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RESEARCH

Enhancing nutraceutical traits in *Brassica oleracea* L. for functional food production

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Abstract

Background: The *Brassica oleracea* complex species (n = 9) represent an important source of genetic diversity for developing new high nutraceutical foods, well known as superfood, as demonstrated by the establishment of the super broccoli F_1 hybrid Beneforte®, widely requested in the UK. Within the EU Horizon project COUSIN (Crop wild relatives utilization and conservation for sustainable agriculture, GA n. 101135314), we grew five crop wild relatives (CWRs), one commercial broccoli, one commercial cauliflower, two Sicilian landraces, thirteen composite cross populations (CCPs), and seven hybrids (UNICTCROSS, F_1) in order to identify elite genotypes combining high bioactive compound content with favorable agronomic behaviors. *Methods*: The trial was conducted under certified organic conditions in Sicily during the 2023–2024 growing season, adopting a completely randomized design with three replicates. Morphological traits, such as plant height, stem width, and leaf number, were assessed following IBPGR Brassica descriptors. Biochemical characterization included glucosinolates profile by ultra-high-performance liquid chromatography (UHPLC) and antioxidant capacity by ABTS and FRAP assays were detected. *Results*: Substantial morphological variation was observed, with plant height ranging from 9.80 to 65.33 cm and leaf number from 7.67 to 29.11 cm. Total glucosinolate content varied from 0.17 to 80.6 µmol/g DW, with hybrid F_1_6 exhibiting the highest value. F_1 hybrids and selected CCPs showed high glucoraphanin content (up to 54.29%), while CWRs displayed high levels of glucoiberin, glucobrassicanapin, and sinigrin. Indolic glucosinolates predominated in specific CWRs and CCPs. Antioxidant capacity ranged from 8.47 to 26.45 µmol TE/g for ABTS and exceeded 40 µmol TE/g for FRAP. Conclusions: The data acquired showed an extensive genetic diversity among the *Brassica oleracea* L., identifying promising F_1 hybrids and CCPs for their high glucosinolate content. These findings support the strategic use of underexploited germplasm for breeding programs targeting improved nutritional quality and agricultural sustainability.

Keywords: superfood, super broccoli, bioactive compounds, *Brassicaceae*, crop wild relatives, organic breeding

Introduction

The *Brassicaceae* family includes over 360 genera and 4000 species, valued for their edible parts, oilseeds, economic importance, and genetic diversity (Jabeen, 2020). Both cultivated species and their crop wild relatives (CWRs), taxa closely related to domesticated crops, are widely distributed, with the Mediterranean region representing a hotspot of genetic diversity. CWRs, adapted to a broad range of ecological conditions, are believed to harbor valuable genetic variability that is crucial for breeding climate-resilient crops (Ford-Lloyd *et al*., 2011; Essoh *et al*., 2020). The interest for the great source of variability expressed by the *Brassica oleracea* complex species (n = 9), widespread in Southern Italian regions, has raised the opportunity to establish a genetic reserve for them (Branca *et al*., 2012; Perrino and Wagensommer, 2022). Beyond their ecological relevance, *Brassicaceae* species are also valued for their phytochemical richness, particularly glucosinolates (GLSs), sulfur-containing secondary metabolites with documented health-promoting properties (Del Carmen Martínez-Ballesta *et al*., 2013; Jo *et al*., 2022). GLSs, when plant tissues are disrupted, are hydrolyzed by myrosinase into isothiocyanates (ITCs), known for their anti-inflammatory, antioxidant, antimicrobial, and chemopreventive activities (Branca *et al*., 2012). Over 200 GLSs have been identified, with Brassica crops typically containing 10–40 types (Eugui *et al*., 2025). GLSs, based on their amino acid precursors, are grouped into aliphatic, indolic, and aromatic classes (Bischoff, 2021). Numerous studies, including clinical and epidemiological evidence, associate ITCs and indoles with anticancer, cardioprotective, and neuroprotective effects (Moreno *et al*., 2006; Higdon *et al*., 2007; Giacoppo *et al*., 2015). As a result, enhancing GLS content in *Brassicaceae* crops has become a growing research focus (Baenas *et al*., 2014; Arena *et al*., 2024). Breeding has traditionally focused on yield and consumer

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preference, often reducing bioactive compounds linked to bitter or pungent flavors (Davis *et al*., 2004). However, recent studies show that these sensory traits can be balanced through targeted selection involving sugars, amino acids, and volatiles (Bell and Wagstaff, 2017). Vegetable quality, from a consumer perspective, integrates nutritional and sensory traits, such as firmness, color, sweetness, and acidity (Al Achkar *et al*., 2024; Sailo *et al*., 2024). These characteristics, along with environmental and agronomic factors such as light, temperature, water availability, and harvest timing, strongly influence the accumulation of bioactive compounds (Lo Scalzo *et al*., 2024; Garcia *et al*., 2025). Nonetheless, natural allelic variation present in wild relatives and landraces offers valuable genetic resources for improving both the content and the composition of GLSs. A key example is Beneforte® broccoli, developed by introgressing alleles from *Brassica villosa* Biv., a wild relative of *B. oleracea* adapted to Mediterranean environments. This introgression significantly enhanced glucoraphanin (GRA) content in commercial F_1 hybrids (Kliebenstein *et al*., 2001). GRA, abundant in cruciferous vegetables, is the precursor of sulforaphane (SFN), a bioactive isothiocyanate known to induce phase II detoxification enzymes, such as NAD(P)H: quinone oxidoreductase (Benzie and Strain, 1996; Cartea *et al*., 2008; Samarth *et al*., 2008; Yadava *et al*., 2022; Castillo-Lorenzo *et al*., 2024). SFN is released from GRA via myrosinase activity, either from the plant or the gut microbiota (Baek *et al*., 2016). The GRA side chain can undergo redox conversion into glucoerucin, yielding SFN analogues like erucin, which also possesses bioactivity. Extensive studies support the chemopreventive potential of SFN, reinforcing the value of breeding strategies aimed at increasing GRA content (Di Gioia *et al*., 2020; Yan *et al*., 2023).

This study aims to screen different populations belonging to the *Brassica oleracea* complex (n = 9), including crop wild relatives, landraces, commercial broccoli and cauliflower, hybrids, and composite cross populations, to identify distinct chemotypes. Morphological and biochemical variability was assessed to characterize both intra- and interspecific diversity, thereby enhancing the use of genetic resources for sustainable agriculture. Ultimately, the aim is to identify elite genotypes with enhanced nutraceutical potential and desirable agronomic performance, facilitating the development of resilient cultivars suited to future climate conditions.

Methods

PLANT MATERIAL AND EXPERIMENTAL DESIGN

The experiment was conducted in the framework of the EU Horizon COUSIN project (Crop wild relatives utilization and conservation for sustainable agriculture, G.A. n. 101135314). The study analyzed twenty-nine genetic materials, including five crop wild relatives (CWRs) populations, one commercial broccoli (*B. oleracea* L. var. *italica*, 'Cavolo Broccolo Ramoso Calabrese', BR_1), one commercial cauliflower (*B. oleracea* L. var. *botrytis,* CV_1), two Sicilian landraces (LRs), 'Broccolo nero' (*B. oleracea* L. var. *italica* Plenck, BR_2) and 'Ciuretto' (*B. oleracea* L. var. *italica* Plenck × *B. oleracea* L. var. *botrytis*, CI), thirteen composite cross populations (CCPs), and seven hybrids (UNICTCROSS, F_1).

