ECPGR Activity Grant Scheme – First Call, 2014

Activity Report

COllection**, CH**aracterization and **EVA**luation of wild and cultivated **BRASS**icas

(**COCHEVA BRAS)**

1 January 2015 – 31 May 2016

F. Branca, P.S. Coelho, A. De Haro, E. Rosa, V.R. Lopes and R. Lo Scalzo

October 2016

CONTENTS

INTRODUCTION

The activities carried out by the *Brassica* Working Group (BWG) in recent years achieved results of interest about the diversity expressed by a set of *Brassica oleracea* complex species (n=9), including several *B. oleracea* crops and landraces and *Brassica* wild relatives (n=9).

During the AEGIS project carried out in Phase VIII of ECPGR, important results were achieved about the characterization of the diversity expressed by *Brassica oleracea* landraces from the Iberian Peninsula and conserved at the Biological Mission of Galicia (*Misión Biológica de Galicia*, MBG, Pontevedra, Spain) and at the Portuguese Gene Bank (*Banco Português de Germoplasma Vegetal,* BPGV, Braga, Portugal), which represent the largest collection of *B. oleracea* landraces of the varieties *acephala, costata* and *capitata* from the Iberian peninsula[1].

At the same time, during Phase VIII, a large set of *Brassica* wild species and *B. rapa* accessions, collected in Europe and provided by several European genebanks, were characterized for their biomorphological and genetic traits by simple sequence repeats (SSRs), showing a large diversity and a great interest to include them as most appropriate accessions (MAAs) in AEGIS (Branca et al. 2013).

In 2014, the BWG successfully submitted an Activity proposal for funding under the ECPGR Activity Grant Scheme, titled "COllection, CHaracterization and EVAluation of wild and cultivated BRASsicas (COCHEVA BRAS)". The main goal of COCHEVA BRAS was to continue the characterization work carried out so far for *Brassica* wild relatives, to continue the characterization of landraces and commercial cultivars of the Iberian and Italian peninsulas, to fill some gaps in the *Brassica* collection for some taxa growing in the Western Mediterranean basin, and to identify sources of resistance for downy mildew (*Hyaloperonospora brassicae*). The accessions were selected among the *Brassica* collections of the BPGV and of the Department of Agriculture, Food and Environment of the University of Catania, Italy (*Dipartimento di Agricoltura Alimentazione e Ambiente, Università degli studi di Catania* [Di3A-UNICT]), on the basis of the previous results obtained and of the AEGIS priority actions agreed upon at the last BWG meeting of Linguaglossa (2010), and on geographic origin and the expression of specific biomorphological, biochemical and genetic traits.

The objectives of COCHEVA BRAS were:

- Identification of eligible accessions to be proposed as MAAs for registration as AEGIS accessions;
- Services for characterization, evaluation and/or phenotyping of AEGIS accessions;
- Insertion of all analysed accessions into the list of AEGIS accessions provided by Associate Members (AMs);
- Safety duplication facilities for AEGIS accessions offered to AMs;
- Collaboration between National Focal Points (NFPs) and collection-holding institutes strengthened;
- Survey of user needs performed and results analysed;
- Effective services to users established;
- Closer link with the conservationists and breeders realized;
- Research partnerships established between genebanks and researchers, including through EU projects.

The Activity proposal including list of partners is available from the COCHEVA BRAS webpage.

MATERIALS AND METHODS

The project started in spring 2015 and involved 41 accessions from the *Brassica* collections of BPGV-INIAV (13 accessions) and Di3A-UNICT (28 accessions).

[1] *Brassica* selection criteria for the identification of the MAAs: relate to the *Brassica oleracea* of Iberian collection (Final report available here).

