of monocytes induced by inflammation 79, going to lower the miRNA levels of the protein Junction Adhesion Molecule-A (JAM-A) thus playing a protective role in the progression of atherosclerotic phenomena. From this scientific evidence it is understood how the study of plant miRNA can lead to new perspectives and opportunities to better understand food nutrition and can facilitate the proper exploitation of plant resources. In this sense, it would be interesting to characterise the Black Broccoli miRNoma both to study more widely the role of miRNA in the physiological and

resistance processes of the plant and to expand the knowledge and possible applications of miRNA plant in the field of health.

6. 6. Final Conclusion

The activities we carried out in the context of the XXXIV cycle of the research doctorate on "Biotechnology" of the University of Catania made it possible to identify and consult numerous bibliographic references related to the bio-morphological, biochemical and genetic traits of *B. oleracea* crops, to the effects of the biotic and abiotic stresses to these crops, to the nutraceutical compounds, to the process and products innovation of the food supply chains, to the new products for ready to eat/ready to cock industry, as such as sprouts, microgreens and baby-leaves.

The study of these bibliographical references made it possible to outline a reference framework on the expressed diversity of the different morpho-types of the *B. oleracea* crops widespread in Europe, on the variation of plant phenotyping in relation to water stress, on the nutraceutical profile of the proposed new products.

The germplasm analysed does not appear to be adequately exploited although it shows evident agronomic and technological traits of value and is mainly relegated to the home and suburban gardens/farms of the European countries of the Mediterranean basin, as it is particularly present in the Iberian and Italian peninsula, as well as in the Balkan countries and in Greece.

In this frame of the first research line, we analysed a set of the Brassica core collection of the BRESOV H2020 project to analyse several crop wild relatives, landraces, advanced lines, and commercial hybrids F1 in order to classify them for their main bio-morphological traits of the stem, leaf and root. The data analysis shows a big diversity both within and among the several accession and the several crops compared.

Of interest, the root traits permitted to distinguish genotypes could be useful for organic breeding for improving the resilience, the efficiency, and the sustainability of the several *B. oleracea* crops. Several morpho-types among the several *B. oleracea* crops showed discriminant traits of interest for the process and product innovation of the vegetable food supply chains.

The availability of source of resistance again biotic and biotic stress was partially investigated by the second research line to increase the resilience of two *B. oleracea* leafy-vegetable crops, as such as kale and kohlrabi. Their modest diffusion of such *B. oleracea* crops is also related to the modest shelf-life of the product, which is however essentially represented by vegetative organs characterised by fast metabolic activities that affect in a few days the dimension of the leaves and the characteristics of the product itself.

The specific studies related to the effects of water stress to kale and kohlrabi were addressed to individuate tools for detecting resistant genotypes to use after the pre-breeding phase in the breeding ones for using them for establishing new recombinant inbred lines to individuate QTLs and genes involved in water stress resistance. The use of physiological, biochemical, enzymatic, and genetic markers could speed up the individuation of elite breeding lines to use in the frame of genetic improvement programs

for each crop. Both for the kale and kohlrabi accessions were individuated by several techniques which showed resistance to water stress. The resistant genotypes individuated showed less reduction of the vegetative biomass in relation to the water stress adopted. We also registered the increase of several antioxidant compounds, such as glucosinolates and polyphenols, increased their amount because of the water stress applied.

With reference to the third research line, we individuated source of resistance to water stress in the population of the *Brassica macrocarpa* which represent a wild relative species which shares with *B. oleracea* some number of chromosomes (2n=18) and an ancient relatives endemic od the Sicilian Egadi archipelagos. The results of the transcriptomic analysis of *B. macroparpa* populations of Favignana and Marettimo islands were compared with two sensible Sicilian landraces of Ciurietti showing a good set of SNPs will be useful to individuate the QTLS and genes involved in water stress resistance

The great relevance of *B. oleracea* products for their nutraceuticals highlighted by the international medical community have suggested for some decades to take in consideration several neglected crops/landraces till now occasionally and locally grown in several European countries.

The scientific community, as well as common people, give recognition to the key role of food and beverage in disease prevention and treatment. Therefore, the production and consumption of functional foods have become very important as these provide a health benefit in addition to basic nutritional value.

New vegetable products are becoming more popular for consumers due to their biochemical and organoleptic traits. In accordance with the literature, the activities carried out in the frame of the third research line allowed us to study information and new data related to new products, such as sprouts, microgreens and baby-leaves, and we confirmed a high amount of antioxidant bioactive compounds, such as glucosinolates, in *B. oleracea* crops. The studied genotype showed different profiles in relation to the initial plant growth phases.

The traditional Sicilian sprouting broccoli landrace Broccolo nero shows greater biomass production, confirming previous studies. The high-quality value of the Broccolo nero could be of interest for organic food chains, especially in traditional rural districts, by establishing specific denomination of origin and of geographic indication labels. On the other hand, as evidenced in this study, and confirmed by previous works, sprouts showed greater quantity of GLSs than more developed plantlets, such as microgreens and baby -leaves. The potential interest for their production and consumption should be focused on innovative and ecologically sustainable production systems, paying particular attention to their sustainability and socioeconomic impact on organic food chains, as well as on the health of the consumers. In addition, these new products could be grown at home and utilized for fresh consumption and/or home processing (beverage, juices, snacks, etc.).

Plants are an integral part to human well-being and for centuries they have been used for their healing properties. Although many mechanisms of action of plant products are still unclarified, new biomolecular evidence are coming out: exosomes and their cargo, miRNAs included, might regulate gene expression with potential therapeutic activities in human chronic diseases. We showed that brassica exosomes, carriers of a plant miRNAs, can significantly reduce levels of target mRNAs and regulate proliferation in cancer cell culture models. Exosomes isolated from Broccolo nero sprouts, microgreens and baby-leaves juice were utilised to inhibit the growth of tumour cell lines, as assayed by in vitro wound scratch test and by 3D cultures and the in-silico study using the *Brassica* miRNA database render possible target genes in the human beings. We demonstrated that MIR414 can directly target Spindlin 1 (SPIN1), a human gene upregulated in many cancer cells, melanoma included, promoting cell proliferation by activation of the Wnt signalling pathway. Although further studies with other miRNA/mRNA targets are required, our data clearly showed that miRNAs, packaged in exosomes, could represent new functional molecules mediating intercellular communications, opening to the possibility to develop new "pharmacological" strategies based on the use of plant-edible nanovesicles. The discovery of plant miRNAs and their roles in the biology of mammalian cells represents remarkable evidence of cross-kingdom transfer of functionally active miRNAs and opens a new avenue to explore miRNA-mediated animal-plant interactions.

7. 7. References

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9. 8. Article Published

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Effects of Growing Cycle and Genotype on the Morphometric Properties and Glucosinolates Amount and Profile of Sprouts, Microgreens and Baby Leaves of Broccoli (*Brassica oleracea* L. var. *italica* Plenck) and Kale (*B. oleracea* L. var. *acephala* DC.)

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Abstract: Some new foods (sprouts, microgreens and baby leaf) of the brassica genus are appreciated for their nutritional and nutraceutical values. The aim of this experimental trial was to improve the nutraceutical traits of these foods by evaluating the effects of the climatic condition, genotype, and plant growth stage on the development of greater quality in relation to these new foods. The morphometric and glucosinolates (GLSs) traits of three traditional Italian cultivars of *Brassica oleracea* crops, such as broccoli (*B. oleracea* var. *italica*), namely the traditional Sicilian landrace 'Broccolo Nero' (BN) and the commercial 'Cavolo Broccolo Ramoso Calabrese' (CR), as well as the commercial kale cultivar 'Cavolo Laciniato Nero di Toscana' (CL) (*B. oleracea* var. *acephala* DC.), were evaluated in an unheated greenhouse in Catania during the end of 2019 and the beginning of 2020. Plant growth was studied at different phenological stages—from seeds to sprouts, microgreens, and baby leaves—over two growing cycles, one in autumn–winter and the other in spring–summer. 'Broccolo Nero' (BN) broccoli showed more rapid growth and biomass production than the other two cultivars evaluated (i.e., weight, hypocotyl length, and leaf width). The GLS profile varied significantly ($p < 0.05$), in relation both to plant's growth stage and to genotype. The highest amount of glucoraphanin was detected for BN microgreens and baby leaves, about 8 µmol g^{-1} d.w., whereas glucobrassicin and its related derivatives were about 14 µmol g^{-1} d.w. in microgreens and baby leaves of CL and about 15 µmol g^{-1} d.w. and 10 µmol g^{-1} d.w. for glucoraphanin in CR, respectively. These new foods can also be produced at home with simple and cheap equipment

Keywords: Brassicaceae; vegetable crops; germplasm exploitation; new foods; greens

1. Introduction

Over the last 30 years, consumers have shown a preference for natural foods (rich in bioactive compounds) while looking for a balanced and healthy diet, and they have moved steadily away from the use of artificial chemicals in foods [1]. Consumers choose foods based not only on taste but also on health and wellbeing benefits, and food supply chains support this trend with new competitive and innovative personalized products, such as edible sprouts, microgreens, and baby leaf salads [2]. A current popular strategy is the improvement of dietary habits to manage and prevent non-communicable diseases by innovating with new products, adding value to the agro-biodiversity at a local level, and thus reducing environmental impacts and providing ready-to-eat options for vegetables [3]. In addition to fresh vegetable consumption, the food industry aims to produce functional new foods for the promotion of health. These products are of great interest due to them

being sources of antioxidants, such as glucosinolates, phenolic compounds, vitamins, and minerals [4]. Edible sprouts (germinating seeds a few days old) and microgreens (young vegetables a few weeks old with the presence of true leaves) are healthy and novel fresh foods due to their abundance of nutrients and bioactive compounds, and they may have positive impacts on human health. In particular, the bioactive compounds in cruciferous foods are inducers of cell antioxidant protection against cancer and different chronic diseases [5]. These characteristics can be improved by growing sprouts under optimal temperature conditions [6–8] or by modulating light conditions (LED lights) and temperature regimes [7,9].

Another widely consumed product in the human diet is leafy vegetables; these are important and sought after because they are highly nutritious and are present in various markets with different characteristics (color, taste, and texture). In recent years, several leafy vegetable genotypes have become more popular as fresh cut or baby leaves. Since the demand for ready-to-eat and convenient fresh foods started to increase [10], species belonging to the Brassicaceae family have become globally popular due to ongoing trends related to healthy eating and the consumption of vegetables [11,12]. Generally accepted as rich sources of health-promoting phytochemicals [13,14], Brassicaceae crops includes vegetables that are consumed worldwide, such as broccoli (*Brassica oleracea* L. var. *italica* Plenck) and kale (*Brassica oleracea* L. var. *acephala* DC), both of which are rich in glucosinolates (GLSs), minerals, carotenoids, and vitamins [15,16].

The GLSs and their degradation products (isothiocyanates, ITCs) have been widely studied as important anticancer compounds, but they are also part of plant defense systems against insects and diseases; they are also, at least in part, responsible for the characteristic 'flavor' (pungency, bitter taste, smell, etc.) of these cruciferous vegetables [17–20]. The chemical structure of the GLSs has a common β-D-glucopyranose residue linked through a sulfur atom to A (Z)-N-hydroximinosulfate ester and to a variable side chain (R) derived from one of the following amino acids: alanine, valine, leucine. isoleucine, phenylalanine, methionine, tyrosine, and tryptophan [21].

The production of GLSs, as well as their contents and profiles in several distinguished chemotypes, is influenced by genotypes, environmental growth conditions, cultivation methods, and interactions among these factors [22]. Individual glucosinolate concentrations also change during plant development and in relation to environmental factors, such as temperature regime [23]. The influence of environmental conditions assures interest because innovative products, such as sprouts, microgreens, and baby leaves, can be obtained all year around in different climatic and crop conditions. The content of bioactive compounds also varies in different phenological phases and in terms of the effects of abiotic stresses, such as salinity level [24].

Another element that can modify the GLS profile is genotype; in this framework, analyses of the GLS composition of underexploited landraces and new varieties of *B. oleracea* crops should be conducted through a more exhaustive evaluation of their agronomic performances and biological value for human health in a wider range of environmental conditions, such as the Mediterranean and Atlantic areas [25,26].

In relation to broccoli and kale specifically, we paid attention to three Italian traditional varieties/landraces within the framework of the H2020 BRESOV project (Available online: www.bresov.eu, accessed on 15 June 2021). Two of these were of broccoli typology, 'Broccolo nero', 'Cavolo Broccolo Ramoso Calabrese', and one of them was kale, 'Cavolo Laciniato Nero di Toscana'. Recent studies have shown that *B. oleracea* landraces are often characterized by a higher concentration of nutrients than commercial cultivars. The protection and the enhancement of agro-biodiversity represent a great opportunity for current challenges in terms of food security with high quality and composition standards. Nevertheless, the adoption of hybrids such as F1 for these crops, due to their rapid growing cycles, high productivity, post-harvest duration, and organoleptic and sensory characteristics, has led to a reduction in their nutritional and phytochemical content [27,28].

'Broccolo Nero' (BN) is a neglected landrace grown in two towns located on the slope of Mt. Etna Sicily [29,30]. This variety is characterized by the absence of plant apical dominance; young shoots provide tender leaves and small inflorescences, and the stem, leaves, and inflorescences are rich in anthocyanins that turn the stems and the leaf midribs and veins a dark violet color, thus making its vegetative and reproductive organs black. 'Cavolo Lacinato Nero di Toscana' (CL), also known as 'Tuscan Kale' or 'Lacinato Kale', is a well-known landrace of kale used in the Italian gastronomic tradition. 'Cavolo Broccolo Ramoso Calabrese' (CR) is a cultivar that produces wide branched inflorescence. All of these cultivars represent rich dietary sources of antioxidants for use in innovations in vegetable production.

In this framework, the objective of this work was to evaluate variations in plant morphometric parameters and the GLS profiles of three studied genotypes, namely BN, CL, and CR, in relation to three plant growth stages and two growing cycles during the autumn-winter and spring-summer seasons. Moreover, we sought to individuate the climatic conditions, genotype, and plant growth stage that were optimal for improving the nutraceuticals traits of these proposed new foods.

2. Materials and Methods

2.1. Plant Material and Experimental Conditions

We compared two cultivars of broccoli, namely the Sicilian landrace of sprouting broccoli (*Brassica oleracea* L. var. *italica* Plenck) 'Broccolo Nero' (BN) of the Di3A active collection of Catania University and the standard commercial cultivar of broccoli 'Cavolo Broccolo Ramoso Calabrese' (CR) provided by the SAIS s.p.a. seed company (S.A.I.S Sementi S.p.a., Cesena (FC), Italy). We also examined one kale cultivar (*B. oleracea* L. var. *acephala*), which was represented by 'Cavolo Lacinato Nero di Toscana' (CL). They are listed in the vegetable germplasm repository of the Department of Agriculture, Food and Environment (Di3A) at the University of Catania (BN = UNICT 4939 BR 354; CR = UNICT 4960 BR 325; CL = UNICT 4961 BH86).

The cultivars were utilized for two established growing cycles, the first of which started during the autumn season (November 2019), while the second began in spring (April 2020). Seeds were sown in cellular trays using organic substrate (Terri® Bio, Agrochimica S.p.A., Bolzano, Italy) placed in an unheated greenhouse in Catania (South Italy, 37°31′10″ N 15°04′18″ E; under natural light, 4.6 to 9.2 MJ m^{-2} d^{-1} during the first growing cycle and the second growing cycle). The mean temperature registered during the first cycle was 15.4 ± 5.8 °C, from November to January, and, during the second cycle, the mean temperature was 22.6 ± 11.4 °C from April to June. The plants were collected at three different stages of their growth: sprouts were collected at the cotyledon disclosure without coats and roots; microgreens were collected at the appearance of the first leaf; and baby leaves were collected at the 3–4th true leaf growth phase. After harvesting, plant samples were frozen at −80 °C and they were freeze-dried and ground to obtain a fine powder. Then, they were stored in glass jars at −20 °C for glucosinolate analysis.

2.2. Morphometric Parameters

The seed lots, previously characterized in [7], were utilized for the two growing cycles, and the plants were characterized for their main morphometric parameters (weight of 10 individuals, hypocotyl length, and cotyledon length, as well as width of the sprouts). In addition, the length and width of the microgreens, as well as the number, length and width of the true leaves, were determined for the baby leaves.

2.3. Glucosinolate Analyses

2.3.1. HPLC-DAD Analyses

Intact glucosinolates analyses were carried out at the CEBAS CSIC laboratory, in the Department of Food and Science Technology (Murcia, Spain). The freeze-dried samples of BN, CL, and CR were used for the extraction of glucosinolates. For each freeze-dried sample,

we utilized 100 mg of powder, which was dissolved in 1.5 mL 70% MeOH (Sigma–Aldrich Chemie GmbH, Steinheim, Germany) at 70 °C for 30 min, mixed by vortex every 5 min to facilitate the extraction, and then centrifuged (10,000 rpm, 15 min, 4 °C). Supernatant was collected, and the methanol was completely removed using a rotary evaporator under vacuum at 37 °C. The dry material obtained was dissolved again in 1 mL of ultrapure water and filtered through a 0.22 µm pore size PVDF syringe filters.

2.3.2. HPLC-DAD-ESI-MSn Analysis of GLSs

For identification and quantification of glucosinolates, we used a HPLC-PDA-ESI-MSn system (Agilent 1200 HPLC-DAD, Barcelona, Spain) coupled with a Bruker MS Ion Trap (Bremen, Germany). Chromatographic conditions and identification and quantification techniques are available elsewhere [31].

2.4. Statistical Analysis

The experimental split plot design considered 3 main plots (genotypes) and 2 sub plots (growing cycle) with 3 replications. A multifactorial ANOVA was performed to evaluate the effects of genotype and growing cycle on morphometric plant characteristics. The mean values associated with the main factors, as well as their interactions, were evaluated using Tukey's test ($p < 0.05$). The significance of differences between glucosinolate compounds for each genotype was evaluated by one-way analysis of variance (ANOVA). Data were reported as mean ± standard error (S.E.). All statistical analyses were performed using CoStat release 6.311 (CoHort Software, Monterey, CA, USA).

3. Results

3.1. Plant Characteristics

The morphometric characteristics of the sprouts are showed in Table 1. The weight of the sprouts did not show any significant difference with regard to either genotype or growing cycle. Hypocotyl length was significantly affected by genotype, as well as by the interaction between growing cycle and genotype. The highest value was observed for CR during the spring growing season (45.32 ± 0.12 mm), while the lowest value was observed for CL during both growing cycles (29.2 ± 3.61 and 31.34 ± 0.40 mm, respectively, for the first and second growing cycle). Cotyledon length was significantly affected by both growing cycle and genotype, as well as by their interaction. The highest values were observed for CR during the spring growing cycle (16.34 ± 0.23 mm), whereas the lowest values were registered for BN and CL during the first growing cycle (10.61 ± 0.24 and 10.30 ± 0.81 mm, respectively). Cotyledon width reached its highest values for CR during the second growing cycle (18.34 ± 0.16 mm), while the lowest value was observed for CL during the first growing cycle (12.2 ± 0.81 mm).

The morphometric characteristics of the microgreens are shown in Table 2. The weight of the microgreens did not show any significant differences in relation to either genotype or experimental growing cycle factors. Hypocotyl length showed a significant interaction between genotype and growing cycle. The highest value was recorded for BN during the second cycle and the lowest values were recorded for CL and CR during the spring cycle. Cotyledon length was only affected by genotype, while BN showed the highest value (33.36 ± 0.49 mm). Leaf length was significantly affected by both experimental factors, as well as by their interaction. The highest value was measured in BN in the second cycle (38.59 ± 1.45 mm). Leaf width increased significantly during the second cycle.