The CCPs and the UNICTCROSS (F_1) described are the dynamic population with MAGIC populations (multiparent advanced generation inter-cross) ones, according to the EU Regulation 848/2018. The CCPs seeds were developed through a 4-year breeding program involving multiple hybrid combinations between cauliflower (*B. oleracea* L. var. *botrytis*) and broccoli (*B. oleracea* L. var. *italica* Plenck). Initial selection was based on a preliminary evaluation (Lo Scalzo *et al*., 2024), followed by a 2-year phase focused on assessing and selecting genotypes for Value for Cultivation and Use (VCU) traits within the EU H2020 BRESOV project (G.A. n. 774244). Seed production took place over two consecutive growing seasons (2021–2022 and 2022–2023) under certified organic conditions at CREA-OF (Council for Agricultural Research and Economics – Research Centre for Vegetable and Ornamental Crops) in Monsampolo del Tronto, Italy (42°53′52.44″ N; 13°47′41.28″ E). The seeds are preserved in the Di3A genbank. A total of 13 CCPs accessions resulting from this breeding scheme are included in the present study. The CCPs include F_3, F_4, and F_5 populations, corresponding to the third, fourth, and fifth generations, respectively; F_2BC_1 populations, a hybrid of second generation and one generation of backcrossing; and Sn generations, referring to selfed lines at various stages of inbreeding. Further details on parental crosses and genetic cycles are provided in Table 1.

The UNICTCROSS (F_1) were developed and preserved at the genbank of the Department of Agriculture, Food and Environment (Di3A) of the University of Catania (UNICT) (Table 1). Among these, one F_1 hybrid (F_1_4) was generated using an F_2 individual of *Brassica macrocarpa*, obtained through an intra-specific cross between two genetically distinct accessions originating from different islands. In a preliminary study (Garcia *et al*., 2025), two *B. macrocarpa* accessions from the Egadi Islands, specifically from Favignana and Marettimo (UNICT 5289 and UNICT 5309, respectively), were evaluated for their glucosinolate profiles and exhibited complementary traits. The intra-specific cross was performed to enhance the quantitative accumulation of sinigrin, a key bioactive compound of interest for breeding purposes.

The seeds were sown on July 20, 2023 in 104-cell seedling trays filled with the organic substrate Brill® Semina Bio (Agrochimica S.p.A., Bolzano, Italy). The plantlets were cultivated in the experimental cold greenhouse of Di3A (Southern Italy, Catania, 37°31′10″ N, 15°04′18″ E; 105 m above sea level). The irrigation was carried out according to standard horticultural practices. The variations in climatic conditions are presented in Fig. 1.

The plantlets with three to four true leaves were transplanted at the end of September 2023 into 10 L pots (20 cm diameter) filled with a 2:1 (v/v) mixture of the same organic substrate and volcanic coarse material. The pots were located at the Istituto Agrario Sperimentale (IAS), an organic farm in Catania, situated at the same site as the experimental greenhouse. The organic farming system is certified according to EU Regulation 2018/848 and the Italian Ministerial Decree No. 220/1995. The pots were arranged in a completely randomized design (CRD), with three replicates, each consisting of ten plants. The plants were characterized throughout the growing cycle for the main bio-morphological traits following the International Descriptors for *Brassica* and *Raphanus* (International Board for Plant Genetic Resources and Commission of the European Communities, 1990). In particular, the parameters included the plant height (PH, cm), plant diameter (PW, cm), vegetative stem length (VSL, cm), vegetative stem width (VSW, mm), and number of leaves (LN, n). The leaf samples at commercial maturity were collected in April 2024, immediately stored at −80°C, then freeze-dried and finely ground using an IKA-A10 mill (IKA-Werke GmbH & Co. KG, Staufen, Germany). The resulting powder was used for biochemical analyses.

GLUCOSINOLATES ANALYSIS (GLSs)

The GLSs were extracted as described by Kliebenstein *et al*. (2001). A total of 10 mg of lyophilized plant material was suspended in 400 µL of methanol, with 10 µL of 0.3 M lead acetate, 120 µL of ultrapure water, and 10 µL of glucotropaeolin, which was used as an internal standard. The mixtures were homogenized for 1.5 min at 25 cycles per second, then incubated at room temperature for 60 min under constant shaking (250 rpm). Following centrifugation at 2250 × *g* for 12 min at room temperature, 45 µL of diethylaminoethyl (DEAE) Sephadex A-25 resin was added to ninety-six-well filter plates.

The filter plates were equilibrated with 300 µL of ultrapure water for 2–4 h, after which the water was removed by applying a vacuum for 2–5 s. Subsequently, 150 µL of the extract supernatant was loaded onto each column, and the step was repeated to reach a

Table 1. List of *Brassica oleracea* complex species (*n* = 9) accessions tested in this work.

UNICT Code	*Species*	Accession	Working code
UNICT3406	*B. rupestris* Raf.	*B. rupestris* Raf.	CWR_1
UNICT5283	*B. rupestris* Raf.	*B. rupestris* Raf.	CWR_2
UNICT4264	*B. hilarionis* Post.	*B. hilarionis* Post.	CWR_3
UNICT5342	*B. incana* Ten.	*B. incana* Ten.	CWR_4
UNICT4803	*B. incana* Ten.	*B. incana* Ten.	CWR_5
UNICT579	*B. oleracea* L. var. *italica* × *botrytis*	*B. oleracea* L. var. *italica* × *botrytis*	CI
UNICT4852	*B. oleracea* L. var. *italica* Plenck	*B. oleracea* L. var. *italica* Plenck	BR_1
UNICT4939	*B. oleracea* L. var. *italica* Plenck	*B. oleracea* L. var. *italica* Plenck	BR_2
UNICT3152	*B. oleracea* L. var. *botrytis*	*B. oleracea* L. var. *botrytis*	CV_1
UNICT5342	*B. oleracea* L. var. *botrytis*	*Botrytis BR41 S C P.A S1 P1 Ix* (Sn)	CCP_1
UNICT5343	*B. oleracea* L. var. *botrytis*	*Botrytis CV52* × *botrytis CV19* (F_3)	CCP_2
UNICT5344	*B. oleracea* L.	*Botrytis CV159* × *botrytis CV136* × *italica BR40* × *CV165* (F_4)	CCP_3
UNICT5345	*B. oleracea* L.	*(botrytis CV159* × *botrytis CV136)* × *(italica BR40* × *botrytis CV165)* (F_4)	CCP_4
UNICT5346	*B. oleracea* L.	*Botrytis CV141* × *(botrytis CV52* × *botrytis CV19)* (F_4)	CCP_5
UNICT5347	*B. oleracea* L.	*(Botrytis CV19* × *italica BR115)* × *italica BR115* (F_2BC1)	CCP_6
UNICT5348	*B. oleracea* L. var. *botrytis*	Botrytis × botrytis (CV52 × CV19) (F_3)	CCP_7
UNICT5349	*B. oleracea* L. var. *botrytis*	*Botrytis CV159* × *botrytis CV136* (F_5)	CCP_8
UNICT5350	*B. oleracea* L.	*Botrytis CV159* × *(italica BR40* × *botrytis CV165)* (F_5)	CCP_9
UNICT5351	*B. oleracea* L.	*Botrytis CV19* × *italica BR115* (F_5)	CCP_10
UNICT5352	*B. oleracea* L.	*[Botrytis CV19* × *italica BR115]* × *italica BR115* (F_2BC1)	CCP_11
UNICT5353	*B. oleracea* L.	*Italica BR15* × [*(botrytis CV19* × *italica BR115)* × *italica BR115*] (F_2BC_1)	CCP_12
UNICT5354	*B. oleracea* L.	*Italica BR15* × [(*botrytis CV19* × *italica BR115)* × *italica BR115*] (F_2BC_1)	CCP_13
UNICTCROSS_1	*B. oleracea* L. var. *italica* × *B. macrocarpa* Guss.	Cavolo broccolo ramoso calabrese × *B. macrocarpa* Guss.	F_1_1
UNICTCROSS_2	*B. oleracea* L. var. *botritys* × *B. villosa* Biv.	*B. oleracea* L. var. *botritys* × *B. villosa* Biv.	F_1_2
UNICTCROSS_3	*B. oleracea* L. var. *italica* × *B. macrocarpa* Guss.	'Broccolo nero' × *B. macrocarpa* Guss.	F_1_3
UNICTCROSS_4	[*B. oleracea* L. var. *italica* × (*B. macrocarpa* Guss. F_2)]	['Broccolo nero' × (*B. macrocarpa* Guss. F_2)]	F_1_4
UNICTCROSS_5	*B. oleracea* L. var. *italica* × *B. macrocarpa* Guss.	*B. oleracea* L. var. *italica* × *B. macrocarpa* Guss.	F_1_5
UNICTCROSS_6	*B. oleracea* L. var. *italica* × *B. macrocarpa* Guss.	Marathon F_1 × *B. macrocarpa* Guss.	F_1_6
UNICTCROSS_7	*B. rupestris* Raf. × *B. villosa* Biv.	*B. rupestris* Raf. × *B. villosa* Biv.	F_1_7