Di3A-UNICT and BPVG-INIAV started to sow the 25 landraces and the 6 commercial cultivars of *B. oleracea* vegetable crops and 10 wild *Brassica* (n=9) accessions during the second part of April 2015. The accessions were sown in cellular trays and grown till they reached the 4-5 leaves stage. Unfortunately, 10 of the 41 accessions sown (5 wild *Brassica* and 5 landraces) did not germinate so new accessions were selected to replace them: 2 wild *Brassica* and 6 landraces of *B. oleracea*. These new accessions were sown in June at Catania and in August at Braga. The plants were then transplanted in the peri-urban field in June for all accessions, except for the new accessions chosen to replace those that had not germinated, which were transplanted at the end of September. The plantlets were transplanted in rows with a 100 x 30 cm spacing (row x plant distance), resulting in a plant density of 3.3 plants/m^2 at Catania, and with a 60 x 60 cm spacing (row x plant distance), resulting in a plant density of 2.8 plants/m^2 at Braga. After transplanting, by 30 September 2015 37 and 39 accessions were growing in both fields in Braga and Catania respectively (Table 1)[2]. However, by the first week of November only 34 accessions were still growing in the field in Braga, because after transplantation the weather was quite rainy and the plantlets of some accessions died in the field.

Accessions which reached the full blooming stage in at least half of the plants in each row in the field were characterized utilizing the same descriptors for morphological characterization previously used by the BWG (Table 2).

Genetic analysis

Two months after transplantation in the characterization field of UNICT, young leaf samples for DNA extraction from ten single plants per accession were collected. DNA extraction was performed by CTAB extraction protocol: from each plant 100 mg of fresh young leaves sample were finely smashed in a tube after addition of 600 µL of 2% CTAB. Tubes were then incubated at 55°C for 15 min, and then centrifuged at 13000 rpm for 10 min. Supernatant extracted by 600 µL of chloroform was transferred in a fresh tube. Then to the supernatant was added 400 µL of isopropanol after washing in 70% of ethanol. DNA concentration and purity of the samples were measured with Nanodrop spectrophotometer at a wavelength of 260 and 280 nm. The primers set utilized (Table 3) was suggested by Carlos Quiros who has worked since several decades to individuate genes involved in glucosinolate (GLS) biosynthesis pathways (Li and Quiros 2001, 2002). The PCR reaction mixture contained 2.5 µL of 10X buffer, 2 µL of dNTPs, 0.75 µL of Forward primer, 0.75 µL of Reverse primer, 0.75 µL of MgCl2, 0.25 µL of bioTaq polymerase and 1 µL of 100 ng template DNA. Amplification was done using a thermocycler INHECO with the following profile: initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 94°C for 1 min, primer annealing at 56°C for 1 min and a phase of elongation at 72°C for 1 min, with a final extension at 72°C for 10 min. For P16 primer we used 58°C as annealing temperature. PCR products were loaded onto 2% agarose gel and run at 100V for 1 h in 0.5 X TBE buffer. For P13 and P14 primers we sequenced their products in order to detect sequence variations.

At BPVG-INIAV of Braga the DNA extraction was performed for 33 accessions (10 plants per accession x 33 accessions collected on 21 July 2015) by DNA extraction E.Z.N.A. SP Plant DNA Kit (OMEGA). The quantification of the DNA was achieved by running the DNA samples on 0.8% agarose gel stained with ethidium bromide by comparing the fluorescent yield of the samples with standards, Lambda HindIII DNA Ladder. The DNA analysis with SSRs was initiated in November 2015 with DNA bulk extraction; the DNA extraction of 10 plants per accession was initiated 1 December 2015 and the SSR PCR analyses were started on 26 January 2016.

- After bibliography research and tests the primers of the 30 SSRs related to chromosomes 5 and 9 on DNA bulks, a group of SSRs was decided. The SSR PCR analysis was done for 10 SSRs related to GLS. PCR protocols were performed at the 20 µL scale in 0.2 mL microcentrifuge tubes and the thermocycler T3 da Biometra.
- The PCR program was: 95°C – 2 min; 95°C – 45 sec, 60ºC – 90 s, 72°C – 90 s – 30 cycles; final extension – 72°C – 5 min, 4ºC – pause. Master Mix: final concentration – 0.2 mM dNTP's, 1.5 mM MgCl2, 0.4 µM per primer (F and R), GoTaq® G2 Flexi DNAPolymerase (1u/ µl) and 20 µl final volume.