Table 1. Morphometric characteristics of the sprouts of 'Broccolo Nero' (BN), 'Cavolo Lacinato Nero di Toscana' (CL), and 'Cavolo Broccolo Ramoso Calabrese' (CR), according to the "cycle" and "genotype" factors, as well as their interactions. Data are reported as mean ± S.E. (n = 3). W = weight of 10 individuals (g); HL = hypocotyl length (mm); S = cotyledon length (mm); CW = cotyledon width (mm).

		W (g)	HL (mm)	S (mm)	CW (mm)
Cycle (C)					
	I	1.20 ± 0.11	35.97 ± 2.25 b	11.71 ± 0.76 b	14.37 ± 0.73 b
	II	1.38 ± 0.09	38.79 ± 2.03 a	13.89 ± 0.64 a	16.67 ± 0.51 a
		n.s.	*	***	***
Genotype (G)					
	BN	1.12 ± 0.12	40.91 ± 0.64 a	11.35 ± 0.36 b	14.81 ± 0.18 b
	CL	1.28 ± 0.10	30.27 ± 1.69 b	11.77 ± 0.75 b	14.37 ± 1.09 b
	CR	1.47 ± 0.13	40.96 ± 2.20 a	15.28 ± 0.72 a	17.37 ± 0.66 a
		n.s.	***	***	**
C × G					
I	BN	1.03 ± 0.11	42.1 ± 0.75 ab	10.61 ± 0.24 de	14.5 ± 0.23 bc
	CL	1.16 ± 0.17	29.2 ± 3.61 d	10.30 ± 0.81 e	12.2 ± 0.81 c
	CR	1.41 ± 0.28	36.6 ± 2.31b cd	14.22 ± 1.22 bc	16.40 ± 1.10 ab
II	BN	1.22 ± 0.23	39.71 ± 0.17 abc	12.09 ± 0.17 bc	15.13 ± 0.11 bc
	CL	1.40 ± 0.12	31.34 ± 0.40 cd	13.24 ± 0.12 bc	16.53 ± 0.75 ab
	CR	1.52 ± 0.07	45.32 ± 0.12 a	16.34 ± 0.23 a	18.34 ± 0.16 a
		n.s.	*	**	***

The mean values associated with the two factors and their interaction were evaluated according to Tukey's test. Values (mean ± S.E.) within each column followed by the same letter do not significantly differ at $p < 0.05$ according to Tukey's test; *n.s.* not significant; * significant at $p < 0.05$; ** significant at $p < 0.01$; *** significant at $p < 0.001$.

Table 2. Morphometric characteristics of the microgreens of 'Broccolo Nero' (BN), 'Cavolo Lacinato Nero di Toscana' (CL), and 'Cavolo Broccolo Ramoso Calabrese' (CR), according to the "cycle" and "genotype" factors, as well as their interaction. Data are reported as mean ± S.E. (n = 3). W = weight of 10 individuals (g); HL = hypocotyl length (mm); S = cotyledon length (mm); CW = cotyledon width (mm); LL = leaf length (mm); LW = leaf width (mm).

		W (g)	HL (mm)	S (mm)	CW (mm)	LL (mm)	LW (mm)
Cycle (C)							
	I	2.99 ± 0.22	33.91 ± 2.63 b	27.19 ± 1.43	19.50 ± 1.14	25.51 ± 1.25 b	17.68 ± 0.87 b
	II	3.49 ± 0.22	42.97 ± 3.83 a	28.61 ± 1.54	20.84 ± 1.43	31.29 ± 2.01 a	20.75 ± 0.39 a
		n.s.	***	*n.s.*	*n.s.*	**	**
Genotype (G)							
	BN	3.48 ± 0.22	50.31 ± 3.15 a	33.36 ± 0.49 a	24.45 ± 0.63 a	32.10 ± 3.15 a	18.97 ± 1.32
	CL	2.81 ± 0.19	30.49 ± 1.32 b	24.29 ± 0.91 b	17.91 ± 0.99 b	24.82 ± 1.04 b	18.50 ± 0.64
	CR	3.43 ± 0.36	34.51 ± 2.94 b	26.05 ± 0.77 b	18.16 ± 1.18 b	28.27 ± 1.44 ab	20.17 ± 1.08
		n.s.	***	***	***	**	*n.s.*
C × G							
I	BN	3.42 ± 0.46	43.30 ± 0.64 b	32.40 ± 0.40	23.10 ± 0.35	25.62 ± 2.36 b	16.43 ± 1.50
	CL	2.72 ± 0.41	29.54 ± 2.62 d	23.47 ± 0.20	17.60 ± 0.98	23.70 ± 1.73 b	17.58 ± 0.95
	CR	2.82 ± 0.28	28.90 ± 3.18 d	25.70 ± 1.62	17.80 ± 2.18	27.20 ± 2.66 b	19.02 ± 2.07
II	BN	3.53 ± 0.17	57.33 ± 0.23 a	34.31 ± 0.35	25.81 ± 0.17	38.59 ± 1.45 a	21.51 ± 0.17
	CL	2.90 ± 0.12	31.44 ± 0.99 cd	25.10 ± 1.85	18.21 ± 1.96	25.94 ± 1.09 b	19.42 ± 0.52
	CR	4.03 ± 0.46	40.12 ± 1.33 bc	26.41 ± 0.46	18.52 ± 1.45	29.34 ± 1.49 b	21.32 ± 0.46
		n.s.	*	*n.s.*	*n.s.*	*	*n.s.*

The mean values associated with the two factors and their interaction were evaluated according to Tukey's test. Values (mean ± S.E.) within each column followed by the same letter do not significantly differ at $p < 0.05$ according to Tukey's test; *n.s.* not significant; * significant at $p < 0.05$; ** significant at $p < 0.01$; *** significant at $p < 0.001$.

The morphometric characteristics of the baby leaves are shown in Table 3. The weight of the microgreens varied according to the genotype, and BN showed the highest value (8.97 ± 0.49 g). Stem length was also significantly influenced by genotype fluctuations

(123.55 ± 2.47 mm for CR and 128.97 ± 3.46 mm for CL). Leaf number was influenced only by growing cycle (Table 3). The leaf length was affected by genotype, and the highest value was measured for BN and CR (125.02 ± 1.03 and 122.02 ± 0.98 mm, respectively). The leaf width was affected the genotype–growing cycle interaction; the highest value was observed for BN harvested at the end of the growing cycle (48.31 ± 0.87 mm) (Table 3).

Table 3. Morphometric characteristics of the baby leaves of 'Broccolo Nero' (BN), 'Cavolo Lacinato Nero di Toscana' (CL), and 'Cavolo Broccolo Ramoso Calabrese' (CR), according to the "cycle" and "genotype" factors, as well as their interaction. Data are reported as mean ± S.E. (n = 3). W = weight of 10 individuals (g); SL = stem length (mm); N = number of true leaf (n); LL = leaf length (mm); LW = leaf width (mm).

		W (g)	SL (mm)	N (n)	LL (mm)	LW (mm)
Cycle (C)						
	I	7.63 ± 0.40	118.59 ± 3.88	3.38 ± 0.14 b	119.43 ± 1.86	36.82 ± 1.58 b
	II	8.24 ± 0.39	122.26 ± 3.49	4.68 ± 0.10 a	121.56 ± 1.59	39.33 ± 2.34 a
		n.s.	*n.s.*	***	*n.s.*	*
Genotype (G)						
	BN	8.97 ± 0.49 a	108.76 ± 2.36 b	4.01 ± 0.28	125.02 ± 1.03 a	45.42 ± 1.49 a
	CL	7.27 ± 0.40 b	128.97 ± 3.46 a	4.21 ± 0.30	114.45 ± 1.05 b	32.92 ± 0.62 c
	CR	7.57 ± 0.2 ab	123.55 ± 2.47 a	3.87 ± 0.38	122.02 ± 0.98 a	35.88 ± 0.60 b
		*	**	*n.s.*	***	***
C × G						
I	BN	8.42 ± 0.64	107.77 ± 3.84	3.51 ± 0.23	124.32 ± 2.08	42.53 ± 1.41 b
	CL	7.05 ± 0.83	125.53 ± 6.90	3.61 ± 0.29	113.28 ± 1.77	32.61 ± 0.87 c
	CR	7.42 ± 0.52	122.47 ± 5.34	3.03 ± 0.04	120.71 ± 1.50	35.31 ± 0.86 c
II	BN	9.52 ± 0.69	109.74 ± 3.47	4.50 ± 0.29	125.72 ± 0.68	48.31 ± 0.87 a
	CL	7.50 ± 0.23	132.41 ± 0.75	4.82 ± 0.06	115.63 ± 1.04	33.23 ± 1.04 c
	CR	7.71 ± 0.16	124.63 ± 0.87	4.71 ± 0.11	123.33 ± 0.92	36.44 ± 0.87 c
		n.s.	*n.s.*	*n.s.*	*n.s.*	*

The mean values associated with the two factors and their interaction were evaluated according to Tukey's test. Values (mean ± S.E.) within each column followed by the same letter do not significantly differ at $p < 0.05$ according to Tukey's test; *n.s.* not significant; * significant at $p < 0.05$; ** significant at $p < 0.01$; *** significant at $p < 0.001$.

3.2. Glucosinolate (GLS) Profile

The analysis of the GLS profiles of the three different genotypes was carried out during the two growing cycles, accounting for seeds, sprouts, microgreens, and baby leaves. The GLSs identified were as follows: aliphatic glucoraphanin (GRA), glucoiberin (GIB), and glucoerucin (GER), the indoles 4-hydroxy glucobrassicin (HGB), glucobrassicin (GBS), 4-methoxyglucobrassicin (MGB), and neoglucobrassicin (NGB). The total content of GLSs is presented as the addition of the individual identified GLSs.

In relation to seeds, total GLSs showed the highest value in CR and CL, whereas the lowest value was observed in BN (Figure 1). MGB and NGB were not detected in the seeds. The major GLS present in the different studied varieties was GRA, while GIB and GBS showed the lowest values. The highest value of total GLSs was found in the sprouts of the second growing cycle of BN, whereas the lowest value was quantified in the microgreens of the first growing cycle (Figure 2). For the sprouts, in both growing cycles, GRA (6.30 ± 0.07 and 7.40 ± 0.03 μmol g^{-1} d.w., respectively, for the first and the second cycle) was the predominant compound. GIB, MGB, and NGB were not detected in the sprouts of the first cycle.

The highest amounts of GBS (3.33 ± 0.31 14.76 ± 0.15 μmol g^{-1} d.w., respectively) were shown in microgreens in both growing cycles. For baby leaves, GBS showed the highest value (13.75 ± 0.69 μmol g^{-1} d.w.) during the spring cycle (Figure 3).

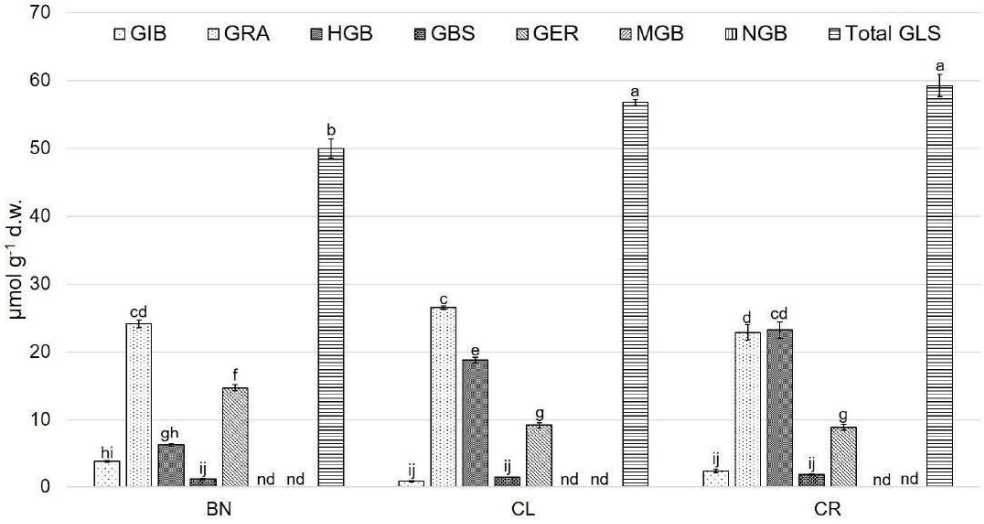

Figure 1. Glucosinolate compounds (µmol g^{-1} d.w.) in seeds of of 'Broccolo Nero' (BN), 'Cavolo Lacinato Nero di Toscana' (CL), and 'Cavolo Broccolo Ramoso Calabrese' (CR). Data are reported as mean ± S.E. Bars with the same letters are not significantly different, as determined by Tukey's test ($p < 0.05$); nd = not detected compound. GIB, glucoiberin; GRA, glucoraphanin; HGB, 4-hydroxyglucobrassicin; GBS, glucobrassicin; GER, glucoerucin; MGB, 4-methoxyglucobrassicin; NGB, neoglucobrassicin; total GLS, total glucosinolates.

For CR, the highest value of GLSs was observed for the sprouts obtained by the spring growing cycle, whereas the lowest ones were observed for microgreens and baby leaves for the spring growing cycle (Figure 4). In the sprouts harvested during the winter growing cycle, NGB (12.28 ± 1.51 µmol g^{-1} d.w.) dominated; however, in the second cycle, a higher amount was observed for GBS (12.43 ± 0.54 µmol g^{-1} d.w.). GIB and MGB were not detected in the sprouts of the first growing cycle.

The microgreens in both growing cycles showed 3.13 ± 0.34 and 14.37 ± 0.57 µmol g^{-1} d.w. of GLSs, respectively. For the baby leaves collected during the first growing cycle, GBS, MGB, and NGB were detected (5.12 ± 0.10, 2.37 ± 0.19 and 3.13 ± 0.10 µmol g^{-1} d.w., respectively). During the second cycle, the highest amount was observed for the GBS (14.18 ± 1.20 µmol g^{-1} d.w.) (Figure 4).

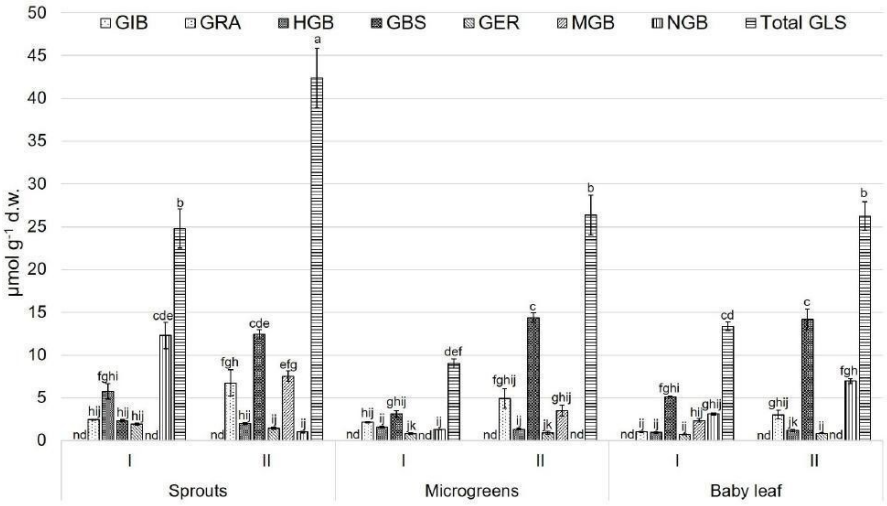

Figure 2. Glucosinolate compounds (µmol g^{-1} d.w.) of 'Broccolo Nero' (BN) at different growth stages and during two growing cycles. Data are reported as mean ± S.E. Bars with the same letters are not significantly different, as determined by Tukey's test ($p < 0.05$); nd = not detected compound. GIB, glucoiberin; GRA, glucoraphanin; HGB, 4-hydroxyglucobrassicin; GBS, glucobrassicin; GER, glucoerucin; MGB, 4-methoxyglucobrassicin; NGB, neoglucobrassicin; total GLS, total glucosinolates.

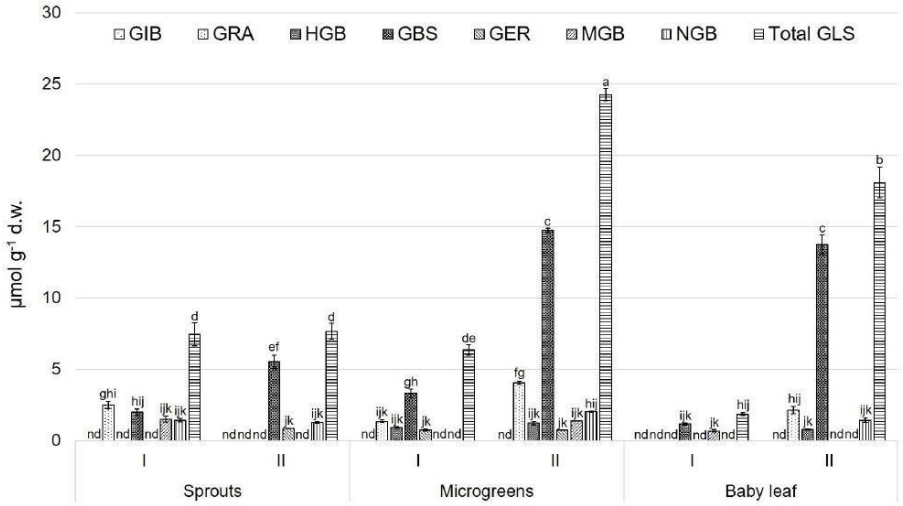

Figure 3. Glucosinolate compounds (µmol g^{-1} d.w.) for 'Cavolo Lacinato Nero di Toscana' (CL) at different growth stages and during two growing cycles. Data are reported as mean ± S.E. Bars with the same letters are not significantly different, as determined by Tukey's test ($p < 0.05$); nd = not detected compound. GIB, glucoiberin; GRA, glucoraphanin; HGB, 4-hydroxyglucobrassicin; GBS, glucobrassicin; GER, glucoerucin; MGB, 4-methoxyglucobrassicin; NGB, neoglucobrassicin; total GLS, total glucosinolates.

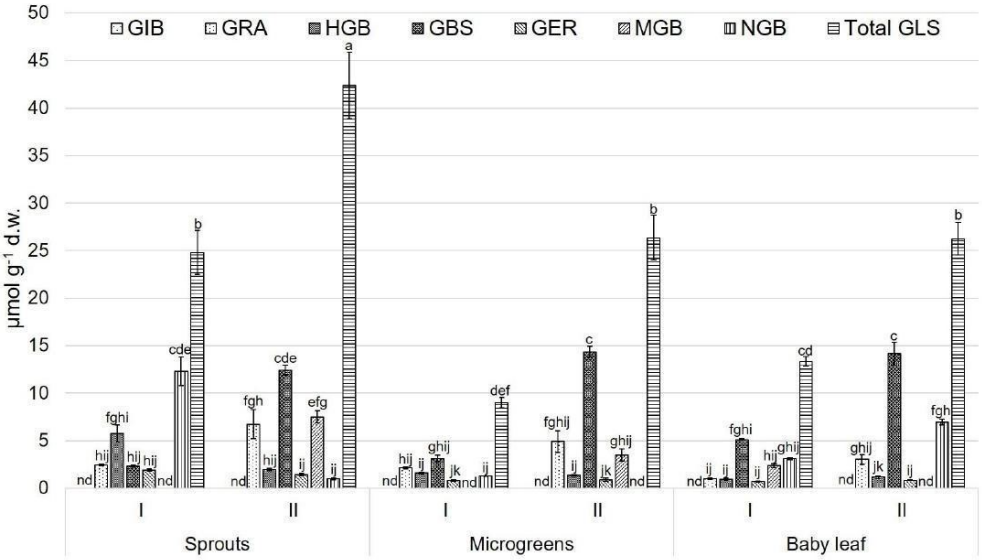

Figure 4. Glucosinolate compounds (µmol g^{-1} d.w.) for 'Cavolo Broccolo Ramoso Calabrese' (CR) at different growth stages and during two growing cycles. Data are reported as mean ± S.E. Bars with the same letters are not significantly different, as determined by Tukey's test ($p < 0.05$); nd = not detected compound. GIB, glucoiberin; GRA, glucoraphanin; HGB, 4-hydroxyglucobrassicin; GBS, glucobrassicin; GER, glucoerucin; MGB,4- methoxyglucobrassicin; NGB, neoglucobrassicin; total GLS, total glucosinolates.