total volume of 300 µL. Then, 10 µL of ultrapure water and 10 µL of sulfatase solution (Sigma-Aldrich, St. Louis, MO, USA) were added to each column. Following overnight incubation at room temperature, the desulfoglucosinolates were eluted twice with 100 µL of 60% methanol (v/v) and twice with 100 µL of ultrapure water. The supernatant was collected and analyzed using an ACQUITY UPLC H-Class PLUS system (Waters, Milford, MA, USA) equipped with a photodiode array (PDA) detector.

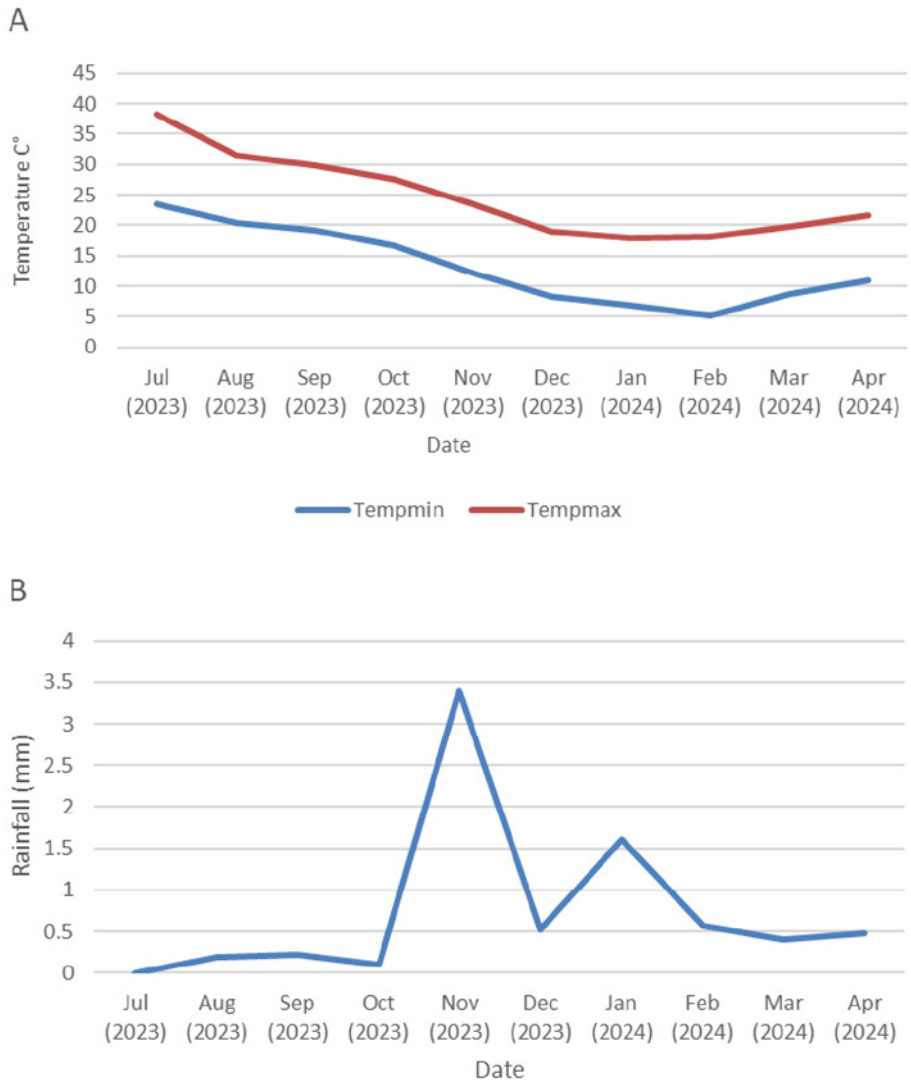

Fig. 1. Variation in climatic conditions during the experimental growing cycle. (A) Maximum and minimum temperatures (°C) recorded from July 2023 to April 2024. (B) Monthly rainfall distribution (mm) observed over the same period.

Chromatographic analyses were performed using ultra-high-performance liquid chromatograph (UHPLC, Nexera LC-30AD; Shimadzu, Kyoto, Japan). Separation was achieved on an ACQUITY UPLC HSS T3 C18 column (50 mm × 2.1 mm, 1.8 µm particle size; Waters), equipped with a corresponding C18 guard column (Waters). The column temperature was maintained at 35°C throughout the analysis.

The mobile phase consisted of solvent A (ultrapure water) and solvent B (25% acetonitrile, v/v), delivered at a constant flow rate of 0.6 mL/min. The gradient elution program was as follows: 95% A at the start, decreasing to 90% A from 0.2 to 1.2 min, 70% A from 1.2 to 2.5 min, 30% A from 2.5 to 4.0 min, and 0% A from 4.0 to 5.4 min. This was followed by an isocratic hold at 0% A for 1.6 min, before re-equilibrating back to 95% A within 1 min.

The GLSs were analyzed with a sample injection volume of 5 µL and detected at 229 nm, with results expressed as µmol/g dry weight (DW). GLS standards (ChromaDex, Santa Ana, CA, USA) included glucoiberin (GIB, RT = 0.35 min, $y = 9.94 \times 10^4$ x), glucoraphanin (GRA, RT = 0.53 min, $y = 1.38 \times 10^5$ x), sinigrin (SIN, RT = 0.64 min, $y = 4.85 \times 10^5$ x), gluconapin (GNA, RT = 1.51 min, $y = 3.53 \times 10^5$ x), glucobrassicanapin (GBN, RT = 2.18 min, $y = 3.58 \times 10^5$ x), glucobrassicin (GBS, RT= 2.49 min, $y = 8.69 \times 10^5$ x), glucotropaeolin (GTP, RT = 2.18 min, $y = 3.55 \times 10^5$ x), and neoglucobrassicin (NeoGBS, RT = 3.03 min, $y = 1.29 \times 10^6$ x). 4-Hydroxyglucobrassicin (OHGBS), and 4-methoxyglucobrassicin (MeOHGBS) were quantified using the calibration curve of NeoGBS.