[2] All tables and figures are grouped as annexes at the end of the report.

- Amplification products of 10 loci SSRs (Table 4) were resolved on 3% agarose gels (CleverGel, Low EEO), 5 volt/cm, 3 h, buffer Tris/Borate/EDTA (TBE), stained with ethydium bromide and bands visualisation were performed using the Doc-It system of image analysis software of Ultra-Violet Products Ltd, UK, with standards, 100 bp and 50 bp Ladders (100bp DNA BioLabs, GeneRuler 50 bp DNA Fermentas, respectively).
- Data analysis: the 10 SSR loci were scored as dominant; profiles were scored according to the presence (1) or absence (0) of a particular band generating a binary matrix. The Jaccard genetic similarities between pairs of accessions were used to generate a dendrogram based on UPGMA analysis, and assess pair-wise similarities between the accessions. Cluster analysis was performed using NTSYS-pc.

Biochemical analysis

Regarding the biochemical analysis, samples from accessions in Catania were collected and they were sent to the CSIC (Cordoba, Spain), UTAD (Vila Real, Portugal) and CREA-IAA (Milano, Italy) for biochemical analysis (total polyphenols and polyphenol profiles, individual and total glucosinolates and their profiles, antioxidant capacity). At Di3A-UNICT samples were collected randomly from three single plants per accession and then immediately freeze-dried. A similar task had to be performed in Braga with two limitations: the biological material available in the field and the unavailable lyophilizer at the time of collecting. Consequently, a new methodology was established considering the biochemical compounds, bibliographic research, experience in this field and the existing conditions: at the time of collection, samples were collected and stored immediately in a specific container filled with liquid nitrogen -170°C; the samples coming from the field were stored at -20°C (freezing took place without formation of ice crystals and therefore we believed that the cell walls were intact and without enzymatic activity); the oven dry was used with ventilation, at 40°C for 6 to 8 hours (as some samples required a longer dehydration) and were immediately ground and placed in desiccator before and after milling. The dehydration and milling of all samples (GLS + AA / PHE) were carried out during two weeks. The process of storing and dehydration took place in the absence of light as much as possible and the samples remained uncut. The last accessions planted on 1 November 2015 at Braga were not collected for biochemical analysis: plants survived, but the plants' phenotype and state of plants could not be compared to the plants' phenotype from the early sown accessions.

Evaluation of downy mildew resistance

INIAV Oeiras sowed a total of 33 accessions, 13 accessions from the *Brassica* collections of BPGV-INIAV and 20 accessions from Di3A-UNICT. Five accessions (four landraces and one wild *Brassica*) did not germinate. Plantlets aged 8-9 days sown in plastic trays (3x3x5-cm cells) were inoculated with four droplets (two per cotyledon) of a suspension of fresh *H. brassicae* conidia. The plants were incubated to stimulate germination of spores in the dark for 24 h (16°C, high RH) and were then transferred to a growth chamber with controlled conditions (20±1°C, 70±10% RH with 19 h photoperiod under cool-white fluorescent light at 250 mmol m-2 s-1). Six days after inoculation, the plants were again incubated in the dark for 24 h (16°C, high RH) to promote asexual sporulation of the pathogen and the plants were individually evaluated, using a visual scale of seven resistance classes. The scale takes into account the level of parasite sporulation on cotyledons and the visible host responses. The resistance of the plants was tested with four isolates Hp006, Hp539, Hp533 and Hp541, which correspond to different *H. brassicae* pathotypes, P1, P2, P3 and P5 respectively (Table 3).

RESULTS

The characterization fields established in Braga and in Catania allowed to better detail the biomorphological, biochemical and genetic traits of the 39 accessions provided by BPGV-INIAV and Di3A-UNICT genebanks, and for some of them their resistance/tolerance against *Hyaloperonospora brassicae* ascertained by INIAV-Oeiras.