4. Discussion

Various typologies of vegetables and herbs, including sprouts, microgreens, and baby leaves, are becoming popular due to their simple production techniques and high nutritional and nutraceutical values [32]. However, increased interest in these new foods is also supported by our results, showing that climatic conditions and plant growth stage could offer the best performance in terms of yield and of GLSs content and profile [6,7]. A considerable variability in the total amount and profile of GLSs was evidenced in this study.

Regarding the plant morphometric characteristics related to the three analyzed growth stages, the results show an increased plant biomass of about 24 times from seeds to sprouts for BN, 32 times for CL and 35-fold for CR, respectively. Comparing sprouts and microgreens, we also observed an increase in plant biomass of three-fold for BN and two-fold for CL and CR. The weight of baby leaves increased during growth by seven- and two-fold for CL and CR, respectively. The baby leaves' biomass increased three-fold for BN and CL and two-fold for CR in comparison to the microgreens, showing similar variation reported by several authors [2,7]. The differences observed among the examined genotypes showed a faster plant growth for BN and CL than for CR. For BN, cotyledon, hypocotyl, and leaf size determined its difference in relation to the other genotypes studied [6,7,24]. Plants during the second growing cycle (spring-summer) showed morphometric characteristics and quantities of total GLSs that were higher than in the first growing cycle (autumn-winter). Several studies have shown that external factors influence GLSs content and composition in Brassicaceae species, thereby enhancing the health-promoting properties of these compounds, which are dependent on quantity and quality [24]. The traditional Sicilian cultivar

of BN and the commercial cultivar of CR showed faster growth in comparison to CL, as demonstrated by the weight of the baby leaves in both genotypes.

Morphometric traits at the different phenological growth phases were associated with the GLSs profiles to favor the availability and consumption of vegetables as sources of health nutrients that can combat cancer and chronic degenerative diseases. The total amount of GLSs was highest (about 55 μmol g^{-1} d.w.) in the seeds of the three cultivars studied, and their values were reduced to less than half in the sprouts, microgreens, and baby leaves, except for the baby leaves of the 'Cavolo Broccolo Ramoso Calabrese' (CR) that was grown during the spring–summer season.

Seed metabolism is activated by germination, which promotes the hydrolysis of carbohydrates through the synthesis of secondary metabolites; this process is of interest in relation to human health [33]. The GLSs amount decreased during the germination process, reaching a high amount until day 4 after cotyledon disclosure, followed by a marked decline between days 4 and 12 (in broccoli, rutabaga, turnip greens, and radish), corresponding to a 50–90% loss of individual GLSs [31,34,35]. In fact, our results highlight a higher content of GLSs in the BN and CR sprouts during the spring–summer cycle. Therefore, sprouts, as stated by many authors, have a greater quantity of antioxidant compounds, mainly glucosinolates [4,36]. According to the literature, our research show that microgreens and baby leaves also have a high content of glucosinolates, but at a lower concentration than that found in sprouts [5,37]. According to several studies, broccoli, cabbage, and kale are good sources of glucosinolates, particularly GRA [7,38]. Therefore, the results of our study are consistent with those previously reported, and they confirm that sprouts are one of the major dietary sources for GRA, which is also present in microgreens and baby leaves of BN; however, GBS is the predominant GLS in CL. The total content of GLSs is driven by the high presence of GRA (4-methylsulfinybutyl-GLS), the precursor of sulforaphane. Moreover, glucoiberin (GIB, 3-methyl-sulfinyl-propyl-GLS) and neoglucobrassicin (NGB, 1-methoxy-3-indolyl-methyl-GLS) were also present in the studied samples; lower amounts of GER, MGB, GBS and HGB were detected; this is a similar trend to that shown in previous studies [39].

The aim of this work was to show that the morphometric traits and GLSs profile were influenced both by the climatic conditions that characterized different plant growth stages and by the genotype taken into consideration [40,41]. In any case, further studies would be needed to better define the relationship between environmental conditions and GLSs profile. Different studies have shown that the GLSs content increases with high temperatures and radiation. According to Shuai-Qi et al. [42], the present research confirms that the GLSs profile is higher in the spring–summer cycle, and therefore increases with high temperatures. Schonhof et al. [43] detected the highest level of major glucosinolates in broccoli by considering the effect of high temperatures; the major compounds found were glucoraphanin and glucobrassicin, confirming what is highlighted in our results. The different GLSs profiles found may be due to the fact that enzymes are influenced by various climatic conditions, such as temperature [44]. The obtained products were influenced by sensory quality and chemical properties, particularly the intensity of sensory attributes such as pungent odor, bitterness and astringency [44]. These characteristics are due to the different concentrations of GLSs; the properties responsible for these concentrations were correlated to the presence of glucoraphanin, which itself had a high concentration in this study.

5. Conclusions

Novel foods are becoming more popular for consumers due to their biochemical and organoleptic traits. In accordance with the literature, the present work allowed us to study information and new data related to new foods, such as sprouts, microgreens, and baby leaves, and we confirmed a high amount of antioxidant bioactive compounds, such as glucosinolates, in *B. oleracea* crops. The studied genotypes showed different profiles in relation to the growth cycle (autumn–winter and spring–summer) and phases of the

examined plants. During the spring–summer growing cycle, we obtained the highest yield of the three new foods, as well as the highest amount of GLSs.

The traditional Sicilian cultivar BN and the commercial cultivar CR showed greater biomass production in comparison to CL in both of the studied climatic conditions. The high-quality value of these genotypes could be of interest for organic food chains, especially in traditional rural districts, by establishing the specific denomination of origin and geographic indication labels. On the other hand, as evidenced in this study, and as confirmed by previous works, sprouts showed a greater quantity of GLSs than more developed plantlets, such as microgreens and baby leaves. The potential interest in their production and consumption should be focused on innovative and ecologically sustainable production systems, paying particular attention to their sustainability and socioeconomic impact on organic food chains, as well as on the health of the consumers. In addition, these new products could be grown at home and utilized for fresh consumption and/or home processing (beverage, juices, snacks, etc.).

Author Contributions: Conceptualization, F.B. and D.A.M.; methodology, F.B., D.A.M. and D.R.; software, S.T.; validation, F.B., D.A.M. and S.T.; formal analysis, S.T.; investigation, M.C.D.B., S.T. and D.A.; resources, F.B. and M.C.D.B.; data curation, M.C.D.B., F.B., S.T. and D.A.; writing—original draft preparation, M.C.D.B. and S.T.; writing—review and editing, F.B., D.R. and D.A.M.; visualization, S.T. and D.R.; supervision, F.B. and D.R.; project administration, F.B.; funding acquisition, F.B. All authors have read and agreed to the published version of the manuscript.

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Morphometric Characteristics, Polyphenols and Ascorbic Acid Variation in *Brassica oleracea* L. Novel Foods: Sprouts, Microgreens and Baby Leaves

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Abstract: In the present study, we investigated the content and profile of polyphenols (PPH), ascorbic acid (AA), the Folin–Ciocalteu index (FCI), and antioxidant activity (1,1-diphenyl-2-picrylhydrazyl (DPPH) and peroxyl radical (ROO)) variation during three different plant growth stages (sprouts, microgreens and baby leaves) of two broccoli types, the traditional Sicilian sprouting broccoli landrace ('Broccolo Nero') and the broccoli standard ('Cavolo broccolo Ramoso Calabrese'), and the standard commercial cultivar of kale ('Cavolo Lacinato Nero di Toscana'). All biomasses collected were freeze-dried for PPH, AA, FCI, DPPH and ROO analysis. The highest polyphenol content was observed for 'Broccolo Nero' (BN) and 'Cavolo Broccolo Ramoso Calabrese' (CR), and generally sprouts showed significantly higher values compared to the microgreens and the baby leaves. The AA, FCI, DDPH and ROO significantly vary with regards to the cultivar and the plant growth stage, showing interaction between the two experimental factors analyzed. The interaction detected showed higher values for the antioxidant traits of the proposed novel food, especially for the two broccoli cultivars in the sprout growth stage in comparison to the microgreens and baby leaves. Our results suggest that the antioxidant activity is partially dependent on kaempferol and apigenin. The PPH compounds showed the highest values of kaempferol and apigenin for 'Broccolo nero', whereas for the other two cultivars studied, only kaempferol was the main compound represented. The data acquired are of interest for increasing the healthy traits of the novel food proposed showing the contribution offered by the neglected LRs until now underutilized and at risk of extinction. The germplasm conserved in several world genebanks could support and diversify the organic vegetable items, providing us with added-value products for organic food supply chains.

Keywords: antioxidants; functional foods; plant growth stage; broccoli; kale; landraces

1. Introduction

Brassica oleracea vegetables are a good source of many phytochemicals with health-related activity, and their dietary consumption is associated with the reduction in the incidence of several human chronic inflammatory diseases [1–4]. Both the profile and the amount of these phytochemicals are strongly affected by the genotype (different species/cultivar/landraces), by the environmental conditions

during plant growth and by the different plant growth stages. Some authors have detected great variation of several antioxidant compounds, as such as glucosinolates, carotenoids, polyphenols and ascorbic acid, and of the related bioactivity of several landraces (LRs) and crop wild relatives (CWRs) of *B. oleracea*, evidencing elite breeding materials useful for improving the nutraceutical traits of the products [5,6].

Among the detected phytochemicals, polyphenols are a large class of organic compounds including several aromatic rings associated with different phenolic groups. Phenolics range from simple and single aromatic-ringed compounds, with a low molecular-weight, to large and complex tannins and derived polyphenols [7,8]. They can be classified based on the number and arrangement of their carbon atoms in flavonoids (flavanols, flavones, flavan-3-ols, anthocyanidins, flavanones, isoflavones and others) and non-flavonoids (phenolic acids, hydroxycinnamates, stilbenes and others) and they are commonly found in plants as conjugated to sugars and organic acids [7].

Polyphenols are often produced in response to biotic or abiotic stress [9] and act as reducing agents, hydrogen donating antioxidants and singlet oxygen quenchers. The multifunctionality of polyphenols is due to their distribution in different tissues and organs of plants at different concentrations. The most widespread and different groups of polyphenols in *Brassica* species are the flavonoids (mainly flavonols but also anthocyanins) and the hydroxycinnamic acids [10,11]. Phenolic compounds play a role in different stages of cancer development, exerting their activity in regulating different signaling pathways involved in cell survival, growth and differentiation of the reproductive organs.

Vitamins are indispensable micronutrients for humans with antioxidant activity. Studies in vitro demonstrated vitamin (E and C) consumption decreases the risks of chronic diseases by acting as direct antioxidants and electron donors [12,13].

Today, new natural products containing high levels of bioactive compounds consumed through food are being researched and studied. Sprouts, microgreens and baby leaves represent a growing market segment within vegetable products, mainly consumed raw for their high nutritional value and sensory traits [14]. Candidate genotypes are expanding based on sensory and health criteria; however, currently, most of the species belong to Brassicaceae, Asteraceae, Chenopodiaceae, Lamiaceae, Apiaceae, Amarillydaceae, Amaranthceae, and Cucurbitaceae families [15].

In the last few years, the request of seeds and sprouts of broccoli and of other Brassicaceae species has become increasingly popular among consumers interested in improving and maintaining their health status by changing dietary habits [16].

The production and commercialization of sprouts are legally defined, and generally they are grown in dark conditions, without growing medium, fertilizers and agrochemicals [14]. Studies carried out on broccoli sprouts exposed to low intensity and high intensity of ultraviolet A (UVA) or ultraviolet B (UVB) demonstrated an enhancement of bioactive compounds, suggesting their exploitation as a functional food with potential industrial applications [17].

A new class of specialty salad crops exploited for their color- and flavor-enhancing phytonutrient content are the microgreens [18,19]. They differ from sprouts for the fully developed cotyledons and the appearance of the first true leaf; they are defined as young and tender vegetables, with a species-dependent production cycle of 1–3 weeks from seed germination, and they are harvested at soil level [20,21]. Compared to their mature-leaf counterparts, microgreens contain higher amounts of important phytonutrients (ascorbic acid, β-carotene, phylloquinone, etc.), minerals and lower nitrate levels [20,22]. Microgreens have been proposed as 'super foods' for their favorable content in micronutrients and bioactive compounds, whose yield can be increased by modulating the blue light proportion in red and blue light-emitting diode lighting [23–25]. Due to their phytonutrient compounds, a recent study supported the idea that microgreens could be considered as a resilient phytochemical factory for the dietary and psychological needs of crew members in orbital flights and platforms [26]. They are thought to be a new food for solving malnutrition problems affecting over two-thirds of the world's people living in countries of every economic status.

Among the ready-to-eat products, the baby leaf vegetable market has been growing and offering to consumers convenient, healthy and appealing items, which may contain useful bioactive compounds. The consumer demand for more convenient fresh food products led to a rapid growth in the fresh-cut industry, which became a multi-billion-dollar sector worldwide in the last decade [27,28]. Fresh-cut vegetables can meet the consumer demands for their relationship among food, healthy lifestyle and convenience [29].

The production of sprouts, microgreens and baby leaves from local varieties and wild edible species may provide novel and nutraceutical vegetables, which can satisfy the demand of modern consumers. Moreover, they represent further expressions of biodiversity in vegetable production, contributing to preserve and to increase the value of many vegetable LRs at risk of genetic erosion or extinction. The phytochemical composition of Brassicaceae varies considerably as a consequence of the plant growth stage and of the species considered [30]. For these reasons, we investigated the content of bioactive compounds at three stages of plant growth (sprouts, microgreens and baby leaves) of a traditional Sicilian broccoli LR and of two commercial standard cultivars, one of broccoli and one of kale.

2. Materials and Methods

2.1. Plants Materials, Seed Morphological Characteristics and Germination Test

Seeds of the standard commercial cultivars of broccoli (*Brassica oleracea* L. var. *italica* Plenck) 'Cavolo Broccolo Ramoso Calabrese' (CR) and of kale (*Brassica oleracea* L. var. *acephala*) 'Cavolo Lacinato Nero di Toscana' (CL) were purchased by the S.A.I.S. S.p.A. seed company (Cesena, Italy). The Sicilian landrace of sprouting broccoli (*Brassica oleracea* L. var. *italica* Plenck) 'Broccolo Nero' (BN) belongs to the Di3A active genebank collection (BR 354, UNICT 4939).

The weight of 1000 seeds, number of seeds per gram, perimeter, longitudinal and transversal lengths were registered. The germination test was carried out at the laboratory of the Department of Agriculture, Food and Environment (Di3A) of Catania University (Italy). Four replicates of 50 seeds for each cultivar (cv) were placed in 90 mm Petri dishes, between two layers of filter paper (Whatman® no. 2), imbibed with 10 mL of distilled water. Petri dishes were placed in dark condition in five incubators (FTC 90E, Refrigerated Incubator, VELP Scientifica, Italy) set at constant temperatures of 5, 10, 15, 20 and 25 °C. At the end of the experiment, the following indices were calculated: germinability (%) = ((N/NT) × 100), where N is the number of germinated seeds and NT is the number of the total seeds utilized; Mean Germination Time (MGT) = $\sum$(ni × di)/n, where n indicates the number of germinated seeds at day i, d is the incubation period in days, and n is the number of the total seeds germinated.

2.2. Sprouts, Microgreens and Baby Leaves Production and Characterization

For the sprouts, microgreens and baby leaf production, seeds were sown in cellular trays placed in cold greenhouse under natural light (4.6 to 9.2 MJ·m^{-2}·d^{-1}) and temperature (15.4 ± 5.8 °C) conditions, from November to December 2017 at Catania (South Italy, 37°31'10" N 15°04'18" E; 105 m above sea level (m a.s.l.) using organic growing practices. We utilized the organic substrate Brill®semina bio (Geotec, Italy) to fill the cellular trays and we treated the baby leaves once by BTK ® 32 WG (Xeda, Italy) based on *Bacillus thuringiensis* sub. *kurstaki* for controlling *Pieris brassicae*. The plantlets were irrigated on the basis of the ordinary techniques. Plants were collected at the three plant growth stages analyzed: sprouts (germinated seeds without coats and roots), microgreens (plantlets with the first true leaf) and baby leaves (plantlets with almost three true leaves), weighted and stored immediately at −80 °C, and then freeze-dried for analyzing them.

Plants were characterized for the main morphological descriptors. Longitudinal and transversal lengths of the cotyledons, number of true leaves, if present, and the hypocotyl length were measured. The morpho-biometric parameters of sprouts, microgreens and baby leaves were acquired using the software IM50 v. 117 (Leica, Germany).

2.3. Polyphenol Analysis

The polyphenol compounds were analyzed in accordance with Soengas et al.'s method [31] with some modifications. For the extraction, 60 mg of lyophilized powder was dissolved in 1.5 mL of a 1:1 v/v mixture of methanol and 0.06 N HCl. The mixture was vortexed and put in an ultrasonic bath for 10 min. After the thermal hydrolysis which was performed by putting samples in hot bath (80 °C for 1 h), the mixture was centrifuged (13,000 rpm, 10 min, 4 °C). The supernatant was stored at −20 °C and analyzed by HPLC Agilent 1200 series system equipped with a diode array detector (DAD). We utilized the analytical column Lichrospher 100RP-18 (240 × 4 mm i.d., particle size = 5 µm). The mobile phase contained water/acetic acid (90:10, v/v, A) and acetonitrile/acetic acid (90:10, v/v, B). Chromatography was performed with 0.6 mL/min flow rate and the following gradient program: 0–7 min 1% B, 7–20 min 30% B, 20–28 min 50% B, 28–33 min 50% B, 33–38 min 1% B, 38–48 min 1% B. The injection volume of sample was 30 µL. Polyphenols were detected by DAD monitoring the absorbance at 280, 310, 325, 350, 520 nm. Hence, the characterization of each phenol compound was based on its characteristic absorption spectra. Each sample was analyzed in triplicate. Quantification was based on the calibration curves of external standards, by comparing each compound through the absorption spectra for apigenin, caffeic acid, chlorogenic acid, cyanidin chloride, gallic acid, coumaric acid, kaempferol and sinapic acid; polyphenols concentrations were expressed in mg g^{-1} d.w.

2.4. Ascorbic Acid Analysis

For the ascorbic acid (AA) measurements, the titration method reported by Thillmans et al. [32] was used. Freeze-dried samples were treated by 3% metaphosphoric acid by shaking. Extracts after filtration on filter paper (size: 110 mm, medium filtration rate, particle retention: 5–13 µm) were titrated by 1.5 mM 2,6-dichlorophenol-indophenol (DCIP) water solution at room temperature. The ascorbic acid contents were quantified by comparing them with the standard curve obtained for the known ascorbic acid concentrations. Each sample was analyzed in triplicate, and the results were expressed as mg g^{-1} d.w.