ANTIOXIDANT CAPACITY

The antioxidant capacity was evaluated using two complementary assays: the Ferric Reducing Antioxidant Power (FRAP) and the ABTS [2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid)] radical scavenging activity tests. For the extraction, 10 mg of lyophilized material was combined with 1 mL of 80% methanol and mixed for 2 h.

For the FRAP assay, in particular, the FRAP reagent was freshly prepared by mixing 10 parts of 300 mM acetate buffer (pH 3.6), 1 part of 10 mM TPTZ (Sigma-Aldrich, St. Louis, MO, USA; Cat. No. 93285-5G) dissolved in 40 mM HCl, and 1 part of 20 mM ferric chloride (Benzie and Strain, 1996). The mixture was incubated at 37°C for 5 min before use. A volume of 50 µL of methanolic extract was added to 250 µL of the freshly prepared FRAP reagent and thoroughly mixed. The mixture was kept at room temperature for 30 min, and the absorbance was determined at 593 nm using a

spectrophotometer (Spectra MR; Dynex Technologies, Chantilly, VA, USA). Quantification was performed using a Trolox standard curve (y = 42.342x + 0.1337; serial dilutions: 2.5, 5, 7.5, 10, 12.5 µM; R^2 = 0.9984). The results were expressed as micromoles of Trolox equivalents per gram of dry weight (µmol TE/g DW).

ABTS radical scavenging activity was assessed according to the method of Samarth *et al*. (2008). The ABTS+ was prepared by oxidizing 7 mM ABTS (Sigma-Aldrich, St. Louis, MO, USA; Cat. No. A1888-2G) with 2.45 mM potassium persulfate in water at room temperature for 16 h. The ABTS+ solution was freshly diluted with water to achieve an initial absorbance between 0.8 and 1 at 734 nm. Fifty microliters (µL) of the methanolic extract was mixed with 250 µL of the ABTS+ solution. The absorbance was measured at 734 nm after 30 min of incubation in the dark at room temperature by a spectrophotometer (Spectra MR; Dynex Technologies, Chantilly, VA, USA). The ABTS values were determined based on a Trolox+ calibration curve (y = −24.498x + 0.9117; serial dilutions: 2.5, 5, 7.5, 10, 12.5 µM; R^2 = 0.9967). The results were expressed as micromoles of Trolox equivalents per gram of dry weight (µmol TE/g DW).

DATA ANALYSIS

A one-way analysis of variance (ANOVA), followed by Tukey's HSD test, was performed using CoStat version 6.311 to evaluate significant differences among genotypes. Data processing and statistical analyses were performed using R software (version 4.4.1; R Core Team, 2023). A barplot was created using the ggplot2 package to illustrate the distribution of selected biochemical parameters. Principal component analysis (PCA) was carried out using the FactoMiner and Factoextra packages to identify patterns of trait variation and accession clustering.

Results and discussion

MORPHOMETRIC CHARACTERIZATION

This study explored the morphological, phytochemical, and antioxidant diversity of 29 accessions of the *Brassica oleracea* complex species, encompassing crop wild relatives (CWRs), cultivated genotypes, F_1 hybrids, and composite crop populations (CCPs). In this context, it is important to note that since the onset of intensive agriculture practices, many crop breeding programs have prioritized yield over nutritional quality, sometimes resulting in a decline in health-promoting compounds in modern cultivars (Davis *et al*., 2004). The results revealed substantial variation across all traits, highlighting the rich diversity present within this panel and its potential for breeding and pre-breeding programs.

The morphometric data are reported in Table S1. PH varied significantly among accessions, ranging from 9.80 cm in F_1_4 to 65.33 cm in CWR_3, with BR_1 also showing a high value of 50.99 cm. Most genotypes had PH values between 20.63 and 65.33 cm. Five genotypes (BR_2, CWR_1, F_1_6, CWR_5, and CCP_4) showed heights below 25 cm, while F_1_2, F_1_3, CV_1, CCP_11, and CCP_6 exceeded 40 cm. The remaining accessions generally displayed heights ranging from 25 to 46 cm. PW also showed considerable variation, ranging from 8.36 cm in CV_1 to 36.46 cm in CWR_5. The average width was approximately 14.6 cm, with most accessions between 13 and 18 cm. The results showed different plant widths, indicating substantial morphological divergence. VSL varied from 8.55 cm in F_1_4 to 44.95 cm in CCP_6, showing significant differences among accessions. VSW also displayed variability, with the lowest values, below 9 mm, recorded in CV_1, CCP_5, and CCP_4. In contrast, F_1_5 had the greatest VSW, reaching 27.04 mm. LN ranged from 7.67 in CCP_10 to 29.11 in F_1_3. The average LN was about 19.8, with most accessions showing values between 16 and 24 leaves. Notably, three accessions (F_1_5, CCP_5, and CI) had more than 25 leaves, while CCP_10 and CCP_6 had fewer than 10. Fig. 2 provides a visual overview of phenotypic variability across accessions. The high variability among CWRs confirms their strategic role in widening the genetic base of crops (Yadava *et al*., 2022; Castillo-Lorenzo *et al*., 2024). Morphometric traits, including plant height (PH), number of leaves (LN), and plant width (PW), exhibited substantial phenotypic variability among the accessions, in line with previous observations (Garcia *et al*., 2025) and further reinforcing the well-documented phenotypic plasticity of the *Brassicaceae* family. These results indicate that incorporating diverse germplasm, including CWRs and CCPs, can support the development of resilient, nutrient-rich varieties.

GLUCOSINOLATES (GLSs) PROFILE AND CONTENT

The total glucosinolate content (TGLSs) varied widely among the 29 accessions analyzed, ranging from a minimum of 0.17 µmol/g DW in CCP_2 to a maximum of 80.6 µmol/g DW in F_1_6 (Fig. 3). The hybrid F_1 exhibited consistently high TGLSs levels. The most prominent values were recorded in F_1_6 (80.6) and F_1_1 (29.3), while accessions such as F_1_2 (7.1) and F_1_3 (3.4) displayed intermediate concentrations. Only F_1_7 showed a notably low content (0.48), indicating possible segregation or genetic variability in glucosinolate accumulation within this group. The hybrid F_1_6 and F_1_1 were developed at the University of Catania through interspecific crosses between cultivated broccoli (*B. oleracea* L. var. *italica*) and *B. macrocarpa* Guss., a crop wild relative endemic to the Mediterranean region. *B. macrocarpa*, native to the rocky coastal areas of Marettimo and Favignana (Egadi Islands, Sicily), is adapted to drought and harsh environments. In addition to their elevated GLS levels and stress tolerance, this wild species is a valuable resource for breeding programs targeting both nutraceutical quality and resilience to abiotic stressors (Perrino and Wagensommer, 2021). This observation aligns with previous findings (Privitera *et al*., 2024), which highlighted the drought tolerance potential of *B. macrocarpa* through comparative transcriptomic analyses under water stress conditions. Of particular interest is the variation in GLSs content observed for F_1_3 and F_1_4, both hybrids of *B. oleracea* L. var. *italica* ('Broccolo nero') crossed with *B. macrocarpa* Guss. While sharing the same cultivated parent, these hybrids displayed markedly different GLS profiles. This divergence may be attributed to the genetic background of the *B. macrocarpa* parental lines: in F_1_4, the wild parent was an F_2 individual derived from an intra-specific cross between two *B. macrocarpa* accessions originating from Marettimo and Favignana. Such genetic recombination may have interfered with or modulated the metabolic pathways involved in glucosinolate biosynthesis, potentially through epistatic or regulatory interactions (Qin *et al*., 2023). Similar phenomena have been reported in previous studies (Velasco *et al*., 1999; Lee *et al*., 2014; Li *et al*., 2021), which described the impact of intra-specific variability and genotype-by-genotype interactions on secondary metabolite profiles in *Brassicaceae.*