In general, the Italian accessions were not well adapted to the Braga climatic conditions and finally only 27 accessions (13 Portuguese and 14 Italian) could be grown and characterized by BPGV, whereas both Portuguese (13) and Italian (26) accessions were well adapted in Catania and all 39 were grown and characterized by Di3A.

The Portuguese accessions were similar among them as they all belong to *B. oleracea* var. *acephala* and var. *costata*, except the three F1 hybrids belonging to the varieties *capitata, italica* and *sabauda* of *B. oleracea.*

The Italian accessions were much more diversified also because they represent several *B. oleracea* crops/landraces and six populations of *Brassica* wild species (n=9).

All accessions grown in Catania were clustered in relation to the biomorphological descriptors in 11 groups showing among them some discriminant traits (Fig. 1).

The biochemical data showed a well detailed pattern in terms of glucosinolates (GLS), polyphenol (PHP) profiles and antioxidant capacity (DPPH) detected in the leaf samples of the accessions characterized (Figs. 2, 3, 4, 5, 6). The GLS amount was higher for the freeze-dried samples than the dried ones and the profiles of the accessions studied varied mainly in relation to their geographical origin (Figs. 2, 3). Leaf samples collected in Catania showed the presence of glucoiberin, progoitrin, sinigrin, glucoraphanin, glucosinalbin, gluconapin, glucoiberverin, 4-hydroxyglucobrassicin, glucobrassicanapin, glucobrassicin and neoglucobrassicin, whereas in the leaf samples of Braga glucoiberin, progoitrin, sinigrin, glucoraphanin, glucobrassicanin, glucotropaolin, glucobrassicin and neoglucobrassicin were detected (Figs. 2, 3). The amount of total GLS varied from 3 µg/g dw to more than 50 µg/g dw. The Portuguese accessions are mainly characterized by glucoiberin, sinigrin and glucobrassicin, the Italian ones by glucoraphanin, glucobrassicin and neoglucobrassicin (Figs. 2, 3). The F1 hybrids showed different GLS profiles in comparison to both Italian and Portuguese landraces and wild species studied. The same trend was observed for the polyphenol and ascorbic acid amounts and for the antioxidant capacity as for GLS (Figs. 2, 3, 4, 5, 6). The level of total phenols ranged in Catania field from 784.6 mg/100 g dw to 2991.1 mg/100 g dw, and the total mean value was 1911.1 mg/100 g dw (Fig. 4). The level of total phenols of dried samples collected in Portugal ranged from 230.7 mg/100 g dw to 1966.1 mg/100 g dw, while it ranged from 165.1 mg/100 g dw to 2283.0 mg/100 g dw collected in Italy (Fig. 5). The total mean values were similar, being equal to 900.4 and 880.1 mg/100 g dw, for Portuguese and Italian dried samples, respectively (Fig. 5). The plant-to-plant variability was similar between Portugal and Italy, with an average CV of 30%. The anthocyanins almost disappeared in dried samples. The DPPH scavenging indexes were higher for the Italian accessions than for the Portuguese ones (Fig. 6). The profiles of the polyphenols varied mainly in relation to the geographic origin; the Italian accessions were characterized by the presence of kaempferol and sinapoyl derivatives and the Portuguese ones by caffeic acid esters. Regarding the genetic analysis, the SSR primers utilized by Di3A were not polymorphic whereas those utilized by BPGV were polymorphic. Among the SSR primers utilized by Di3A the attention was pointed to GLS-ELONG and the PCR products of the studied accessions were sequenced. We obtained 37 readable sequences which were aligned with the *Brassica oleracea* var. *italica* GenBank accession AC149635 (region 40,000-49,000). Several sequence variations were observed and six single nucleotide polymorphisms (SNPs) were found, one base insertion and one 30 base pairs per cassette deletion (Fig. 7). In particular, the same homozygous condition observed for the reference accession AC149635 is expressed by UNICT3944, UNICT3513, UNICT4780 and UNICT4781, which represent two Sicilian broccoli landraces and two Sicilian wild *Brassica* populations (Fig. 7). Slight variation for a cassette GAP or HeteroGAP was ascertained for several UNICT accessions of broccoli and kale, represented by several F1 hybrids of broccoli and by two kale accessions (one Italian and one Portuguese). Several other UNICT accessions and some BPGV ones showed variation from the reference accessions by two SNPs and by a cassette deletion (Fig. 7). The highest variation for BoGLS-ELONG PCR products were found in a Portuguese kale accession and a Sicilian wild *Brassica*