2.5. Folin–Ciocalteu Index

The Folin–Ciocalteu index (FCI) was calculated on methanolic extracts as described by Meda et al. [33], with slight modifications. Twenty milligrams of freeze-dried material were dissolved in 0.5 mL of 80% ethanol (EtOH). The mixture was vortexed and put in ultrasonic bath for 10 min and then centrifuged (13,000 rpm, 10 min, 4 °C). The supernatant was collected and the remaining pellet was re-extracted as described above. The collected supernatant was pooled. Twenty microliters of the extract were diluted with 1580 µL of distilled water and 100 µL of Folin-Ciocalteu reagent and then we left it at room temperature for 5 min. The solution was then treated with sodium carbonate (20%, 300 µL) and incubated in the dark for 2 h at room temperature. The absorbance of the blue-colored solution was measured at 750 nm. Each sample was analyzed in triplicate. The total polyphenol content was expressed as gallic acid equivalents (GAE mg g^{-1} d.w.) after using a standard calibration curve obtained by the known concentrations of the gallic acid standard.

2.6. Antioxidant Activity

The antioxidant capacity was measured by 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical quenching and peroxyl radical (ROO) scavenging methods. For the sample preparations, 100 mg of lyophilized powder was extracted by 1.5 mL of a 1:1 v/v mixture of ethanol (EtOH) and 0.06 N HCl, homogenized and centrifuged at 25,000 g for 5 min at 4 °C. The DPPH was measured using electronic paramagnetic resonance (EPR) with a MiniScope MS200 Magnettech (Berlin, Germany) following the protocol detailed in Picchi et al. [34]. The antioxidant activity towards ROO was determined spectrophotometrically by measuring the enzymatic peroxidation of linoleic acid after the addition of lipoxygenase, as described in Lo Scalzo et al. [35].

2.7. Statistical Analysis

The experiments were performed using a completely randomized design. The significance of differences between the main factors of morphometric characteristics was evaluated by one-way analysis of variance (ANOVA). Data were reported as mean ± standard error (S.E.). To evaluate the effect of the genotype and of the stage of growth, the results of the Folin–Ciocalteu index, ascorbic acid and antioxidant activity were analyzed through a multifactorial ANOVA. The mean values associated with the main factors as well as their interactions were evaluated using Tukey's test ($p < 0.05$). All statistical analyses were performed using CoStat release 6.311 (CoHort Software, Monterey, USA).

3. Results

3.1. Germination Process, Seed and Plant Characteristics

The seed characteristics of BN, CR and CL are shown in Table 1. The weight of 1000 seeds was higher for BN (4.5 g) and lower for CR (3.9 g); the number of seeds per gram showed the statistically lower value for BN (223.6) in comparison to CL (242.1) and CR (254.4). No significant differences for the other morphometric parameters were registered (Table 1).

Table 1. Seed morpho-biometric characteristics of 'Broccolo Nero' (BN), 'Cavolo Laciniato Nero di Toscana' (CL) and 'Cavolo Broccolo Ramoso Calabrese' (CR).

Characteristics	BN	CL	CR	*p* Value
Weight of 1000 seeds (g)	4.5 ± 0.1a	4.1 ± 0.0b	3.9 ± 0.0c	***
Seeds per gram (n)	223.6 ± 2.5c	242.1 ± 2.7b	254.4 ± 2.4a	***
Perimeter (mm)	6.0 ± 0.1	6.1 ± 0.1	5.8 ± 0.1	ns
Longitudinal length (mm)	1.8 ± 0.0	1.8 ± 0.0	1.8 ± 0.1	ns
Transversal length (mm)	2.0 ± 0.0	2.1 ± 0.1	2.0 ± 0.1	ns

Data are reported as mean ± S.E. Means were compared using Tukey test (*** $p < 0.001$; ns: $p > 0.05$). Values in the same row followed by the same letter are not significantly different at $p < 0.05$.

For all genotypes no germination occurred at 5 °C. In this sense, the genotypes seem to be more sensitive to low temperatures than high ones, during germination; in fact, all genotypes showed the highest germinability (100%) at highest temperature (25 °C) (Figure 1).

MGT was significantly affected by temperature for all the genotypes studied (Table 2); the number of days for germination decreased by increasing the temperature (from 4.5 days at 10 °C to 2.1 days at 25 °C). Among the genotypes studied, CL was the fastest to germinate at 25 °C (2.1 days) while the slowest was CR (2.6 days), showing a significant interaction between the two experimental factors analyzed.

With regards to the morphometric characteristics of the sprouts, significant differences were observed among the cultivars (cvs) studied (Table 3). The sprouts were collected seven days after the sowing when all of them showed the cotyledons well disclosed without any seed coat. The weight of ten sprouts varied from 1.0 (BN) to 1.4 g (CR), showing a very sensible increment from the weight of the seed. The weight of the sprouts did not show any significant difference among the cvs, despite the size of the cotyledons and of the hypocotyl varying among the genotypes (Table 3).

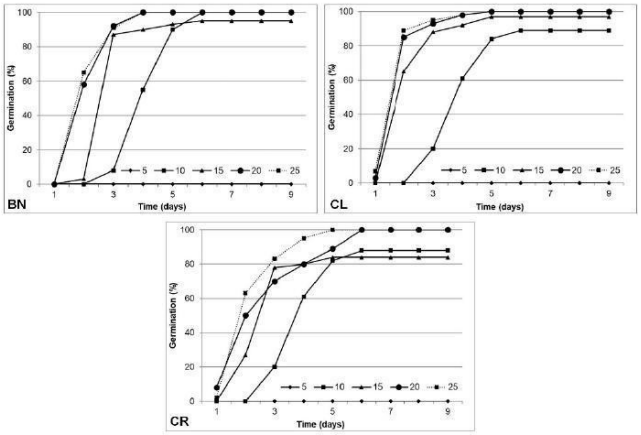

Figure 1. Progress of germination percentage (%) of BN, CL and CR seeds in relation to temperature (5, 10, 15, 20 and 25 °C). Each point represents the mean of four replications of 50 seeds.

Table 2. Effect of growing temperature on the mean germination time (MGT) of studied genotypes.

MGT (days)	10 °C	15 °C	20 °C	25 °C
BN	4.5 ± 0.04a	3.1 ± 0.04a	2.5 ± 0.04b	2.4 ± 0.05b
CL	4.0 ± 0.06b	2.5 ± 0.04c	2.2 ± 0.11c	2.1 ± 0.08c
CR	4.1 ± 0.04b	2.8 ± 0.05b	3.0 ± 0.24a	2.6 ± 0.03a

Data are reported as mean ± S.E. Means were compared using Tukey test. Values in the same column followed by the same letter are not significantly different at $p < 0.05$.

The microgreens were harvested 19 days after the sowing when the first true leaf of all the individuals reached the minimum size of 20 mm. The weight of ten microgreens varied from 2.7 (CL) to 3.4 g (BN), increasing by about three-fold compared to the sprouts (Table 3). The weight of the microgreens did not show any significant difference among the cvs (Table 3), despite the differences in hypocotyl and cotyledon length (Table 3). The first true leaf showed a similar size among the cvs (Table 3).

The baby leaves were harvested 39 days after the sowing when the plants showed about 3–4 true leaves. The weight of ten baby leaves varied from 7.0 to 8.4 g, respectively, for CL and BN, and the length of the stem ranged from 107.8 to 125.15 mm for BN and CL, respectively (Table 3).

The weight of baby leaves increased more than two-fold in comparison to the microgreens (Table 3). The number of the true leaves varied from 3.0 to 3.6 and their length and width ranged significantly from 113.5 to 124.3 mm and from 32.6 and 42.5 mm, respectively, for CL and BN (Table 3).

The stem, the cotyledons and the leaf midribs and veins were well distinguishable from both CL and CR ones for their reddish color dues to the high content of anthocyanins (Figure S1).

Four grams of seeds, on the average of the three genotypes studied, produced 120 g of sprouts, 300 g of microgreens and 760 g of baby leaves after 7, 19 and 39 days from sowing, respectively. The great increment of the weight of these products in so short a time offers an idea of the high added value of these novel foods that could be of great interest for seed companies.

Table 3. Morpho-biometric characteristics of the sprouts, microgreens and baby leaves of the three cultivars studied. Data are reported as mean ± S.E. (n = 10).

Characteristics	Sprouts				Microgreens				Baby leaves			
	BN	CL	CR	Means	BN	CL	CR	Means	BN	CL	CR	Means
Weight of 10 individuals (g)	1.0 ± 0.1a	1.2 ± 0.2a	1.4 ± 0.3a	**1.2 ± 0.0**	3.4 ± 0.5a	2.7 ± 0.4a	2.8 ± 0.3a	**3.0 ± 0.1**	8.4 ± 0.7a	7.0 ± 0.8a	7.4 ± 0.5a	**7.6 ± 0.5**
Hypocotyl length (mm)	42.1 ± 0.8a	29.2 ± 3.6b	36.6 ± 2.3ab	**36.0 ± 1.7**	43.3 ± 0.6a	29.5 ± 2.6b	28.9 ± 3.2b	**33.9 ± 1.5**	-	-	-	**-**
Cotyledon length (mm)	10.6 ± 0.2ab	10.3 ± 0.8b	14.2 ± 1.2a	**11.7 ± 0.8**	32.4 ± 0.4a	23.5 ± 0.2b	25.7 ±1.6b	**27.2 ± 0.6**	-	-	-	**-**
Cotyledon width (mm)	14.5 ± 0.2b	12.2 ± 0.8b	16.4 ± 1.1a	**14.4 ± 0.8**	23.1 ±0.4a	17.6 ±1.0a	17.8 ±2.2a	**19.5± 1.2**	-	-	-	**-**
Stem length (mm)	-	-	-	**-**	-	-	-	**-**	107.8 ± 3.9a	125.5 ± 6.9a	122.4 ± 5.3a	**118.6 ± 3.9**
Number of true leaf (n)	-	-	-	**-**	1.0 ± 0.0a	1.0 ± 0.0a	1.0 ± 0.0a	**1.0 ± 0**	3.5 ± 0.2a	3.6 ± 0.3a	3.0 ± 0.0a	**3.4 ± 0.1**
Leaf length (mm)	-	-	-	**-**	25.6 ± 2.4a	23.7 ± 1.7a	27.2 ± 2.7a	**25.2 ± 2.0**	124.3 ± 2.1a	113.5 ± 1.8b	120.7 ± 1.5ab	**119.5 ± 0.6**
Leaf width (mm)	-	-	-	**-**	16.4 ± 1.5a	17.6 ± 1.0a	19.0 ± 2.0a	**17.7 ± 0.9**	42.5 ± 1.4a	32.6 ± 0.9b	35.3 ± 0.9b	**36.8 ± 0.7**
Weight of 10 individuals (g)	42.1 ± 0.8a	29.2 ± 3.6b	36.6 ± 2.3ab	**36.0 ± 1.7**	43.3 ± 0.6a	29.5 ± 2.6b	28.9 ± 3.2b	**33.9 ± 1.5**	-	-	-	**-**
Hypocotyl length (mm)	10.6 ± 0.2ab	10.3 ± 0.8b	14.2 ± 1.2a	**11.7 ± 0.8**	32.4 ±0.4a	23.5 ± 0.2b	25.7 ±1.6b	**27.2 ± 0.6**	-	-	-	**-**

Means between genotypes (BN, CL and CR) for each growth stages were compared using Tukey test ($p < 0.05$). The statistical analysis was performed via one-way ANOVA. Values in the same row followed by the same letter are not significantly different at $p < 0.05$.

The data show the variation of the size of the plant during the first growing stages, which depends on the average of the genotypes utilized, mainly for the cotyledon length and width in the sprouts, on the enlarged cotyledons and on the first true leaf size for the microgreens, and on the number and size of the true leaves for the baby leaves (Table 3).

3.2. Multifactorial ANOVA

The multifactorial ANOVA showed that the Folin–Ciocalteu index (FCI), ascorbic acid (AA) and antioxidant activity (DPPH and ROO) were significantly affected by genotype and the stage of plant growth, as well as by their interaction (Table 4). The highest FCI values were measured for BN and CR, and generally sprouts showed significantly higher values compared to microgreens and baby leaves. Similarly, AA content was higher for BN and CR compared to CL, and it tended to increase with the growth stage, since its level was higher for microgreens and baby leaves compared to the sprouts.

Table 4. Results of the multifactorial ANOVA for Folin–Ciocalteu index (FCI), ascorbic acid (AA), 1,1-diphenyl-2-picrylhydrazyl (DPPH) and peroxyl radical (ROO) scavenging activity according to the factors "genotype" and "stage of growth" and their interaction.

			FCI	AA	DPPH	ROO
Genotype (G)						
		BN	39.06a *	6.67a	3.29b	82.55a
		CL	34.48b	5.48b	2.91c	80.19a
		CR	40.02a	6.34a	3.81a	70.53b
			$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$
Stage of growth (S)						
		Sprouts	41.9a	5.7c	3.73a	86.89a
		Microgreens	36.9b	6.1b	3.09b	72.28b
		Baby leaves	34.8c	6.3a	3.20b	74.11b
			$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$
G x S						
		Sprouts	42.9b	6.4b	3.22c	93.28ab
	BN	Microgreens	37.2cd	7.5a	3.23c	89.66b
		Baby leaves	37.1d	6.1bc	3.43bc	64.71d
		Sprouts	35.9de	4.8d	3.54b	99.40a
	CL	Microgreens	34.2ef	5.9c	2.61d	63.23d
		Baby leaves	33.3f	5.8c	2.59d	77.95c
		Sprouts	46.9a	6.1bc	4.41a	67.99d
	CR	Microgreens	39.2c	6.5b	3.43bc	63.94d
		Baby leaves	34.0ef	6.5b	3.59b	79.66c
			$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$

* The mean values associated with the two factors and their interaction were evaluated according to Tukey's test. Means significantly different are indicated by different letters.

With regard to the antioxidant activity, the DPPH quenching capacity provided similar results of the FCI analysis, since it was higher for CR and BN and it decreased during the growth stage. The ROO quenching capacity showed the highest values for BN and CL and it decreased during growth, similarly to what was observed for DPPH analysis.

3.3. Total Polyphenol Content

The FCI showed the highest value for the CR sprouts, whereas the lowest ones were ascertained for the microgreens of CL and the baby leaves of CL and CR (Figure 2). The FCI of microgreens did not significantly differ for BN and CR, while with regard to the baby leaves, BN showed significant higher value compared to CL and CR. CL showed the lowest FCI value in all growth stages (33.3, 34.2, 35.9 mg GAE g^{-1} d.w., for the baby leaves, microgreens and sprouts, respectively) (Figure 2).

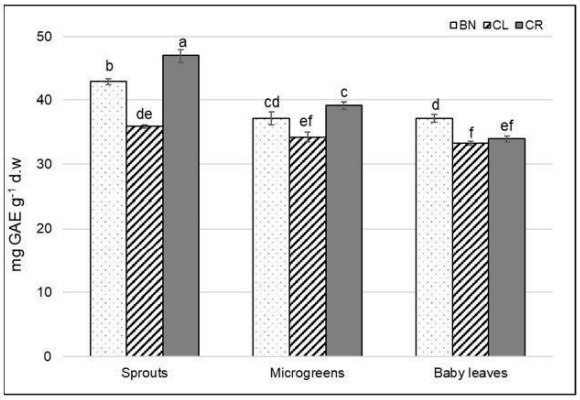

Figure 2. Folin–Ciocalteu index in sprouts, microgreens and baby leaves of BN, CL and CR. Data are reported as mean ± S.E. Bars with the same letters are not significantly different, as determined by Tukey's test ($p < 0.05$).

3.4. Polyphenol Analysis

The seeds of the three cultivars showed significant difference of the polyphenols content. HPLC analysis showed the main PPH compounds of the cultivars studied are represented by sinapyl and gallic acid derivatives (Table 5). The highest content of gallic acid derivate was found in CL (0.525 mg g^{-1} d.w.) while the lowest one was found in BN (0.206 mg g^{-1} d.w.). The highest content of sinapyl acid derivates was observed for CR (3.572 mg g^{-1} d.w.) and the lowest for BN (2.826 mg g^{-1} d.w.).

Table 5. Polyphenol content in seeds of BN, CR and CL.

Genotypes	Gallic Acid Derivate (mg g^{-1} d.w.)	Sinapyl Acid Derivate (mg g^{-1} d.w.)	Total Polyphenol Content (mg g^{-1} d.w.)
BN	0.206 ± 0.021b	2.826 ± 0.051b	3.032 ± 0.252b
CL	0.525 ± 0.062a	3.413 ± 0.251a	3.937 ± 0.313a
CR	0.261 ± 0.047b	3.572 ± 0.412a	3.832 ± 0.456a

Data are reported as mean for each genotype ± S.E. The statistical analysis was performed via one-way ANOVA. Values in the same column followed by the same letter are not significantly different at $p < 0.05$ (Tukey's test).

The content of the main polyphenols compounds of the three cultivars are reported in Figures 3–5. The PPH compounds showed a significant interaction between the cultivars and the plant growth stage.

The phenolics identified by HPLC were caffeoyl, chlorogenic acid, cyanidin, gallic acid, kaempferol, p-coumaric acid, sinapyl, and apigenin derivates. They were identified by their UV-VIS DAD spectral properties, which were compared with the used external standards (Figures 3–5).

For BN, for all the plant growth stages studied, kaempferol (15.1, 20.8 and 50.6 mg g^{-1} d.w. In sprouts, microgreens and baby leaves, respectively), and apigenin derivate (17.9, 30.6 and 43.8 mg g^{-1} d.w. sprouts, microgreens and baby leaves, respectively) predominated. The chlorogenic and p-coumaric acid was not detected in BN baby leaves, and it was observed in higher amount for sprouts than for the other two stages (Figure 3).

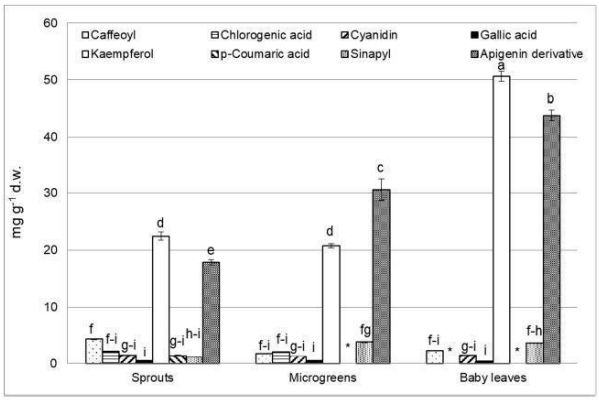

Figure 3. Polyphenol compounds for BN at different growth stages. Data are reported as mean ± S.E. Bars with the same letters are not significantly different, as determined by Tukey's test ($p < 0.05$). Symbol * = not detected compound.

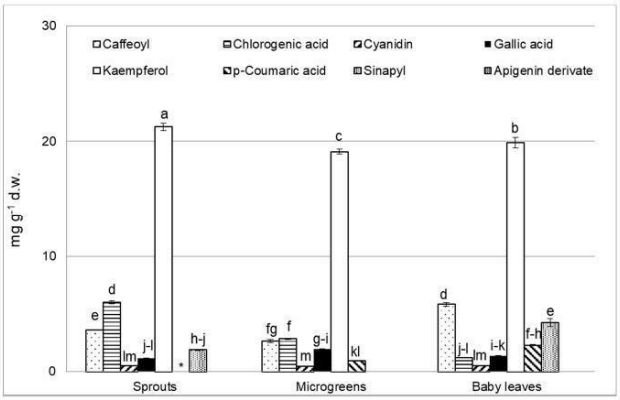

Figure 4. Polyphenol compounds for CL at different growth stages. Data are reported as mean ± S.E. Bars with the same letters are not significantly different, as determined by Tukey's test ($p < 0.05$). Symbol * = not detected compound.