About the CCPs, considerable intra-group variation was observed. While CCP_2 (0.17) and CCP_12 (0.25) recorded the lowest TGLSs levels in the entire dataset, others, like CCP_7 (14.7), CCP_8 (13.0) and CCP_4 (12.4), achieved the highest concentrations within the group. These results support previous concerns that choosing plants for yield or taste might unintentionally lower the levels of beneficial compounds.

Among the CWRs, CWR_1 stood out with a relatively high value (17.2 µmol/g DW), while CWR_3 (9.4) and CWR_5 (9.6) also showed intermediate to elevated levels, supporting their potential role in breeding programs aimed at nutritional quality improvement (Kell *et al*., 2007). In contrast, CWR_4 exhibited one of the lowest values overall (0.18), underscoring the natural variability even within wild genetic resources. As for the other varieties, CV_1 displayed the highest TGLSs content in this group (11.9), followed by BR_1 (4.3) and BR_2 (1.2).

A comparative analysis across groups clearly shows that F_1 hybrids achieved the highest average TGLSs content, followed by selected CCPs and CWRs. In contrast, cultivated varieties consistently exhibited the lowest values, reinforcing the relevance

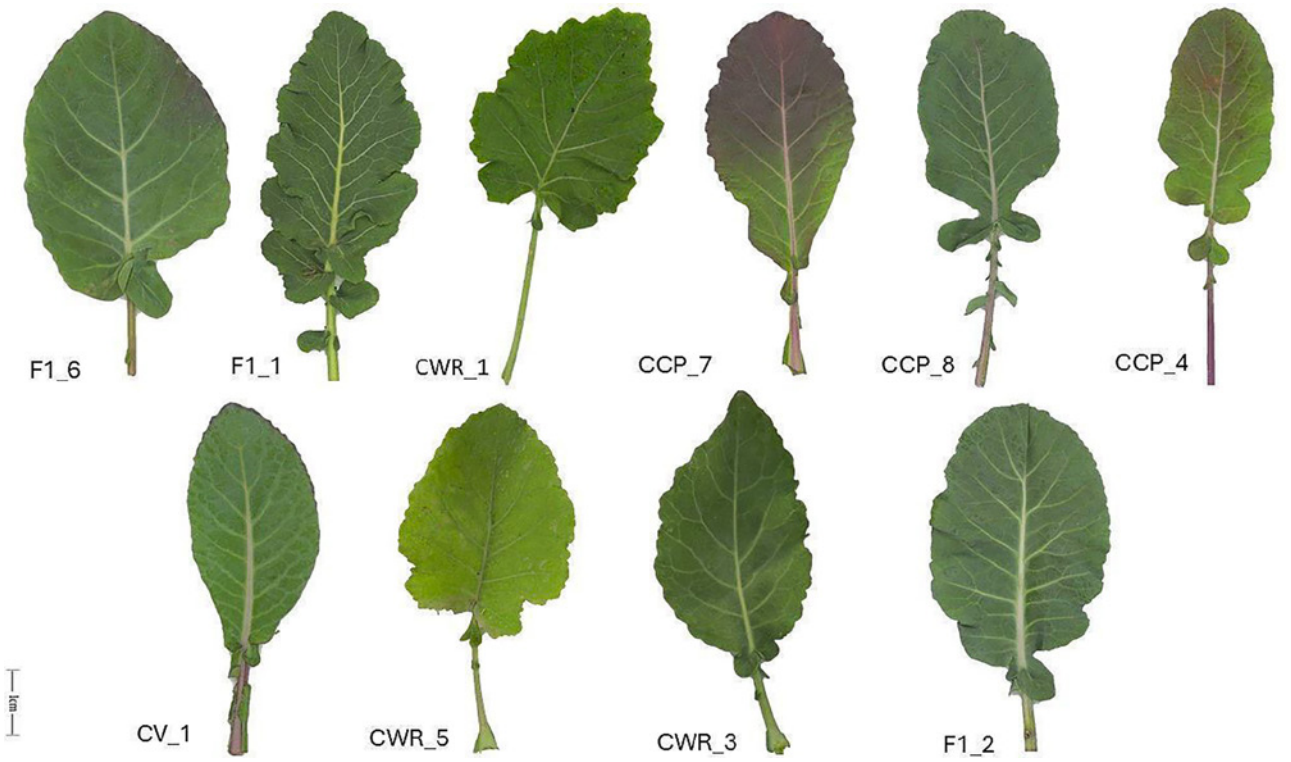

Fig. 2. Representative leaf morphology of ten *Brassica* genotypes listed by total glucosinolate content, from highest to lowest (F_1_6, F_1_1, F_1_2, CWR_1, CWR_3, CWR_5, CCP_4, CCP_7, CCP_8, and CV_1). The leaves display evident variability in shape, margin type, size, and colour among genotypes. Scale bar = 1 cm.

of underutilized germplasm such as hybrids, wild relatives, and synthetic populations as promising sources for enhancing bioactive compound levels in modern breeding programs. The observed high variability in glucosinolate content and composition highlights the need to consider both quality and quantity in breeding. While some studies have proposed selecting genotypes based on total GLS content, this strategy can be overly simplistic (Cartea *et al*., 2008; Baek *et al*., 2016). Not all GLSs yield bioactive hydrolysis products with proven health benefits or acceptable sensory attributes. In fact, certain GLSs may even exert negative effects when consumed in excess (Di Gioia *et al*., 2020). Moreover, developing breeding populations in non-model species remains challenging due to limited genomic resources and long generation times, which hinder the detailed characterization of biosynthetic pathways and allelic variation (Yadava *et al*., 2022).