With regard to CL, kaempferol (~20.0 mg g^{-1} d.w.) was the main component in all stages of growth. The p-coumaric acid was not detected in sprouts, and sinapyl derivates were present only in sprouts and baby leaves (Figure 4).

CR was characterized by the predominance of caffeoyl, kaempferol, gallic acid, and sinapyl derivatives, and a very small amount of other compounds. Chlorogenic acid was not found in all the plant growth stages studied (Figure 5).

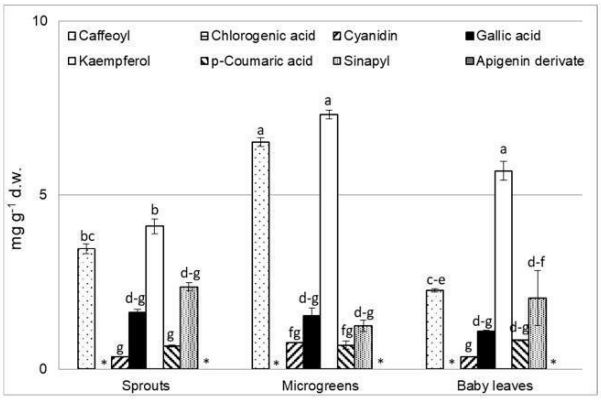

Figure 5. Polyphenol compounds for CR at different growth stages. Data are reported as mean ± S.E. Bars with the same letters are not significantly different, as determined by Tukey's test ($p < 0.05$). Symbol * = not detected compound.

3.5. Ascorbic Acid Content

Ascorbic acid (vitamin C) is an essential nutrient for the human body, acting as an antioxidant. The vitamin C content, measured by acid ascorbic (AA), is shown in Figure 6. Significant differences in the content of AA at individual stages of plant growth were noticeable. The highest amount of AA was ascertained for the microgreens of BN (7.5 mg g^{-1} d.w.) while the lowest for the sprouts of CL (4.8 mg g^{-1} d.w.) (Figure 6). A significant increment of the AA amount was observed from the sprouts to the microgreens of BN and CL whereas for CR the value was stable for all the three plant growth stages (Figure 6).

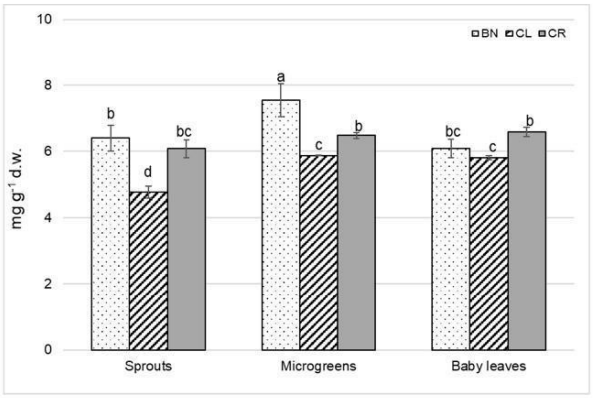

Figure 6. Ascorbic acid (mg g^{-1} d.w.) in BN, CL and CR in sprouts, microgreens and baby leaves. Each value represents mean ± S.E. of three replicates. Bars with the same letters are not significantly different, as determined by Tukey's test ($p < 0.05$).

3.6. Antioxidant Activity

The DPPH quenching activity of CR was significantly higher ($p < 0.001$) compared to BN and CL (Figure 7, top). The data of DPPH scavenging ranged from 2.6 to 4.5 mmol AA eq/100 g dw. In particular, CR sprouts were distinguished for the highest DPPH activity (4.41 mmol AsAeq/100 g d.w.) which was 37.0% and 24.4% higher compared to BN and CL sprouts, respectively (Figure 7), with similar values for BN for all three stages. With regard to microgreens and baby leaves, CR and BN showed similar values, while for CL and CR, both microgreens and baby leaves were characterized by lower DPPH quenching activity. In this case, the evolution of the DPPH antioxidant capacity was similar to the Folin–Ciocalteu index (Figure 2; Figure 7) In fact, the two parameters were significantly correlated ($r = 0.68$, $p < 0.001$), and both had a tendency to decrease from sprouts to baby leaves, particularly for CL and CR.

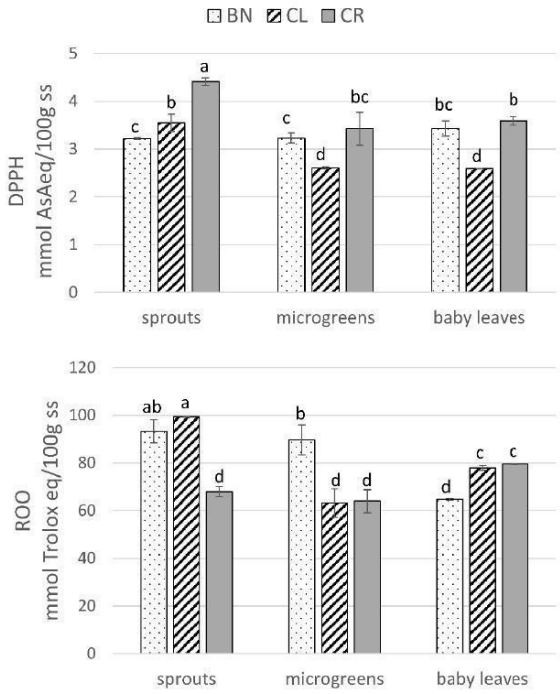

Figure 7. DPPH (mml AsAeq/100 g^{-1} d.w.) and ROO (mmol trolox equ/100 g^{-1} d.w.) in BN, CL and CR in sprouts, microgreens and baby leaves. Each value represents mean ± S.E. of three replicates. Bars with the same letters are not significantly different, as determined by Tukey's test ($p < 0.05$).

The data of ROO scavenging ranged from 57 to 100 mmol Trolox eq/100 g dw. As for the ROO scavenging capacity, the highest values were found in sprout samples of the BN and CL genotypes (around 100 mmol Trolox eq/100 g d.w.), while the values tended to decrease during the plant growth, particularly for BN, for which the baby leaves reached values of around 60 mmol Trolox eq/100 g d.w. (Figure 7, bottom). On the contrary, for CL and CR, we ascertained a slight but significant increment of the ROO scavenging activity values from microgreens (around 60 mmol Trolox eq/100 g d.w.) to the

baby leaves (around 80 mmol Trolox eq/100 g d.w.), not detected in DPPH scavenging data, for which we registered similar scavenging values.

4. Discussion

Several authors focused their attention on the optimization of the germination process in order to maximize the content in health-promoting compounds of the sprouts [36,37]. Seeds can germinate at a wide range of temperatures, but the germinability is drastically reduced at extreme temperatures [38]. Previous investigation showed a good germinability at 30 °C but with a higher MGT values in comparison to 25 °C [37]. That has been observed in our study in which no germination occurred at 5 °C for all the genotypes analyzed.

The phenolic profile of vegetables can be influenced by intrinsic factors related to the plant growth stage and to genetic variability, also within the same species. In seeds, the differences between varieties and genotypes suggested that the genotype was the main factor of variation; in fact, our results show that the main content in polyphenols was shown for CL and CR. During the germination process, reactivation of seed metabolism takes place, promoting the hydrolysis of storage proteins and carbohydrates by the synthesis/accumulation of metabolites with health-promoting properties [39].

According to Paśko et al. [16], higher total phenolic content in sprouts compared to seeds suggest the synthesis of phenolic antioxidants during germination may occur. The strong correlation found between total polyphenol content and antioxidant activity also suggests that this content is a good predictor of the in vitro antioxidant activity. It is thought that seeds mainly act as a reservoir for the development of the sprouts [40]. The PPH compounds increased significantly for the three cultivars considered, from the seeds to the baby leaves stage, showing significant differences among them (Figures 3–5).

Some of the health-promoting factors may be present at a ten times higher level in sprouts than in mature vegetables [41,42]. This is the case of the flavonoids content and of the other phenolic compounds that clearly contribute to the antioxidant potential [43].

Germination determines significant changes in the phenolic content, mainly due to activation of endogenous enzymes and to the complex biochemical metabolism of seeds during this process [44]. For this reason, germination may be a suitable process to obtain functional food with high antioxidant capacity. Sprouts are produced by the seed germination process and they represent an interesting novel food and a valuable alternative to increase the consumption of different seeds in human nutrition [45]. Sprout phenolic composition depends on genotype as well as on numerous environmental factors, including temperature, light or dark condition, humidity and sprouting time [46] and also water quality and salt content [42].

With regard to the cvs analyzed, BN showed the highest increment of the kaempferol and apigenin from the seed to the baby leaves growth stage, whereas for CL and CR, we ascertained the increment of the PPH compounds, mainly represented by kaempferol, caffeoyl and chlorogenic, just after germination process with no significant differences among the three plant growth stages considered (Figures 4–6).

According to the literature data, *Brassica* plants are rich in anthocyanins, phenolic acids and flavanols, in particular, kaempferol and quercetin [47]. In different studies conducted by Cartea et al. [11,48] on qualitative flavonoid evaluation in *B. napus*, quercetin and kaempferol glycosides were found as the major compounds in *Brassica* samples, in full qualitative accordance of previous data [11,48] with the here presented ones. Moreover, a quantitative survey by Velasco et al. [48] on kale and leaf rape reported a total phenol amount of 3–5 mg g^{-1} d.w., very close to the here presented data in seeds (Table 5), and lower than the data in vegetative parts reported in the present work, especially for BN samples (Figure 3).

According to Pająk et al. [49] and Velasco et al. [48], a range of phenolic acids have been identified in sprouts (chlorogenic, gallic, sinapyl, ferulic, p-cumaric). Broccoli sprouts are very rich in phenolic compounds, more than commercial broccoli florets; among them, free phenolic acids, caffeic, chlorogenic

and gallic acids occurred in the largest amounts; the main free phenolic acids found in broccoli sprouts were ferulic and sinapic acids [49]. Elevated levels of flavonoids and phenolic acids, which are highly bioavailable, were observed in a study conducted by Gawlik-Dziki et al. [50] on broccoli sprouts; this is in accordance with our results where sprouts of BN showed the highest content of kaempferol and apigenin derivates.

Phenolic compounds and vitamin C are the major antioxidants of *Brassica* vegetables. The content of vitamin C among *Brassica* vegetables varies significantly among and within each species and interspecific entity [1]. The cause of the reported variations in vitamin C content might be related to the different genotypes studied [51,52]. In particular, in the study by Vallejo et al. [52], the content of vitamin C was very irregular among the genotypes analyzed; the content ranged from 43.1 mg per 100 g fw in Lord (commercial cultivar) to 146.3 mg per 100 g fw in SG-4515 (experimental cultivar). In our work, the highest content in vitamin C was found in BN microgreens. Vitamin C is an important water-soluble dietary antioxidant, which significantly decreases the adverse effects of the free radicals can cause oxidative damage to macromolecules such as lipids, DNA and proteins, which are in turn implicated in chronic diseases, like cardiovascular disease, stroke, cancer, neurodegenerative diseases and cataractogenesis [53].

The average levels of DPPH and ROO scavenging activity were highest in sprouts and tended to decrease with growth (Figure 7). In particular, as regards to the DPPH quenching activity, CR and CL sprouts showed higher values compared to microgreens and baby leaves, and a similar trend was observed for ROO antioxidant activity, even if with different results depending on the cultivars. Our findings partially agree with the results of Ebert et al. [54], who found that the antioxidant activity for amaranth sprouts was much higher than for microgreens, but not for the fully expanded leaves plant growth phase. Indeed, higher levels of the Folin–Ciocalteu index were found in sprouts (Figure 2), while the highest level in AA was found in microgreens (Figure 6) and single polyphenols usually increased in baby leaves, particularly for BN (Figure 3). These results suggest the presence of other antioxidants than kaempferol and apigenin or AA in Brassica sprouts, which may enhance the antioxidant capacity of the sprouts compared to the microgreens and the baby leaves. Interestingly, the Sicilian landrace BN of broccoli was distinguished for the highest ROO scavenging activity both at the stage of sprouts and of microgreens, but not at the stage of baby leaves, for which its value significantly decreased. On the other hand, for CL and CR, the ROO antioxidant capacity of the baby leaves increased in comparison to microgreens, being finally higher by around 20% compared to the BN baby leaves.

5. Conclusions

The activities carried out provide additional data related to the novel foods proposed, such as sprouts, microgreens and baby leaves. *B. oleracea* crops, and in this case broccoli and kale, are shown to represent good sources of antioxidant compounds, such as polyphenols and ascorbic acid, confirmed also by their high antioxidant capacities. The cultivars utilized showed different polyphenol profiles among them and also in relation to the plant growth stages proposed, correspondent to the three novel foods investigated.

The total polyphenols showed the highest values for the sprouts of both the two cvs of broccoli utilized. The Sicilian landrace of 'Broccolo Nero' showed the highest amount of kaempferol and apigenin in late growth stages, whereas the former represents the main polyphenol compound for the other cultivars of broccoli and of kale studied. Among the plant growth stages analyzed, the baby leaves showed high values of kaempferol and apigenin. The ascorbic acid varied significantly both in relation to the cultivar and to the plant growth stage and the highest value was observed for the microgreens of 'Broccolo Nero'. The antioxidant capacity showed in general the highest values for the sprouts for all the three cultivars analyzed.

The experimental factors analyzed offer the opportunity to improve the knowledge related to these novel foods of interest for diversifying the vegetable items by the exploitation of the *B. oleracea*

landraces. Of interest are the data of the antioxidant traits acquired for implementing the nutraceutical and organoleptic traits of the novel foods proposed for improving the horticultural organic food supply chains.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4395/10/6/782/s1, Figure S1: Different growth plant stages of the Sicilian landrace 'Broccolo nero' (BN): from the top to bottom, sprouts, microgreens and baby leaves.

Author Contributions: The following statements should be used "Conceptualization, F.B. and D.R.; methodology, F.B., D.R., R.L.S., V.P.; software, S.T., V.P.; validation, F.B., S.T., R.L.S.; formal analysis, S.T., V.P.; investigation, A.N., M.C.D.B., V.P.; resources, F.B.; data curation, F.B., S.T., M.C.D.B., V.P.; writing—original draft preparation, F.B., S.T. M.C.D.B., V.P.; writing-review and editing, F.B., D.R., S.T.; supervision, F.B.; project administration, F.B.; funding acquisition F.B. All authors have read and agreed to the published version of the manuscript.

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Evaluation of Italian and Spanish Accessions of *Brassica rapa* L.: Effect of Flowering Earliness on Fresh Yield and Biological Value

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Abstract: A comparative study for evaluating Italian and Spanish accessions of *Brassica rapa* var. *rapa* L., including turnip greens and turnip tops, was carried out at different locations with a view to determine the effect of earliness on crop production, antioxidant activity, glucosinolates amount, and profile (GLSs) and total phenolics content. The accessions evaluated were represented by two turnip top local varieties (one Italian variety and a Galician one), four new synthetic varieties established by Misión Biológica de Galicia (MBG-CSIC), and three commercial varieties widely used by growers in Galicia and in Italy. The results showed a great variability regarding flowering time, fresh and dry weight of the leaves and flower buds, and the branch number per plant. The highest turnip greens production was found in two synthetic varieties ("SIN07" and "SIN01") for both countries. Local varieties "BRS550" and "CM39" were also suitable for turnip greens production in Spain and Italy, respectively. For turnip tops, the highest production was found for "SIN07" in Spain, for "CM39" in Italy and for "BRS550" in both countries. We found a high diversity in the total and individual glucosinolate, phenolic content, and antioxidant activity among genotypes, geographical origins, and the different parts of the plant (leaf and flower). Varieties "SIN01" and "SIN07" showed the highest values in total GLSs, total aliphatic and gluconapin contents in turnip greens followed by the two commercial varieties. For turnip tops, the highest values in gluconapin, aliphatic, and total GLSs contents were found in "SIN01" and "BRS550". Even though different varieties stand out over the rest depending on the location, "SIN01", "SIN07", "CM39", and "BRS550" could be recommended for turnip greens production because of its high antioxidant activity. The study showed that the latest varieties are more productive and show higher bioactive compounds than the earlier ones and that it is possible to improve genotypes for different growing cycles. Therefore, these varieties could be proposed for further breeding programs for *B. rapa* production.

Keywords: turnip greens; turnip tops; synthetic varieties; fresh production; glucosinolates; phenolic compounds; antioxidant activity

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1. Introduction

Species belonging to the Brassicaceae family support one of the world's most economically important crop groups. They range from noxious weeds to leaf and root vegetables and to oilseed and condiment crops. *Brassica rapa* is an important oil and vegetable crop in many parts of the world, with seeds being used for oil, and leaves, flowers, stems, and roots being used as vegetables [1]. The cultivation of this species for many centuries has caused a large variation in the plant organs that are consumed, which represent the results of human selection of different morphotypes, depending on local preferences [1,2]. Based on their morphological characteristics, there are three well defined groups of *B. rapa*:

(1) the oleiferous type of which canola is a specific form, having low erucic acid levels in its oil and a low glucosinolate (GLSs) content in its meal protein; (2) the rapiferous type, comprising the rapifera group (turnip, rapini), and the ruvo group (turnip broccoli, Italian turnip, "cima di rapa") and (3) the leafy type, including the rapa group (turnip greens), the chinensis group (pak-choi), the pekinensis group (Chinese cabbage), and the perviridis group (tendergreen) [3].

Vegetable *B. rapa* crops, including rapifera and leafy types, are widely grown in Asia and Europe. In Europe, they are notably popular in Portugal, Italy and Spain [4–6], where they play an important role in traditional farming and in the diet. In these countries, *B. rapa* includes two main crops, turnip greens and turnip tops, as vegetable products. Turnip greens are the young leaves harvested trough the vegetative period, while turnip tops are the young inflorescences with the flower buds and their surrounding leaves, which are consumed before opening and while they are still green In Italy, turnip tops (or "cima di rapa") is a typical vegetable grown mainly in the Southernmost Italian regions, such as in Apulia, where it is sold at local markets nearly all year round. They are commonly consumed as boiled vegetables, being used in the preparation of soups and stews and they have a slightly spicy flavor like mustard greens [6]. Turnip greens and turnip tops have a good commercial potential in both countries, and the number of industries producing *B. rapa* canned products has been increasing in the last years.

A collection of vegetable *B. rapa* from Spain is currently kept at Misión Biológica de Galicia (CSIC, Spain). Agronomical and nutritional evaluations of this collection were previously performed concerning genetic diversity [6] and the GLSs amount and profile in leaves [7,8] under specific environmental conditions in NW Spain. These works highlighted the enormous diversity existing in Galician *B. rapa* local varieties and their use to produce improved new varieties that are adapted to modern consumer habits and to a wide range of environmental conditions.

Despite the wide consumption of *B. rapa* in Italy and Spain, little information about the genetic resources preserved and used in these countries is available. With a view to expand the use of underexploited local varieties/landraces and new varieties, a more exhaustive evaluation of their agronomic performance and biological value on human health in a wide range of environmental conditions, like the Mediterranean and Atlantic areas, becomes necessary.

The high genetic diversity described within *B. rapa* species [2,9] would be useful to select varieties with a high production and an improved nutritional value. One of the biggest concerns on *B. rapa* production is the harvest period. Turnip greens are harvested throughout the vegetative period (autumn), while, in the case of turnip tops, it is in late winter, as they require a prolonged cold exposure before flowering. Turnip tops have a very short harvest period between the appearance of the first flower buds and before bolting. Therefore, the development of turnip greens and turnip tops varieties with different earliness cycles will be important, not only to have a continuous production through autumn–winter for fresh consumption but also to have varieties adapted to requirements of the agro-food industry. These vegetables are consumed as fresh vegetables and as processed foods (frozen and canned). Developing new *B. rapa* varieties with new attributes would be interesting for both fresh and industrial markets. The introduction of new *B. rapa* varieties in fresh and agro-food markets can render a profit, not only for producers but also for consumers, due to the possibility of including new vegetables of high biological value in their diet.

Most efforts made in *B. rapa* breeding have been focused on the oleiferous types, where canola varieties were successfully developed in the 1980s. However, breeding for vegetables types like turnip greens or turnip tops was scarcely developed, and so far, no programs have been carried out for obtaining new varieties with different earliness cycles. Except for oil seed types, most major types of *B. rapa* are self-incompatible, and pure line selection is complicated, labor-intensive, and it often leads to severe inbreeding depression. Therefore, most cultivars are produced by mass or family selection. Consequently, there is a

strong interest in developing new cultivars by using simple recurrent selection procedures to select local varieties with a benefit for consumers and producers. Varieties with a higher production and a wide range of adaptability to edaphic and climatic conditions are essential for breeding programs. The evaluation of different genotypes at different locations across the years will provide us with valuable information about their agronomic performance and will allow us to identify superior cultivars on several environments.

Brassica crops are considered to be important vegetables due to several evidences of their health-promoting effects, such as a reduction in the risk of chronic diseases, particularly cardiovascular diseases and several types of cancer [10–15]. The beneficial effects that are associated to *B. rapa* consumption have been linked to the presence of phenolics and GLSs in these plants and to many constituents having a strong antioxidant activity [7,16–18]. GLSs and their degradation products have been extensively researched for their role in cancer prevention and plant defense [12,19,20]. On the other hand, antioxidant compounds are being extensively researched due to their potent antioxidant activity, their ability to wipe up harmful free radicals, and the health benefits associated to them. The antioxidant properties of some *Brassica* vegetables such as cabbage, broccoli, cauliflower, kale, and turnip have been studied extensively [9,16,21–23]. However, relatively little is known about the antioxidant properties of *B. rapa* var. *rapa* leaves and floral buds, which are common foods in some European Mediterranean countries.

In a previous study carried out by our group, the effect of earliness and plant habit in the total and individual GLSs content was studied from a set of local varieties of *B. rapa* [8]. Authors suggest that the variation on GLSs concentrations was affected by earliness. Early varieties had the lowest gluconapin content, although the result was dependent on the environment. Therefore, further exhaustive studies will be necessary with other genotypes and environments in order to verify these first results.

To the best of our knowledge, few information is available on (i) the effect of earliness of *B. rapa* genotypes on fresh production in turnip greens and turnip tops or (ii) the effect of earliness on bioactive compounds and antioxidant capacity. Thus, the objectives of the present study were to evaluate the agronomic attributes of new *B. rapa* genotypes with different earliness when compared to *B. rapa* commercial varieties under different environments and (ii) to evaluate the effect of the genotypes and the origin on the GLSs profile and content, total phenolic content, and the antioxidant activity.

2. Materials and Methods

2.1. Plant Material

Seven varieties of *B. rapa* var. *rapa* from Galicia (Spain) and two varieties from Apulia (Italy) were chosen for this study (Table 1). Out of these ones, four synthetic varieties (denominated "SIN01", "SIN05", "SIN06", and "SIN07") were developed at Misión Biológica de Galicia (MBG-CSIC, Galicia, NW Spain). Synthetic variety "SIN01" was obtained after four cycles of mass selection according to its agronomic performance for turnip greens fresh production in a typical production area for *B. rapa* crops (Oroso, A Coruña, Spain). Synthetic varieties "SIN05", "SIN06", and "SIN07" were obtained after three cycles of mass selection and were designed to obtain three synthetic turnip tops varieties with different earliness (early, medium, and late, respectively). Each synthetic variety was obtained in 2011 (cycle 0) after cross-pollination of a set of Galician local varieties kept at the MBG *Brassica* germplasm bank and selected depending on the abovementioned traits for each synthetic variety. In 2011, approximately 300 plants from cycle 0 (C0) for each synthetic variety were transplanted in the field. For synthetic "SIN01", the best 60 plants for turnip greens production were selected (≈20% selection intensity). For synthetic varieties "SIN05", "SIN06", and "SIN07", the 60 earliest, medium, and latest plants, respectively, were selected (≈20% selection intensity). The non-selected plants were removed before flowering, and cross-pollination among the selected plants in each plot was obtained by using bumblebees (*Bombus terrestris*). In 2012, seeds were taken from the selected plants belonging to each synthetic variety to create cycle 1. From 2013 to 2015, this process was repeated for

three successive generation cycles for "SIN05", "SIN06", and "SIN07" and for four cycles (from 2013 to 2016) for "SIN01". Two commercial varieties ("Grelos de Santiago" and "Nabo Globo de Lugo") of turnip greens that are widely used in Spain and a local variety ("BRS0550") used by canning companies for turnip greens production were included as checks for comparison with the new synthetic varieties. The two accessions used in Italy are Nabo sessantino ("CM39"), an extra-early variety, and Broccoletto di Rapa Sessantino Riccio San Marzano ("CM24"), an Italian commercial variety (Pagano sementi) that shows a dark green lamina, while the petiole is whitish.

Table 1. Description of *Brassica rapa* varieties studied and their classification according to their flowering earliness.

Variety Name	Description	Origin	Source [1]	Flowering Earliness
SIN05 C3	Synthetic	Spain	MBG	Early
SIN06 C3	Synthetic	Spain	MBG	Medium
SIN07 C3	Synthetic	Spain	MBG	Late
SIN01 C4	Synthetic	Spain	MBG	Medium
BRS0550	Landrace	Spain	MBG	Extra-late
Nabo globo de Lugo	Commercial	Spain	Rocalba	Medium
Grelos de Santiago	Commercial	Spain	Rocalba	Late
Broccoletto di Rapa sessantino Riccio San Marzano ("CM24")	Landrace	Italy	UNICT 817	Extra-early
Nabo sessantino ("CM39")	Commercial	Italy	UNICT 3272	Extra-early

[1] MBG: Germplasm Bank at the Misión Biológica de Galicia, Pontevedra, Spain; UNICT: active genebank collection at the University of Catania, Italy.

2.2. Experimental Design

These varieties were evaluated for 2 years (2016–2017 and 2017–2018) in Spain, at two representative locations, Oroso (A Coruña) (43°1′ N, 8°26′ W, 280 m.a.s.l.), and Salcedo (Pontevedra) (42°24′ N, 8°38′ W, 20 m.a.s.l), and in Catania (Sicily, Italy) (37°31′ N, 15°4′ E 105 m.a.s.l.). In Spain, transplanting dates were 23 September 2016 in Oroso, and 10 October and 27 October 2017 in Oroso and Pontevedra, respectively. In Catania, transplanting dates were 14 February 2016 and 9 November 2017. Varieties were planted in multipot-trays, and seedlings were transplanted into the field at the five-six leaf stage. Varieties were transplanted in a randomized complete block design with three replications. The experimental plots consisted of two rows with 15 plants per row. Rows were spaced 0.8 m apart and plants within rows were spaced 0.5 m apart. Irrigation was done after transplanting into the field and when it was required, depending on the rainfall, by drip irrigation. Cultural operations, fertilization, and weed control were made according to local practices. For pest control, Force® (Syngenta, Basel, Switzerland) was added at the time of transplantation to combat soil insects, Pyganic 1,4 (Biograd, Grassobbio (BG)) for aphids' control, and BTK ® 32 WG (Xeda, Forlì, Italy) based on *Bacillus thuringiensis* sub. *kurstaki* for controlling *Pieris brassicae*.

2.3. Biomorphometric Traits

Morphological and agronomical traits were recorded for turnip greens and turnip tops related to earliness and fresh production, along with the maturity cycle of varieties. Traits measured (Table 2) were adapted from the International Board for Plant Genetic Resources *Brassica* L. and *Raphanus* L. descriptors list [24].

2.4. GLSs Identification and Quantification

Two sample types were collected and analyzed: leaves (turnip greens), three months after sowing, and flower buds (turnip tops), taken sequentially depending on the maturity of each genotype, just after flower bud formation and before flower opening. Five samples of healthy leaves and young shoots from five plants per plot were used. Samples were frozen in situ on dry ice, immediately transferred to the laboratory, and frozen at −80 °C. All samples were lyophilized (BETA 2–8 LD plus, Christ, GmbH, Osterode

am Harz, Germany) for 72 h. The dried material was powdered by using an IKA-A10 (IKA-Werke GmbH & Co. KG, Staufen, Germany) mill, and the fine powder obtained was used for GLS analysis. Sample extraction and desulfation were performed according to Kliebenstein et al. [25] with minor modifications. Ten microliters of the desulfo-GLSs extract were used to identify and quantify GLSs. Chromatographic analyses were carried out on an Ultra-High-Performance Liquid-Chromatograph, UHPLC Nexera LC-30AD (Shimadzu, Kyoto, Japan) equipped with a Nexera SIL-30AC injector (Shimadzu, Kyoto, Japan) and one SPD-M20A UV/VIS photodiode array detector (Shimadzu, Kyoto, Japan). The UHPLC column was a XSelect HSS T3 XP Column C18 protected with a C18 guard cartridge (Waters Corporation, Milford, MA, USA). The oven temperature was set at 30 °C. Compounds were separated by using the following method in aqueous acetonitrile, with a flow of 0.5 mL min^{-1}: 1.5 min at 100% H_2O, an 11 min gradient from 0% to 25% (*v*/*v*) acetonitrile, 1.5 min at 25% (*v*/*v*) acetonitrile, a minute gradient from 25% to 0% (*v*/*v*) acetonitrile, and a final 3 min at 100% H_2O. Data were recorded on a computer with the LabSolutions software (Shimadzu, Kyoto, Japan). All GLSs were quantified at 229 nm by using glucotropaolin (GTP, monohydrate from Phytoplan, Diehm & Neuberger GmbH, Heidelberg, Germany) as an internal standard and quantified by comparison to purified standards. GLSs are reported as μmol g^{-1} dry weight (dw).

Table 2. Agronomic traits used in the evaluation of varieties of *Brassica rapa* from northwestern Spain and South Italy.

Agronomic Traits	Description
Time to flowering (d)	Days from transplanting until 50% of plants have the first flower
Turnip greens fresh production (g)	Average fresh weight of 30 leaves per plot
Turnip greens moisture (%)	Leaf water content
Branch number (n)	Average number of secondary stems per plant at first flower opening of five plants per plot
Leaf per plant (n)	Average number of leaves per plant at first flower opening of five plants per plot
Turnip tops fresh production (g)	Average fresh weight of the turnip top of five plants per plot
Turnip tops moisture (%)	Turnip top water content

2.5. Evaluation of Antioxidant Activity: ABTS Assay

Freeze-dried and ground samples (10 mg) were extracted with 1 mL of 80% aqueous methanol in dark maceration for 24 h. After centrifugation (3700 rpm, 5 min), methanolic extracts were employed in order to determine the antioxidant activity by [2,2′-azino-bis (3-ethylbenzothiazoline-6-sul-fonic acid)] (Sigma–Aldrich Chemie GmbH (Steinheim, Germany) cation assay (ABTS). Three technical replications were analyzed for each sample. A standard prepared with different concentrations of Trolox (0, 0.008, 0.016, 0.024, 0.032, 0.040 mM) (Sigma–Aldrich Chemie GmbH (Steinheim, Germany) was also measured. The antioxidant activity was normalized to Trolox equivalents per gram (g) of dry weight (dw). The method of decolorization of free radicals ABTS employed was a modified version of that used by Samarth et al. [26] and initially reported by Re et al. [27]. ABTS was generated by oxidation of ABTS 7 mM with potassium persulphate 2.45 mM in water, at room temperature for 16 h. For each analysis, the ABTS solution was freshly diluted with water in order to obtain an initial absorbance around 0.8 at 734 nm. An aliquot of 10 μL methanolic extract for the sample was added to 250 μL of ABTS solution. Absorbances were measured at 734 nm after 30 min of incubation in the dark at room temperature.

2.6. Estimation of Phenolic Content

Phenolic content was estimated according to the phenolic colorimetric method described by Dewanto et al. [28]. The same methanolic extracts employed for antioxidant activity assays were employed to determine phenolic content. Extracts were oxidized with

50 mL of 0.5 M Folin reagent (Sigma–Aldrich Chemie GmbH (Steinheim, Germany). After 5 min, 200 mL of a 20% Na_2CO_3 solution were added to neutralize the reaction. Then, the absorbance was read at 760 nm after 2 h of incubation in the dark at room temperature. Standards prepared with different concentrations of gallic acid (Sigma–Aldrich Chemie GmbH (Steinheim, Germany) (0, 0.008, 0.016, 0.024, 0.032, and 0.04 mM) were also measured. Results were expressed as micromoles of gallic acid equivalents per gram of dry weight.

2.7. Statistical Analysis

Each location × year interaction was considered as an environment. We conducted a mixed model ANOVA according to a randomized complete block design where the main effects of environments, varieties, and plant organs and their interactions were considered as fixed factors. Blocks and their interactions with varieties, environments, and plant organs were considered as random factors. As we found a significant variety × environment interaction, we also performed these analyses individually for each environment, considering the variety as a fixed effect and block, and the interaction between block and variety as random factors. Means comparisons were done with Fisher's protected least significant difference (LSD) at a 0.05 level of probability [29]. Analyses were made by using the GLM procedure of SAS 2007 statistical software (SAS Institute, Cary, NC, USA).

3. Results

3.1. Agronomical Traits

The combined analysis of variance showed significant differences among environments and varieties for most traits ($p < 0.01$). The variety × environment interaction was highly significant for most of them. Because of the significance of the interaction, individual analyses by environment were carried out for each trait. When comparing the results from the individual analyses for the most relevant agronomic traits, different varieties stand out over the rest, depending on the country but not on the location. Then, both magnitude and rank changes contributed to these interactions and analyses of variance were combined across countries. Therefore, comparisons were made between countries.

The means of combined data across environments for each country are shown in Table 3. These traits will allow us to define the most appropriate varieties for turnip greens and turnip tops fresh production. Turnip greens fresh matter ranged from 7.23 g to 28.38 g, the best varieties being "BRS0550", "SIN01", and "SIN07"in both countries, along with "CM39" in Catania (Table 3).

All these varieties were significantly better than the three commercial varieties in most environments.

Varieties flowered earlier in Italy than in Spain (148 days and 91 days, respectively) and turnip tops production was higher in Spain than in Italy (51.3 g and 28.2 g, respectively). No significant differences between countries ($p > 0.05$) were found for turnip greens fresh production and moisture. In conclusion, plants performed similarly in both countries for turnip greens production, but varieties had a better agronomic performance for turnip tops production in Spain.

The potential of these genotypes for turnip tops fresh production was evaluated at two locations in Spain and at one location in Italy, all of them during the 2017–2018 period because of these varieties' problems to produce this plant organ at the other environments. Branch number and earliness are useful traits to select varieties that are suitable for turnip top production. Varieties "SIN07" and "BRS0550" along with commercial variety "Grelos de Santiago" in Spain and two local varieties ("BRS0550" and "CM39") in Catania showed the highest turnip tops fresh production (more than 60 g per branch), differing significantly from all other varieties (Table 3). Variety "CM39" was under environmental conditions and growing seasons in Spain too early. Data for turnip tops fresh yield were recorded only at one environment in Spain, as plants performed badly and reached bolting when they were still small

Table 3. Means of several agronomical traits of nine *Brassica rapa* genotypes evaluated at three environments in Northwestern Spain and two environments in Italy.

Country	Varieties	Time to Flowering (d)	Turnip Greens Fresh Production(g)	Turnip Greens Moisture (%)	Branch Number[1] (n)	Leaf per Plant (n)	Turnip Tops Fresh Production [1] (g)	Turnip Top Moisture [1] (%)
Spain	BRS550	205.67 ± 2.36	27.73 ± 1.25	89.95 ± 0.62	15.72 ± 1.26	65.21 ± 10.2	65.27 ± 6.95	91.43 ± 2.56
	CM24	70.33 ± 1.33	8.30 ± 1.57	91.27 ± 0.30	7.21 ± 0.84	18.10 ± 2.9	24.66 ± 1.75	91.71 ± 1.65
	CM39	84.32 ± 3.11	7.23 ± 0.87	82.43 ± 3.41	6.53 ± 1.54	24.13 ± 7.5	20.52 ± 2.10	85.81 ± 0.78
	SIN01C4	145.11 ± 4.16	21.15 ± 1.68	90.34 ± 0.90	12.77 ± 0.79	63.84 ± 10.2	54.16 ± 6.65	85.05 ± 1.05
	SIN05 C3	117.00 ± 3.34	11.21 ± 1.29	89.65 ± 3.19	8.54 ± 1.84	42.51 ± 5.3	29.69 ± 4.73	79.38 ± 0.88
	SIN06 C3	140.00 ± 4.69	17.92 ± 1.63	90.08 ± 1.18	11.39 ± 1.41	55.14 ± 8.9	47.98 ± 5.69	83.85 ± 1.22
	SIN07 C3	152.11 ± 2.19	21.02 ± 1.93	90.55 ± 0.78	14.74 ± 0.59	72.23 ± 11.5	65.67 ± 12.71	84.06 ± 2.02
	Grelos de Santiago	161.33 ± 3.26	18.28 ± 1.38	89.84 ± 1.11	13.26 ± 0.89	63.61 ± 6.3	65.36 ± 13.45	85.92 ± 0.92
	Nabo Globo Lugo	156.44 ± 2.79	18.07 ± 1.36	89.77 ± 0.99	13.32 ± 1.21	69.32 ± 8.9	61.42 ± 8.94	84.72 ± 1.25
	LSD (5%)	3.84	3.52	1.08	2.95	26.31	11.73	3.68
Italy	BRS550	-[2]	16.50 ± 4.23	87.41 ± 1.05	6.33 ± 0.88	26.78 ± 5.9	74.00 ± 26.00	87.25 ± 2.19
	CM24	58.50 ± 6.50	9.81 ± 2.06	84.94 ± 3.00	6.13 ± 0.81	23.10 ± 5.6	20.00 ± 0.99	82.14 ± 1.12
	CM39	64.60 ± 0.40	26.52 ± 4.02	85.61 ± 0.53	7.83 ± 0.46	16.78 ± 2.8	60.40 ± 7.60	83.15 ± 0.43
	SIN01C4	82.67 ± 5.20	23.03 ± 2.42	87.09 ± 0.89	4.89 ± 0.61	46.89 ± 18.9	20.42 ± 4.08	86.29 ± 0.99
	SIN05 C3	78.83 ± 4.03	15.22 ± 2.32	87.96 ± 1.11	6.78 ± 0.83	26.89 ± 7.5	18.43 ± 2.00	83.16 ± 0.91
	SIN06 C3	84.25 ± 6.36	14.80 ± 2.33	88.43 ± 1.50	4.56 ± 0.38	45.8 ± 10.2	17.67 ± 3.84	86.52 ± 1.20
	SIN07 C3	92.50 ± 3.48	28.38 ± 4.10	86.62 ± 0.53	7.22 ± 0.89	41.71 ± 8.9	20.88 ± 8.08	83.62 ± 0.73
	Grelos de Santiago	111.17 ± 0.65	15.81 ± 1.95	85.55 ± 0.82	6.44 ± 0.71	53.22 ± 11.9	22.72 ± 5.90	81.55 ± 0.92
	Nabo Globo Lugo	130.00 ± 1.15	20.90 ± 3.66	86.61 ± 0.95	8.44 ± 1.71	55.50 ± 25.6	31.60 ± 10.28	84.61 ± 0.85
	LSD (5%)	4.52	8.35	3.62	4.18	23.59	30.61	4.25

[1] Data for turnip tops were recorded in the growing cycle 2017–2018 for two locations in Spain and one location in Italy; [2] Flowering data were not recorded for this variety since it did not get to bloom in this country.