The heatmap (Fig. 4) illustrates the percentage composition of the GLS profile among the accessions, showing significant variation in both total GLSs content and the relative proportion of individual compounds. The corresponding mean values ± standard errors are reported in Table S2. Aliphatic glucosinolates (GIB, SIN, GRA, GNA, GBN) were predominant in several F_1 hybrids and CCPs lines, with particularly high GRA levels in CCP_11 and CCP_13. These findings are consistent with prior reports (Mocniak *et al*., 2023; Yan *et al*., 2023), indicating that certain composite populations can preserve or even enhance health-promoting compounds through recombination and selection. Notable variation among aliphatic glucosinolates was observed across accessions. Specifically, F_1_2 was dominated by GIB (47.83%), whereas F_1_6 exhibited a profile including 16.03% GIB, 14.15% SIN, and 54.29% GRA. High levels of GRA were observed for CCP_11 and CCP_13, reaching 71.71 and 69.34%, respectively, with CCP_5, CCP_6, and CCP_9 also showing GRA content above 40%, suggesting a conserved chemotype within the CCP group. GNA was not detected in relevant amounts in any accession, while GBN reached 68.99% in CWR_3. Among the indolic glucosinolates (GBS, OHGBS, MeOHGBS, and NeoGBS), GBS showed the highest concentrations in CCP_3, CCP_4, and CCP_12, with values of 46.53, 61.62, and 47.25%, respectively, and NeoGBS was particularly abundant in CWR_2, accounting for 61.28%. Neoglucobrassicin (NeoGBS) and 4-methoxyglucobrassicin (MeOHGBS) accumulated predominantly in CWR_2 and CV_1, respectively, suggesting specific metabolic specialization.

ANTIOXIDANT CAPACITY

The ABTS assay showed high variability among accessions (Fig. 5A). The lowest values were observed for F_1_3 and CCP_9, with 8.47 and 8.89 µmol TE/g DW, respectively. In contrast, several genotypes exhibited high ABTS values exceeding 20 µmol TE/g DW, including CCP_12, CCP_2, CCP_10, and BR_2, with the highest value recorded in CCP_6 (26.45 µmol TE/g DW). All accessions showed high antioxidant capacity for the FRAP assay, with values exceeding 40 µmol TE/g DW (Fig. 5B). However, the ranking of the highest values differed from the ABTS results, with BR_2 (85.60 µmol TE/g DW), CCP_2 (88.29 µmol TE/g DW), and CCP_6 (92.61 µmol TE/g DW) showing the greatest reducing power. Antioxidant activity, assessed via ABTS and FRAP assays, showed marked differences among accessions. Notably, CCP_6 ranked among the highest-performing genotypes in both assays, highlighting the potential of genetically diverse breeding populations to combine agronomic performance with enhanced bioactive properties.

PRINCIPAL COMPONENT ANALYSIS

Principal component analysis (PCA) was employed to evaluate the distribution of genotypes along the main axes of variation. The first two principal components accounted for 56.20% of the

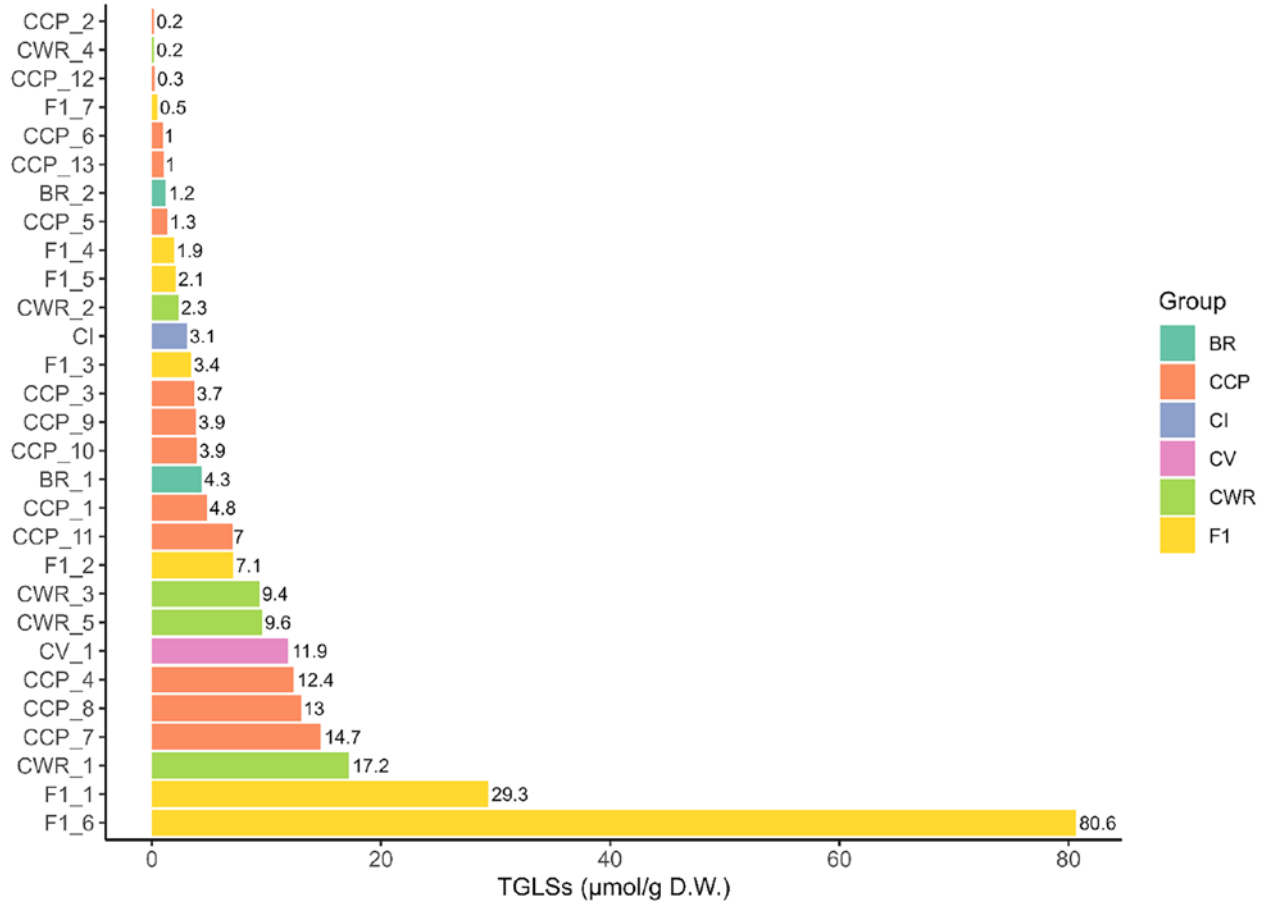

Fig. 3. Total glucosinolate content (TGLSs, µmol/g DW) among different varietal groups, with each group represented by a different color: Crop wild relatives (CWRs), the Sicilian landrace 'Ciurietto' (CI), broccoli (BR), cauliflower (CV), cross composite populations (CCPs), and UNICTCROSS hybrids (F_1). F_1 hybrids exhibited the highest levels, particularly F_1_6 (80.6 µmol/g DW) and F_1_1 (29.3 µmol/g DW). Some crop wild relatives, such as CWR_1 and CWR_5, also showed relatively high values.

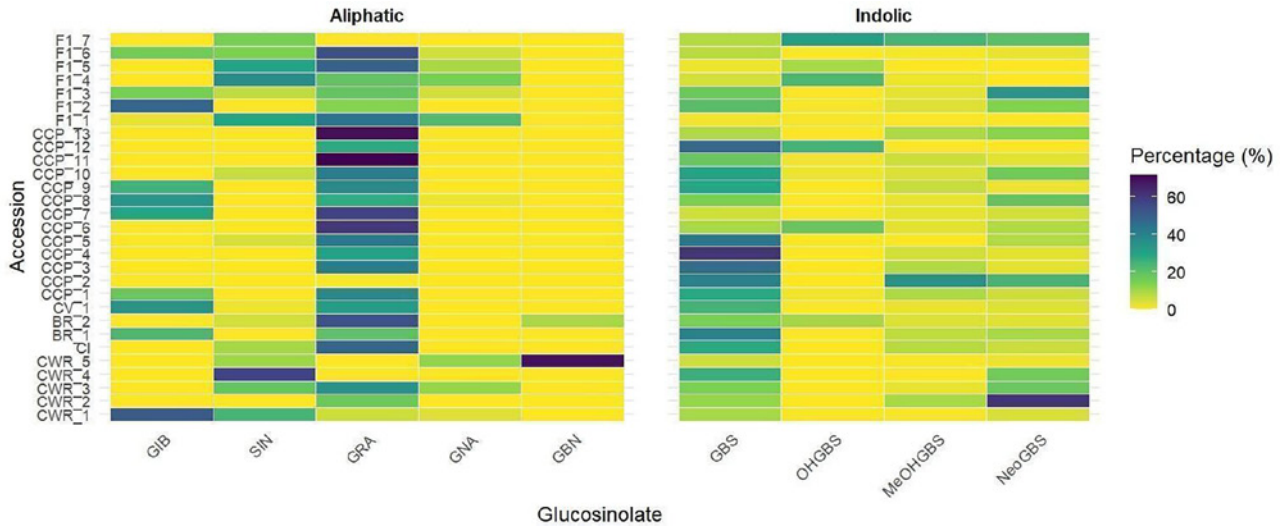

Fig. 4. Heatmap showing the relative composition (%) of individual glucosinolates (GLSs) observed among the tested varietal groups: Crop wild relatives (CWRs), Sicilian landrace 'Ciurietto' (CI), broccoli (BR), cauliflower (CV), cross composite populations (CCPs), and UNICTCROSS hybrids (F_1). Glucosinolates are grouped into two main classes: Aliphatic, which include GIB (glucoiberin), SIN (sinigrin), GRA (glucoraphanin), GNA (gluconapin), GBN (glucobrassicanapin); and indolic, which comprise GBS (glucobrassicin), OHGBS (4-hydroxyglucobrassicin), MeOHGBS (4-methoxyglucobrassicin), NeoGBS (neoglucobrassicin). Each cell represents the percentage of a given GLSs relative to the total glucosinolate content (TGLSs) within each accession. Color intensity reflects relative abundance, from low (yellow) to high (purple).

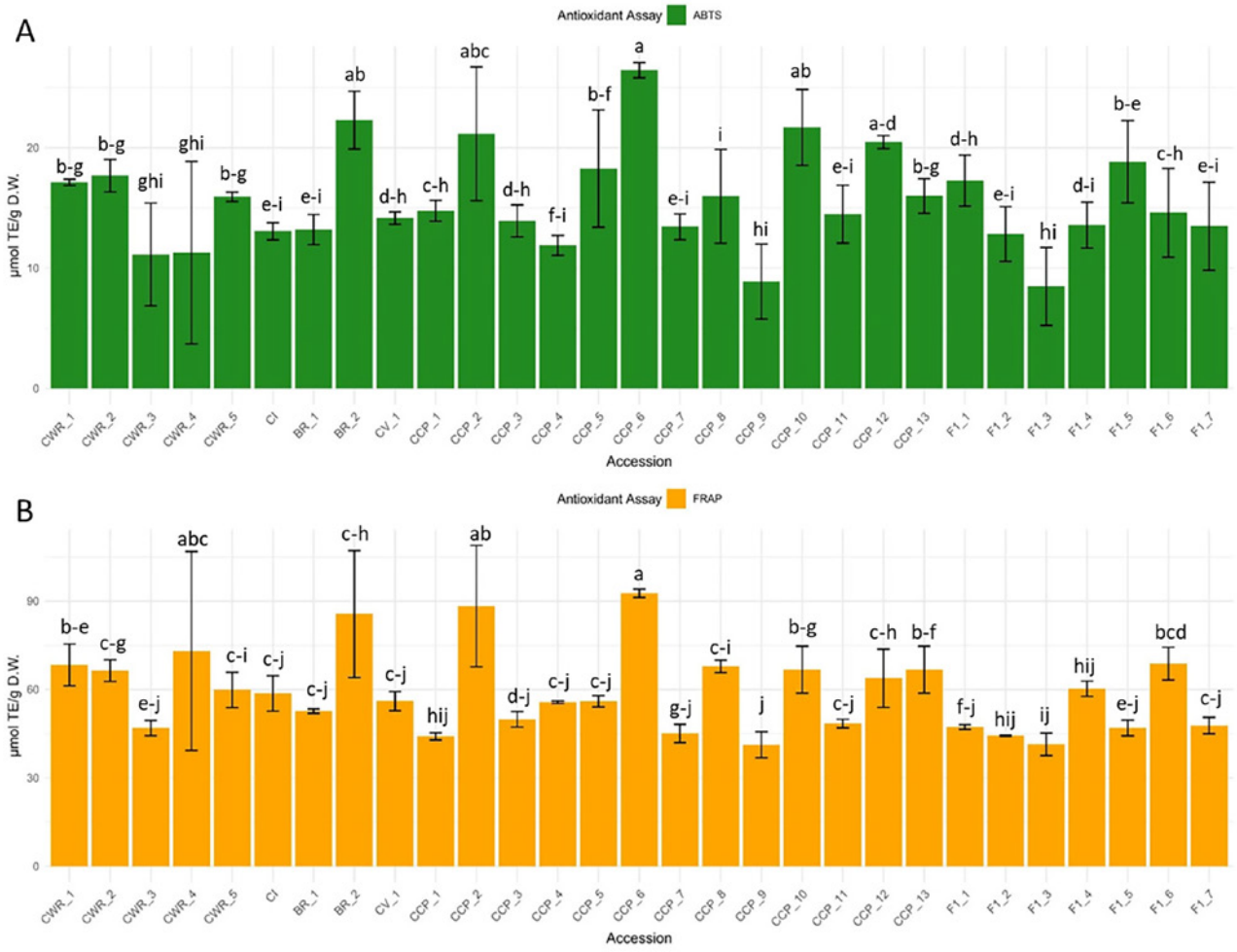

Fig. 5. Antioxidant capacity measured by ABTS (A) and FRAP (B) (µmol TE/g DW) assays across the evaluated accessions: Crop wild relatives (CWRs), Sicilian landrace 'Ciurietto' (CI), broccoli (BR), cauliflower (CV), cross composite populations (CCPs), and UNICTCROSS hybrids (F_1). Data are reported as the mean ± S.E. (n = 3). Different letters indicate statistically significant differences as determined by Tukey's HSD test ($p < 0.05$). Substantial variability was observed among accessions, with several CWRs and CCPs genotypes displaying the highest antioxidant activities, particularly in the FRAP assay.