From the point of view of the farmer-producer, flowering time is an important agronomic trait for *B. rapa* crops. Variety "BRS0550" showed the longest time to flowering (206 days in Spain and it was too late for Catania, where this variety did not bloom). On the contrary, the two Italian varieties, "CM24" and "CM39", were the earliest ones in all environments (70 and 84 days to flowering in Spain, and 59 and 65 days to flowering in Catania), differing significantly from all other varieties.

Besides flowering, branch number is another relevant trait to select varieties that are suitable for turnip tops production, since this trait may greatly affect the yield. Two varieties, "BRS550" and "SIN07", along with the two commercial varieties, "Grelos de Santiago" and "Nabo Globo de Lugo", presented the highest branch number (more than 13) in Spain. Similar results were found for varieties evaluated in Italy, where "CM39", "SIN07" and commercial variety "Nabo Globo de Lugo" showed more than 7 branches per plant, differing significantly from all other varieties (Table 3). All these varieties, except for "CM39", performed well for branch number and they were among the latest varieties, thus indicating that earliness was inversely related with fresh production.

Means for traits related to turnip greens and turnip tops production at each environment are shown in Figure 1. Significant differences among environments ($p < 0.001$) were found for turnip greens fresh production, being Pontevedra and Catania in 2017 the locations where varieties had the highest values (Figure 1a). Regarding turnip tops production, data were taken at three locations in 2017. Once again, Pontevedra was the location where varieties performed better for branch number and turnip tops fresh production (Figure 1b).

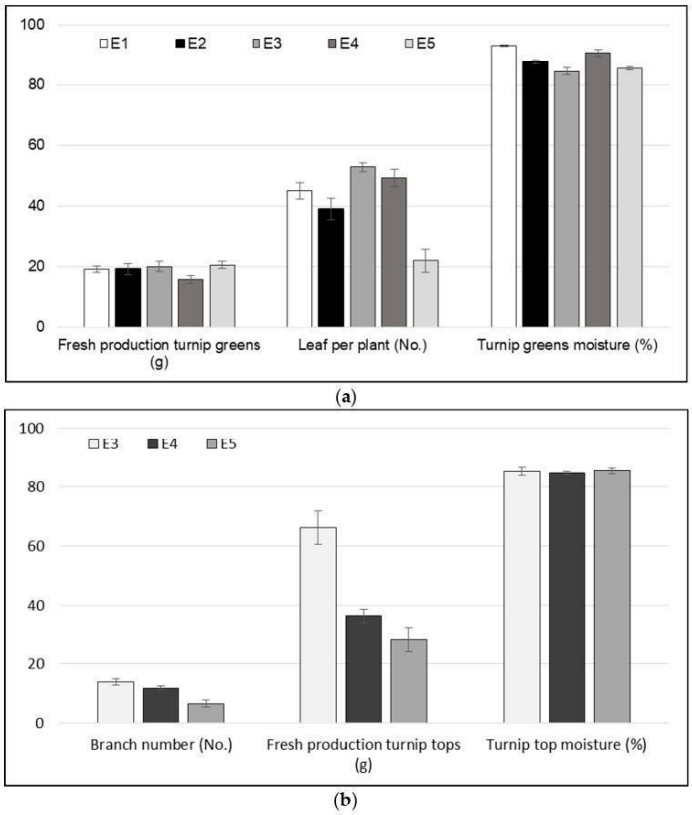

Figure 1. (**a**) Means for traits related to turnip greens fresh production: fresh production turnip greens (g), leaf per plant (No.), and turnip greens moisture (%) at the five environments where varieties were grown (E1 = Pontevedra 2016; E2 = Catania 2016; E3 = Pontevedra 2017; E4 = Oroso 2017; E5 = Catania 2017). (**b**) Means for traits related to turnip tops fresh production: branch number (No.), fresh production turnip tops (g) and turnip top moisture (%) at the three environments where varieties were grown (E3 = Pontevedra 2017; E4 = Oroso 2017; E5 = Catania 2017).

3.2. Variation of GSLs among Varieties, Locations, and Plant Organs

The chemical profile of *B. rapa* varieties studied in this work was composed of nine GLSs belonging to the three chemical classes: four aliphatic (progoitrin, glucoraphanin, gluconapin, and glucobrassicanapin), four indolic (glucobrassicin, 4-hydroxyglucobrassicin, methoxyglucobrassicin and neoglucobrassicin) and one aromatic (gluconasturtiin). Other aliphatic GLS such as glucoiberverin and gluconapoleiferin were found in minor quantities (Tables 4 and 5). GLS analyses for turnip greens were performed at five environments from Spain and Italy, whereas GLSs for turnip tops were evaluated at two Spanish locations during the 2017–2018 growing season, where plants were able to produce floral buds.

Table 4. Mean (μmol gram^{-1} dw) glucosinolate content in turnip greens from the *B. rapa* varieties evaluated at three environments in Northwestern Spain and two environments in Italy.

Variety	PRO	GRA	GNA	GBN	GBS	OHGBS	MGBS	NGBS	ALIPH	INDOL	AROM	Total GLSs
SIN01C4	0.80 ± 0.13	0.03 ± 0.01	40.14 ± 2.63	1.95 ± 0.22	1.68 ± 0.18	5.60 ± 0.63	1.34 ± 0.13	0.41 ± 0.06	42.97 ± 2.69	9.02 ± 0.84	0.78 ± 0.15	52.77 ± 3.29
SIN05C3	1.74 ± 0.22	0.07 ± 0.04	21.63 ± 2.43	1.23 ± 0.21	1.17 ± 0.14	3.23 ± 0.51	0.83 ± 0.11	0.20 ± 0.04	24.67 ± 2.64	5.42 ± 0.73	0.35 ± 0.10	30.45 ± 3.32
SIN06C3	1.20 ± 0.16	0.15 ± 0.06	30.91 ± 2.81	2.10 ± 0.36	1.43 ± 0.16	6.32 ± 0.94	1.16 ± 0.11	0.34 ± 0.05	34.50 ± 2.87	9.25 ± 1.15	0.61 ± 0.11	44.36 ± 3.56
SIN07C3	1.49 ± 0.20	0.02 ± 0.02	36.14 ± 2.08	3.61 ± 0.45	1.60 ± 0.14	6.81 ± 0.85	1.33 ± 0.13	0.33 ± 0.03	41.44 ± 2.16	10.07 ± 1.04	0.98 ± 0.14	52.50 ± 2.83
BRS550	2.59 ± 0.19	0.23 ± 0.57	32.49 ± 2.50	2.57 ± 0.30	1.60 ± 0.10	9.18 ± 0.90	1.92 ± 0.12	0.19 ± 0.02	37.98 ± 2.63	12.85 ± 1.05	0.69 ± 0.14	51.52 ± 3.24
CM24	0.09 ± 0.05	0	5.35 ± 0.87	6.33 ± 1.24	0.79 ± 0.19	1.01 ± 0.17	0.29 ± 0.02	0.21 ± 0.04	12.09 ± 2.17	2.30 ± 0.37	1.36 ± 0.29	15.74 ± 2.76
CM39	1.58 ± 0.32	0	6.58 ± 0.51	8.73 ± 0.75	1.16 ± 0.18	2.00 ± 0.49	0.71 ± 0.09	0.39 ± 0.06	18.71 ± 1.38	4.25 ± 0.66	0.97 ± 0.15	23.94 ± 1.88
Grelos de Santiago	0.21 ± 0.10	0.32 ± 0.08	33.62 ± 2.22	1.56 ± 0.24	1.39 ± 0.12	5.37 ± 0.55	1.16 ± 0.13	0.40 ± 0.06	35.85 ± 2.31	8.32 ± 0.74	1.35 ± 0.25	45.52 ± 2.93
Nabo G. Lugo	0.44 ± 0.19	0.04 ± 0.02	33.97 ± 2.70	1.21 ± 0.17	1.51 ± 0.16	4.48 ± 0.35	1.20 ± 0.13	0.32 ± 0.04	35.84 ± 2.72	7.51 ± 0.57	0.85 ± 0.14	44.20 ± 3.24
LSD (5%)	0.46	0.12	3.89	1.01	0.30	1.45	0.20	0.12	4.04	1.74	0.29	5.13

PRO, progoitrin; GRA, glucoraphanin; GNA, gluconapin; GBN, glucobrassicanapin; GBS, glucobrassicin; OHGBS, 4-hydroxyglucobrassicin; MGBS, methoxyglucobrassicin; NGBS, neoglucobrassicin; ALIPH, total aliphatics; INDOL, total indolics; AROM, total aromatic glucosinolates; total GLSs, total glucosinolates; LSD, least significant difference.

Table 5. Mean (μmol gram^{-1} dw) glucosinolate content in turnip tops from the *B. rapa* varieties evaluated at two environments in Northwestern Spain at the growing season 2017–2018.

Variety	PRO	GRA	GNA	GBN	GBS	OHGBS	MGBS	NGBS	ALIPH	INDOL	AROM	Total GLSs
SIN01C4	1.00 ± 0.55	0.01 ± 0.01	68.93 ± 4.27	2.04 ± 0.41	2.00 ± 0.16	0.12 ± 0.07	0.99 ± 0.08	0.34 ± 0.10	71.98 ± 3.98	3.46 ± 0.27	2.01 ± 0.14	77.44 ± 4.09
SIN05C3	3.98 ± 0.42	0.45 ± 0.11	36.13 ± 3.32	0.85 ± 0.12	2.02 ± 0.24	3.30 ± 0.83	0.49 ± 0.12	0.13 ± 0.03	41.43 ± 3.55	5.95 ± 1.15	2.20 ± 0.19	49.57 ± 4.51
SIN06C3	2.10 ± 0.74	0.25 ± 1.71	46.91 ± 5.37	1.96 ± 0.46	2.16 ± 0.31	0.20 ± 0.07	0.66 ± 0.08	0.20 ± 0.03	51.23 ± 5.51	3.20 ± 0.35	1.51 ± 0.19	55.94 ± 5.73
SIN07C3	1.77 ± 0.46	0.04 ± 0.02	59.91 ± 4.45	2.84 ± 0.63	1.89 ± 0.20	0.68 ± 0.15	0.59 ± 0.11	0.20 ± 0.05	64.62 ± 4.24	3.36 ± 0.30	2.15 ± 0.28	70.14 ± 4.34
CM24	0	0	8.93 ± 0.93	9.49 ± 0.75	0.94 ± 0.14	2.80 ± 0.30	0.09 ± 0.05	0.29 ± 0.07	19.51 ± 1.71	4.13 ± 0.48	3.13 ± 0.34	26.77 ± 2.35
BRS0550	5.64 ± 0.73	0	62.51 ± 3.34	1.71 ± 0.18	0.79 ± 0.04	1.48 ± 0.05	0.42 ± 0.05	0.19 ± 0.03	69.94 ± 3.29	2.88 ± 0.11	0.94 ± 0.03	73.76 ± 3.41
Grelos de Santiago	0.04 ± 0.02	0.44 ± 0.10	53.00 ± 2.35	1.33 ± 0.28	1.34 ± 0.08	0.30 ± 0.08	0.62 ± 0.10	0.18 ± 0.03	54.83 ± 2.38	2.44 ± 0.16	2.04 ± 0.25	59.29 ± 2.36
Nabo Globo Lugo	0	0.10 ± 0.04	57.47 ± 3.54	0.96 ± 0.25	1.30 ± 0.13	0.57 ± 0.10	0.50 ± 0.11	0.24 ± 0.07	58.68 ± 3.49	2.61 ± 0.20	1.42 ± 0.23	62.71 ± 3.49
LSD (5%)	1.32	0.26	11.06	1.21	0.56	0.43	0.26	0.18	10.7	0.87	0.57	11.09

PRO, progoitrin; GRA, glucoraphanin; GNA, gluconapin; GBN, glucobrassicanapin; GBS, glucobrassicin; OHGBS, 4-hydroxyglucobrassicin; MGBS, methoxyglucobrassicin; NGBS, neoglucobrassicin; ALIPH, total aliphatics; INDOL, total indolics; AROM, total aromatic glucosinolates; Total GLSs, total glucosinolates; LSD, least significant difference.

GLS quantification showed that aliphatic GSLs were predominant, representing 79 and 91% of the total GLS content in turnip greens and turnip tops, respectively. As it was expected for *B. rapa* crops, gluconapin was by far the most abundant GSL in Spanish varieties. However, Italian varieties had a different profile, where glucobrassicanapin was the main GSL in both plant organs. Gluconapin levels represented 67% and 83% of the total GLSs content in turnip greens and turnip tops, respectively. The mean value of gluconapin was 26.8 μmol g^{-1} dw in turnip greens and ranged from 5.4 μmol g^{-1} dw in "CM24" to 40.1 μmol g^{-1} dw in "SIN01". In turnip tops, the mean value of gluconapin was 49.3 μmol g^{-1} dw and ranged from 8.9 μmol g^{-1} dw in "CM24" to 68.9 μmol g^{-1} dw in "SIN01". In turnip greens, the second GLS in abundance was hydroxyglucobrassicin, representing 12% of the total GLS content. The mean value of hydroxyglucobrassicin in this plant organ was 4.9 μmol g^{-1} dw. In turnip tops, the second GLS in abundance of the total GLS content was glucobrassicanapin, representing 4.5% of the total GLS content. The mean value for glucobrassicanapin was 2.8 μmol g^{-1} dw.

The indolic group of GLSs represented between 19% and 6% of the total GLS content in turnip greens and turnip tops, respectively. Means for total indolic GLSs were 7.7 μmol g^{-1} dw in turnip greens and 3.50 μmol g^{-1} dw in turnip tops. Gluconasturtiin was the only aromatic GLS found in a concentration of 0.9 μmol g^{-1} dw and 1.90 μmol g^{-1} dw in turnip greens and turnip tops, respectively Aromatic GLSs were minor and ranged between 2% and 3% of the total GLS content in turnip greens and turnip tops, respectively.

Leaves of all varieties were harvested before bud opening at the same date, regardless of their earliness to reduce the effects due to different environmental factors, which notably affects the final GLS content. At this period, early varieties are close to the flowering stage; the medium ones are at the pre-flowering stage with floral bud formation starting, and late ones are at the vegetative stage. Because turnip tops are the other edible part that is very appreciated for food purposes, GLS content was also evaluated in trials carried out 2017 at two locations in Spain. Flower buds were collected according to the earliness of each variety.

In the individual analysis of variance performed for each plant organ, significant differences ($p \leq 0.001$) were found among varieties and environments for most individual and total GLS content in both plant organs. For turnip greens, varieties were significantly different for the total GLS content, as well as for most of the individual GLS content, except for glucobrassicin, glucoraphanin, neoglucobrassicin, and glucoiberverin (all of them minor GLSs in this crop). Differences among environments were significant ($p < 0.01$) for gluconapoleiferin (a minor GLS in this species).

Similar results were found in turnip tops. Varieties were significantly different for most of the individual and total GLS content, except for neoglucobrassicin, and differences among environments were significant for the two major GLSs (gluconapin and glucobrassicanapin) and three minor ones (glucoraphanin, 4-hydroxyglucobrassicin and gluconasturtiin).

Total and most individual GLS content showed a significant variety × environment interaction in both organs. Therefore, individual analyses of variance for these compounds were made at each environment. Comparing the results from the individual analyses, varieties showed a similar performance across environments, i.e., varieties with extreme values were the same at different locations for most traits. Then, magnitude changes rather than rank changes contributed to these interactions. For this reason, data are presented as means of the two locations.

Our results showed high variability for many of the components and for total glucosinolates among different genotypes in both plant organs. Varieties "SIN01" and "SIN07" showed the highest values on total GLS, total aliphatic and gluconapin contents in turnip greens, followed by the two commercial varieties (Table 4). For turnip tops, the highest values on gluconapin, aliphatic and total GLS contents were found for "SIN01" and local variety "BRS550" (Table 5). No data for turnip tops were taken for this last variety "BRS550" at Oroso because this variety did not flower at this location. Therefore, results for this

variety should be taken cautiously because of the effect of the environment in GLS values. The two commercial varieties also had high GLS levels, but they were significantly lower than in synthetic varieties "SIN01" and "SIN07".

For indolic GLSs, varieties with the highest contents were different depending on the plant organ. In turnip greens, "BRS550" and "SIN07" had the highest indolic GLS content, whereas in turnip tops, "SIN05" and "CM24" showed the highest indolic GLS content. For aromatic GLSs, Italian commercial variety "CM24" had the highest values for gluconasturtiin content both in turnip greens and in turnip tops.

Furthermore, significant differences ($p < 0.01$) were found between plant organs (turnip greens and turnip tops) for most GLSs analyzed. Similar to what happened with varieties, the location × plant organ interaction was significant for most GLSs (data not shown). In spite of these interactions, varieties and plant organs showed similar behaviors across locations for gluconasturtiin and total GLS content, and therefore, a combined analysis of variance across locations was made. As it has been already reported, the concentration of GLSs in *B. rapa* varies across stage development.

Comparisons between the two plant organs were carried out at two Spanish locations where plants could produce turnip greens and turnip tops. Data show that total GLS and total aliphatic and gluconapin content were higher in turnip tops than in turnip greens (Figure 2). In turnip greens, the total GLS content ranged from 20 μmol g^{-1} in CM24 to 64.2 μmol g^{-1} dw in "SIN01", with a mean value of 51.9 μmol g^{-1} dw. In turnip tops, the total GSL content ranged from 26.8 in "CM24" to 77.4 μmol g^{-1} dw in "SIN01" with a mean value of 58.9 μmol g^{-1} dw (Figure 2).

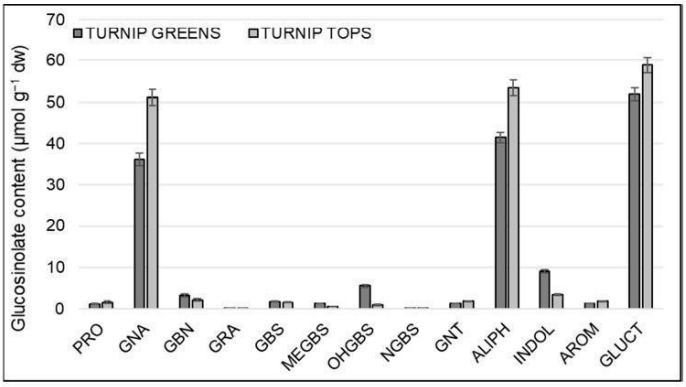

Figure 2. Total and individual glucosinolate content (μmol g^{-1} dw) measured in leaves (turnip greens) and flower buds (turnip tops) of nine *B. rapa* varieties grown at two locations from Spain in 2017–2018 growing season.

3.3. Variation of Phenolic Content and Antioxidant Activity among Varieties, Locations, and Plant Organs

For turnip greens, the combined analysis of variance across environments showed significant differences ($p < 0.01$) among varieties and environments for DPPH assays and for the total phenolic content. The environment × variety interaction was also highly significant. Therefore, individual analyses of variance for these compounds were made at each environment. When environments were analyzed by year and by location, varieties showed similar behaviors for all compounds across years for each location. Therefore, data are presented as means of the two years for each location (Figure 3).