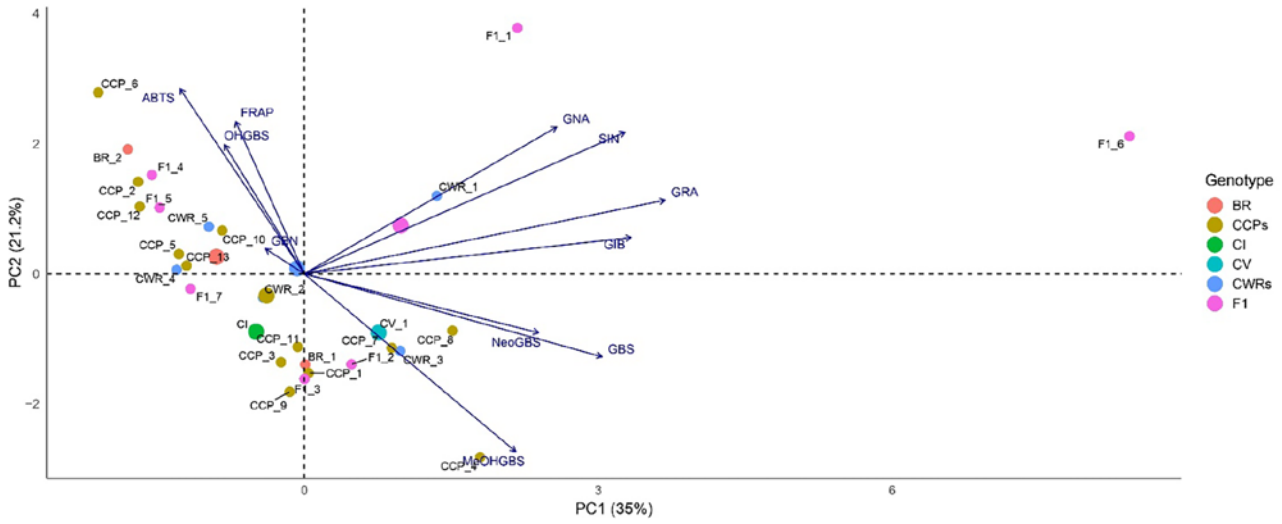

Fig. 6. Principal Component Analysis (PCA) was performed on 29 accessions grouped by genetic background: Crop wild relatives (CWRs), Sicilian landrace 'Ciurietto' (CI), broccoli (BR), cauliflower (CV), cross composite populations (CCPs), and UNICTCROSS hybrids (F_1). The PCA including the individual glucosinolates and antioxidant capacity (ABTS and FRAP), across all genotypes. The first two principal components (PC1 and PC2) explain 56.2% of the total variance (PC1: 35.0%, PC2: 21.2%). For clarity, only the most representative glucosinolates and antioxidant traits were selected and displayed as vectors in the biplot, with their direction and length indicating the strength and orientation of their influence on the distribution of genotypes in the multivariate space.

total variance (Fig. 6). The PCA provided insight into the extent of differentiation among genetic groups and highlighted associations between specific metabolite signatures and potential agronomic or nutritional traits of interest. Specifically, F_1_6 derived from *B. oleracea* L. var. *italica* (Marathon F_1) × *B. macrocarpa* Guss., was clearly separated along PC1, indicating a pronounced enrichment in aliphatic glucosinolates. This metabolic shift may reflect enhanced activity or differential regulation of biosynthetic genes such as AOP2, MAM1, or GSL-ALK inherited from the wild parent (Mitreiter and Gigolashvili, 2021). In addition, F_1_1 derived from *B. oleracea* L. var. *italica* (Cavolo broccolo ramoso calabrese) × *B. macrocarpa* Guss., also appeared distinct, but to a smaller extent. Both hybrids, F_1_6 and F_1_1, were associated with high levels of aliphatic glucosinolates, such as GNA, GRA, and SIN.

The CCPs were more widely distributed, indicating their genetic variability. Notably, CCP_6, derived from a backcross of the F_1 hybrid (*botrytis* CV19 × *B. oleracea* var. *italica* BR115), was located close to the ABTS and FRAP vectors, indicating elevated antioxidant capacity. This positioning suggests enrichment in phenolic and redox-active compounds. The observed biochemical variability likely stems from allelic segregation, residual heterosis, and genetic recombination affecting glucosinolate and antioxidant-related pathways (Bellostas *et al*., 2007).

Among the CWRs, CWR_1 was distinguished by high levels of GNA and SIN. Regarding the other groups, the landrace BR_2 clustered in the upper left quadrant, suggesting an enriched phytochemical composition with potential nutritional or pharmacological value (Syed *et al*., 2023).

In contrast, the CI was located in the lower left quadrant, indicating shared morpho-biochemical features. In contrast, the CV was mainly distributed along the positive side of PC1. In particular, CV_1 aligned closely with the NeoGBS and MeOHGBS, suggesting a higher content of these indolic glucosinolates.

These results highlight the genetic complexity of biochemical traits and the value of diverse populations for uncovering metabolite–genotype associations relevant to both agronomic and nutritional traits. The observed metabolic heterogeneity also reflects the adaptive potential and allele reshuffling typical of composite cross populations, especially in early backcross generations under selection pressure (Scossa *et al*., 2016; Fujimoto *et al*., 2018; Swarup *et al*., 2021).

While this study provides valuable insights into the morpho-phytochemical diversity across a wide panel of Brassica accessions, certain limitations should be acknowledged. First, the analysis was conducted under controlled environmental conditions, which may not fully capture the interactions of genotype × environment that influence trait expression under field conditions. Future studies will be conducted across multiple environments to assess the stability and environmental responsiveness of these traits. Secondly, the scope of phytochemical profiling focused primarily on glucosinolates and antioxidant capacity, thereby overlooking other relevant classes of bioactive compounds such as polyphenols, carotenoids, and vitamins, which may also contribute to the nutritional and functional quality of the genotypes. Moreover, the absence of genomic data limits the resolution of the genetic architecture underlying the observed traits, particularly with regard to glucosinolate biosynthesis and regulation. Despite these limitations, the study offers valuable applicability for pre-breeding and crop improvement programs. These results provide a basis for using metabolically rich genotypes in breeding programs and could guide the development of *Brassicaceae* cultivars with enhanced nutritional quality and stress resilience, supporting the production of functional foods. The datas*et al*so serves as a reference for future multi-omics studies aimed at dissecting the genetic and metabolic networks underlying health-promoting traits in Brassica crops.

Conclusion

The results highlight the strategic value of crop wild relatives, landraces, F_1 hybrids, and composite crop populations as reservoirs of metabolic diversity and as promising resources for biofortification and climate-resilient breeding. The identification of elite genotypes such as F_1_6, F_1_1, and CCP_6, which exhibit the highest levels of glucosinolates compounds and antioxidant capacity, confirms the feasibility of integrating nutritional traits into pre-breeding pipelines without compromising agronomic performance. Moreover, the divergent glucosinolate profiles observed among genetically related F_1 hybrids highlight the complexity of metabolic regulation in cole crops and related CWRs and point to the importance of the parental origin effects by recombination. The incorporation of CWRs traits such as for *B. macrocarpa*, known for its glucosinolate richness and drought stress tolerance, offers a dual advantage for the development of functional foods and resilient crops, especially under the climatic change in acts. The underutilized genetic and phytochemical diversity of the *B. oleracea* complex species, which is widespread across Mediterranean countries, together with the derived dynamic populations, represent a promising resource for breeding next-generation *B. oleracea* cultivars with improved agronomic performance and enhanced nutraceutical properties, potentially suitable as superfoods.

CONFLICT OF INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this article.

ETHICS STATEMENT

The authors confirm that the research meets any required ethical guidelines, including adherence to the legal requirements of the study country.

AUTHOR CONTRIBUTIONS

All authors contributed equally to the development of this article.

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DATA AVAILABILITY

N/A.

SUPPLEMENTARY MATERIAL

The supplementary material is available in the online version of this article.

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