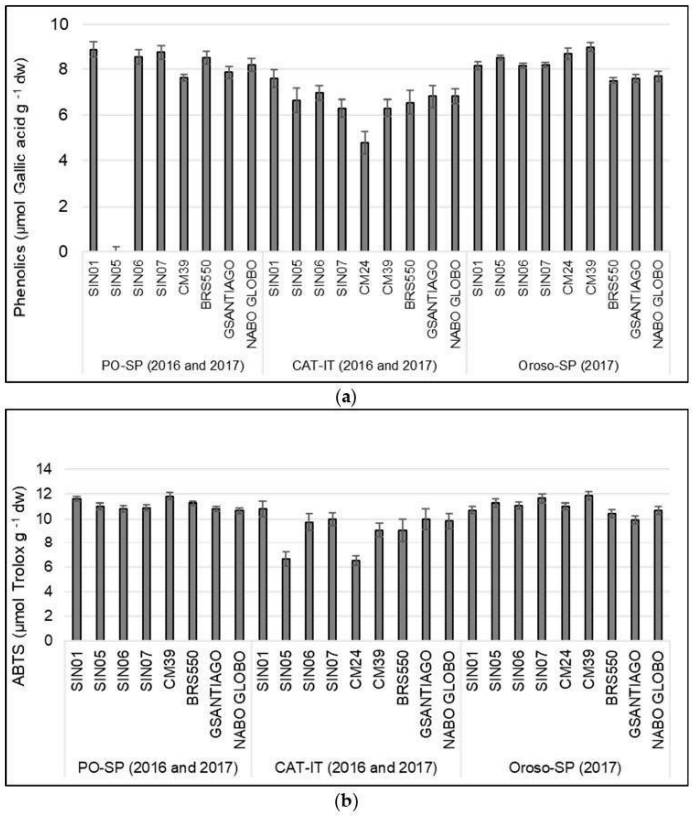

Figure 3. Phenolic content (**a**) and antioxidant activity measured with ABTS assays (**b**) in leaves (turnip greens) of nine *B. rapa* varieties grown at five environments in North Weast Spain and in South Italy for two years. Significant differences among environments ($p < 0.001$).

In this work, different varieties stand out over the rest, depending on the location. In Pontevedra variety "SIN01" had significantly higher leaf phenolic content than the other varieties (8.85 µmol Gallic acid g^{-1} dw), followed by "SIN07" (8.72 µmol Gallic acid g^{-1} dw), which significantly differ from the rest of varieties (Figure 3a). Synthetic "SIN01", along with the two commercial varieties, stand out over the rest in Catania, while "CM24" showed the lowest values for total phenolic content in leaves. The two Catania varieties, "CM24" and "CM39", showed the highest values for total phenolic content in Oroso, whereas Galician local variety "BRS550" and "Grelos de Santiago" had the lowest values (Figure 3a).

Variety "SIN01" had significantly higher antioxidant activity in leaves than the other varieties for ABTS in Pontevedra (11.6 µmol Trolox g^{-1} dw) and Catania (10.8 µmol Trolox g^{-1} dw), and it did not differ significantly from the varieties with the highest ABTS content in Oroso (Figure 3b). For this assay, "SIN01" was followed by "CM39" and "BRS550" in Pontevedra and by the two commercial varieties in Catania, which significantly differ from the rest of crops for both ABTS assays.

When comparing the antioxidant activity and the total phenolic content of the consumed organs (leaves and flowering buds) (Figure 4), no significant differences ($p > 0.05$) were found between plant organs for the compounds analyzed. In the combined analyses of variance across locations, the location × plant organ interaction was significant for the total phenolic content and ABTS assays. Varieties showed a different performance at each location, and therefore, data are shown for each location.

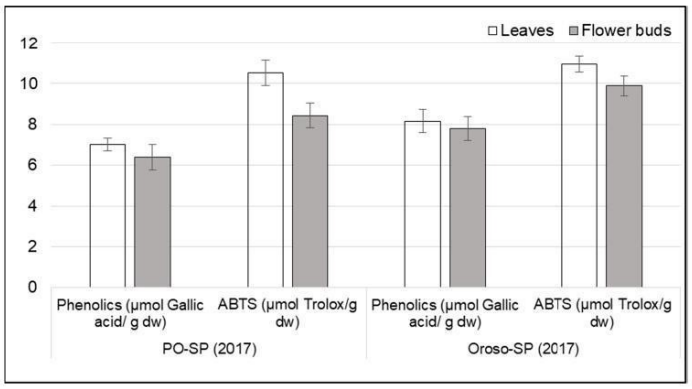

Figure 4. Phenolic content (µmol Gallic acid/g dw) and antioxidant activity measured with ABTS assays (µmol Trolox/g dw) of nine *B. rapa* varieties measured in leaves (turnip greens) and flower buds (turnip tops) in two locations from Spain in 2017–2018 growing season. Significant differences among environments ($p < 0.001$).

The earliest varieties "SIN05" and "CM24" had the lowest levels of antioxidant activity in leaves, whereas the latest varieties such us "SIN07" and "BRS550" showed the highest total phenolic content, thus suggesting a relationship between earliness and the antioxidant activity once again. However, this effect was not noticed in turnip tops, where variety "SIN05" had the highest total phenolic content and ABTS values in Pontevedra, while BRS550 and "SIN07" had the lowest values.

Total phenolic content showed a mean value of 6.99 and 6.37 µmol Gallic acid g^{-1} dw in Pontevedra in turnip greens and turnip tops, respectively. These values were similar in Oroso, where the total phenolic contents for turnip greens and tops were 8.15 and 7.79 µmol Gallic acid g^{-1} dw, respectively (Figure 4). For ABTS assays, contents ranged from 10.5 in turnip greens to 8.42 µmol Trolox g^{-1} dw in turnip tops grown in Pontevedra, whereas we found values from 11 µmol Trolox g^{-1} dw in turnip greens to 9.9 µmol Trolox g^{-1} dw in turnip tops in Oroso (Figure 4).

4. Discussion

Brassica rapa spp. *rapa* is an important vegetable species. Several plant parts, including turnip tubers, leaves and turnip tops, are important for human consumption. In the present study, agronomic value and bioactive compounds were determined for nine genotypes with different genetic background. Genotypes included landraces, synthetic and commercial varieties with different geographical origins and earliness. The overall goal of this study was to compare the agronomic value of synthetic varieties and local varieties versus commercial varieties and to select high yielding *B. rapa* varieties suitable for growing in different environments.

Due to the importance of a continuous turnip tops and turnip greens production throughout the autumn and winter for the fresh market and the processing industry, we developed three synthetic varieties (early, medium, and late). Late varieties delay turnip greens production, which can be very interesting for producers and agro-industrial

purposes, while early varieties allow offering turnip tops on the market before their usual dates. Besides, the earliest varieties have greater flexibility to grow in different seasons due to lower cold and photoperiod requirements, and they facilitate the product arrival in the markets very soon, which is desirable for the consumer. The convenience of one or the other will depend on the market and producers' decisions.

The best plants for turnip greens production should have a high leaf number, large leaves and high fresh weight content. The best varieties for turnip greens fresh yield were local variety extra-late BRS0550 and two synthetic varieties developed at MBG ("SIN01" and "SIN07"). Besides, agronomic performance of these genotypes was stable in both countries. The Italian variety "CM39" also showed good turnip greens production under the environmental conditions of Sicily, because it is well adapted to local conditions. Furthermore, all these varieties were significantly better than both commercial varieties "Grelos de Santiago" and "Nabo Globo de Lugo" in most environments tested in Spain and Italy.

Because turnip tops are the other plant organ that is highly appreciated for human consumption, its fresh production was also evaluated in trials performed in 2017. The highest turnip tops fresh production was noticed for extra-late variety BRS0550 in both countries. Synthetic "SIN07" and commercial variety "Grelos of Santiago" were suitable varieties for turnip tops in Pontevedra, whereas local variety "CM39", as it was noticed for turnip greens production, stood out over the other varieties in Catania once again.

Local varieties ("BRS550" and "CM39") could be valuable resources for turnip greens production since they are adapted to the climatic conditions of the area. In our work, "BRS550" was a very extra-late variety under the environmental conditions and sowing cycles used in each country, and it did not flower in some environments after more than six months had passed. In a previous evaluation carried out by Francisco et al. [7], authors also found that "BRS0550" was appropriate for turnip greens production, although it was very late for turnip tops production. An extra-late variety could be desirable for canning companies, as leaf production occurs for a long time. However, late varieties mean that a variety growing out of the usual dates is not able to produce edible turnip tops.

Regarding "SIN01", this variety is derived from four cycles of mass selection by turnip greens fresh production in NW Spain, and therefore, it is expected to have a good agronomic performance. On the other hand, "SIN07" was developed after three cycles of mass selection by lateness in selecting and crossing the latest plants at each cycle, but no selection for fresh yield was performed.

Flowering time is a complex trait controlled by multiple loci in *Brassica* species [30]. We found an effect of the earliness on traits related to yield (leaf number, branch number, and fresh weight of leaves and flower buds). The earliest varieties like "SIN05" or "CM24" had the lowest turnip greens production, whereas the latest varieties like "SIN07" and "BRS550" were the best in most environments. However, "SIN01" had a medium cycle. Similar results had previously been reported by various authors evaluating germplasm of *B. rapa* from Galicia, where they found a relationship between plant habit and earliness with fresh production [8].

Considering the yield contributing traits in connection with the earliness, it might be concluded that varieties "BRS0550", "SIN07", and "SIN01" are suitable for both turnip greens and turnip tops production. Therefore, they could be proposed for further breeding programs for *B. rapa* production. On the other hand, early local variety "CM39" was also suitable for turnip tops and turnip greens production in Catania but not for Atlantic areas like those in NW Spain where trials were performed. These varieties could be used for further breeding programs in each country.

Trials were performed at two geographical sites: Galicia, in NW Spain, and South Italy (Sicily). These sites represent Atlantic coast and Mediterranean conditions. On the Atlantic coast, the weather is often cloudy with frequent rainfall. On the Mediterranean coast, the weather is mild with rain in spring and autumn. These differences in temperatures and rainfall clearly affect the performance of varieties for flowering and turnip tops fresh

production. Varieties flowered earlier in Italy than in Spain, but no significant differences were found between countries for turnip greens fresh production. In conclusion, plants performed similarly in both countries for turnip greens production, but varieties had a better agronomic performance for turnip tops production in Spain.

Bioactive Compounds

Firstly, our goal was to be able to select turnip greens and turnip tops genotypes of high agronomic potential and superiority to current cultivars and, secondly, to maintain their GLS and phenolic contents and antioxidant activity in order to preserve their beneficial health properties. Therefore, identification of varieties with high levels of these compounds provides a value-added opportunity for marketing these crops.

GLS profile analyses in leaves and flower buds showed that aliphatic ones were the most abundant, being gluconapin the major of them in both plant organs. Yang and Quiros [31] studied the GLS variation in more than 80 crops of *B. rapa,* and they found that the major compound was gluconapin. The identities of the main GLS compounds in *B. rapa* reported here were different in leaves and flower buds. The GLS profile in turnip tops was the expected for this species, with GNA being the major one, followed by another aliphatic one, that is, glucobrassicanapin. However, gluconapin was the most abundant GLS in leaves, followed by the indolic hydroxyglucobrassicin, which differs with the GLS profile detected in previous studies [6,7,32]. These authors reported two aliphatic GLSs, gluconapin and glucobrassicanapin, as the most abundant both in leaves and in flower buds.

The most promising varieties for future breeding purposes would be those with the highest total GLS content and profile, with beneficial effects related to human health. In our work, we found that the new *B. rapa* varieties developed in our group, like "SIN01" and "SIN07", as well as local Galician variety "BRS550", could be used as a source of GLSs in our diet. However, "BRS550" is too late for the environmental conditions of NW Spain and S Italy. Considering the two organs analyzed, synthetic variety "SIN01" has stood out as the best variety to have *B. rapa* crops enriched in GLS.

A significant environmental influence on locations for total and individual GLS content was found, even though varieties with the highest and lowest contents were stable in most environments. This result agrees with other works where GLS content in *B. rapa* varieties was dependent upon the crops and genotypes [7,31,33,34]. In this work, GLS levels varied depending on the edible part evaluated, and they were higher in flower buds than in leaves, as it was already reported by other authors [7,35,36]. The high GLS and gluconapin levels found in turnip tops content could be related with the pungent and bitter flavor attributed to this vegetable [37,38]. Considering the beneficial effects of GLSs, turnip greens and turnip tops, which contain a relatively high content of GNA and aliphatic GLSs, are of high dietary value.

Because phenolic compounds are also important as health-protective agents in human nutrition, the development of varieties with an improved nutritional value would be useful [39]. In this sense, the *B. rapa* varieties evaluated in this work had different total phenolic content in turnip greens, hence showing that the environmental effect is important for these compounds, as well as it was for GSL content. The influence of the genotype and the environment on the total and individual phenolic contents in *B. rapa* crops was previously reported by Francisco et al. [7]. Authors concluded that both hydroxycinnamic acids and flavonoids are highly influenced by the environment and the genotype × environment interactions.

The antioxidant activity of *B. rapa* crops, including measurements of antioxidant potential on different plant organs, has not been conveniently studied yet, and these crops may be promising subjects in the field of antioxidant activity [17]. Variations in the content of phenolic compounds in *Brassica* spp. are affected by differences in genetics, environmental conditions, and plant organs [7,40,41]. In this work, the antioxidant activity was more dependent on the environmental effects rather than the plant part. Besides,

different varieties stand out over the rest depending on the location. Unlike what was observed for GLSs, the concentration of total phenolic content and the antioxidant activity did not vary for plant organs. However, other authors found that the antioxidant activity depends on the plant development stage, and it reaches its maximum when plants are young [42,43]. Varieties with the highest levels of total phenolic compounds in leaves were the two synthetic varieties, "SIN01" and "SIN07", and the two Italian varieties, "CM39" and "CM34". Thus, these varieties could be recommended for turnip greens production because of its high antioxidant activity.

5. Conclusions

In conclusion, analyses from nine *B. rapa* genotypes showed considerable variation in agronomical traits, total GLS, phenolic content and antioxidant activity among genotypes (landraces, synthetic, and commercial varieties), the different parts of the plant (leaf and flower buds), and the geographical origin, which confirmed the relationship between the content of these traits and the environmental and genetic factors. The study showed that the latest genotypes are more productive than the earlier ones and that it is possible to improve genotypes for different growing cycles. Thus, varieties selected in our work have not only good commercial perspectives for fresh production, but they also may provide considerable amounts of bioactive compounds and constitute an important natural source of dietary antioxidants.

We demonstrate that the mass selection carried out in *B. rapa* was successful. The development of synthetic varieties such as the four selections evaluated in this work offers us new *B. rapa* crops with good agronomic performance, preserving their positive health effects based on their antioxidant potential and their GLS and phenolic compounds content. As such, they will be useful material for the agro-food sector or could be used as parents in breeding programs to develop new varieties for future releases.

Author Contributions: M.E.C. conceived the study, carried out the field experiments in Spain, performed statistical analysis of the data and wrote the manuscript; F.B. and M.C.D.B. carried out the field experiments in Italy; P.V. carried out the glucosinolate analysis and discussed the results; P.S. carried out the antioxidant analysis and discussed the results; F.B. and S.T. reviewed and edited; F.B. conceived the study and discussed the results. All authors read, edited, and approved the final version of the manuscript.

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10. 9. List of publication

1. Di Bella M. C., Toscano S., Arena D., Moreno D. A., Romano D., Branca F. (2021). Effects of growing cycle and genotype on the morphometric properties and glucosinolates amount and profile of sprouts, microgreens, and baby leaves of broccoli (Brassica oleracea l. var. italica plenck) and kale (b. oleracea l. var. acephala dc.). Agronomy, vol. 11, 1685, ISSN: 2073-4395, doi: 10.3390/agronomy11091685.
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9.1 List of publication in press

1. Timpanaro G., Branca F., Cammarata M., Di Bella M.C., Foti V.T., Scuderi A. Biodiversity enhancement for sustainability organic seed production of Broccoli (*Brassica oleracea* var. *italica* Plenk) (2022 in MPDI Sustainability press)
2. Di Bella M.C.*, Melilli M.G., Treccarichi S., Argento S., Tribulato A., Arena D., Ruffino A., Branca F. - Influence of irrigation regime on productive and qualitative traits of kale (Brassica oleracea var. acephala DC) under organic farming system. (2022 in Acta Horticulturae press).
3. Detterbeck A.*, Nigro, S., Lefebvre du Prey V., Infurna G.M., Di Bella M.C., Branca F. - The effect of microorganism application on organic seed production of broccoli and cauliflower cultivars grown in Sicily. (2022 in Acta Horticulturae press).
4. Ben Ammar H.*, Sdouga D., Di Bella M.C., Treccarichi S., Calì R., Rosa E., De Castro I., Marghali S., Branca F. - Detection of glucosinolate metabolite pathway using SSR markers of Brassica oleracea complex species (n = 9) core collection (2022 in Acta Horticulturae press).
5. Branca F., Di Bella M.C., Arena D.*, Tribulato A., Kusznierewicz B., Parchem K., Bartoszek A. - Chemical characterization of wild populations of Brassica oleracea complex species (n=9) for the content of their bioactive compounds (2022 in Acta Horticulturae press).
6. Lo Scalzo R.*, Bianchi G., Picchi V., Campanelli G., Ficcadenti N., Dattoli M.A., Sestili S., Di Bella M.C., Branca F. - Agrobiodiversity in organic Brassica crops: relationship between pigment composition and antioxidant properties (2022 in Acta Horticulturae press).
7. Treccarichi S., Di Bella M.C.*, Arena D., Nicotra R., Mazzaglia A., Melilli M.G., Bartoszek A., Kusznierewicz B., Parchem K., Branca F.– Evaluation of Sicilian landraces of broccoli (Brassica oleracea var. italica Plenck) for quality traits. (2022 in Acta Horticulturae press).
8. Scandurra S., Branca F., Sollima L., Argento S., Di Bella M.C., Melilli M.G.* - First results of agronomic and chemical evaluation of yacon (Smallanthus sonchifolius) in Mediterranean environment (2022 in Acta Horticulturae press).

10. List of participations to congress

- III International Organic Fruit Symposium and I International Organic Vegetable Symposium.
  14^{th}-16^{th} December, online from Catania, Sicilia, 2021.
  (ORAL PRESENTATION AND POSTER)

- XIII Convegno Nazionale sulla Biodiversità. Dipartimento di Scienze Agrarie, Alimenti, Risorse Naturali e Ingegneria (DAFNE) dell'Università degli Studi di Foggia, in collaborazione con l'Accademia delle Scienze della Biodiversità Mediterranea (ASBM) e altre Università e Istituti di ricerca pugliesi. 7-9 settembre, online da Foggia, Puglia, 2021.
  (POSTER)

- XIII Giornate Scientifiche SOI *"I traguardi di Agenda 2030 per l'ortoflorofrutticoltura italiana" Dipartimento* di Agricoltura, Alimentazione e Ambiente.
  Università degli Studi di Catania 22-23 giugno 2021
  (POSTER)

- International Conference on breeding and Seed Sector Innovations for Organic Food Systems by Eucarpia Section Organic and Low Input Agriculture jointly with Liveseed, Bresov, Ecobreed, Flpp projects and Eco-pb. 08^{th} – 10^{th} march, online from Latvia ,2021.
  (POSTER)
  WINNER OF POSTER SECTION FOR THE INNOVATIVE RESEARCH.

- X International Symposium on Artichoke, Cardoon, and their Wild Relatives. $12t^{th}$-15^{th} march, Orihuela, Spain, 2019
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Marico'