population. Finally, six landraces and two F1 hybrids among the Portuguese accessions showed two SNPs variation and a cassette of GAP/HeteroGAP (Fig. 7). The amplifications obtained with primer P14 will be analysed in the near future, since two bands are present (Fig. 8). The 489 bp product derives from the *Brassica oleracea* 2-oxoglutarate-dependent dioxygenase gene (AY044425.1), while the 565 bp product is obtained from a null-allele, as template, already described in Genbank (AY044424.1). On this basis, we clustered the accessions studied in relation to the P13 primers SNPs and the insertion found (Fig. 9). In particular, the same homozygous condition observed for the reference accession AC149635 is expressed by UNICT3944, UNICT3513; UNICT4780 and UNICT4781, which represent the group D. Theseare respectively two Sicilian broccoli landraces and two Sicilian wild *Brassica* populations (Fig. 9). Slight variation for a cassette GAP or HeteroGAP was ascertained for several UNICT accessions of broccoli and kale, represented by several hybrids F1 of broccoli and by two kale accessions (one Italian and one Portuguese), grouped in E and F (Figs. 7, 8, 9). Several other UNICT accessions and some BPGV ones showed variation from the reference accessions by two SNPs and by a cassette deletion (Figs. 8, 9). The highest variation of the P13 PCR product sequence in comparison to reference accessions was found for the group B which is represented by a Portuguese kale accession and a Sicilian wild *Brassica* population (Fig. 9). Six landraces and two F1 hybrids among the Portuguese accessions showed two SNPs variations and a cassette of GAP/HeteroGAP and were grouped in G and H (Fig. 9).

All 27 accessions grown in Braga showed among them high variability concerning the biomorphological descriptors. The Portuguese accessions are located in a specific cluster, the landraces, wild accessions and the F1 hybrids have genetic dissimilarity, so the accessions were grouped in five clusters (Fig. 10). The average taxonomic distance (coefficient 0.33) and their origin seem to be important: Portuguese accessions and Catania accessions were grouped with more genetic similarity between them, than the material from other origins (Fig. 10).

Among the SSR primers utilized by BPGV-INIAV of Braga, the cluster analysis for the 10 loci of the 33 accessions showed a dendrogram with a not very significant cophenetic coefficient (r=0.64; Fig. 11).

Findings from the SSR analysis carried out by BPGV were the following:

i. a total of 172 alleles were amplified in 33 x 10 plants accessions;
ii. the average number of alleles per locus was 17.2;
iii. the BRMS020 locus was the most polymorphic with 32 alleles;
iv. between the Portuguese and Italian accessions there is genetic polymorphism (average number of alleles per locus was 28.6 for the Portuguese and 43.25 for the Italian accessions);
v. in the Portuguese collection, the average number of alleles per locus was 33.6 for var. *acephala* and 24.5 for var. *costata;*
vi. in the Italian collection, the average number of alleles is higher for var. *italica* than for var. *botrytis* (47.5:41.4); the average number of alleles for commercial cultivars was 37.2;
vii. the average number of alleles locus^{-1} accession^{-1} was 37.

The downy mildew (DM) data showed no differential responses to the four *H. brassicae* pathotypes, but high differences in DM response between accessions. Since there were no differences in virulence between pathotypes, to facilitate comparisons between accessions, the accessions were sorted by resistance level according to the mean disease index (DI), calculated with the evaluations performed with the four *H. brassicae* pathotypes (Table 5). Some accessions showed a low germination capacity. The accessions can be separated in three resistance groups. Seven accessions were included in the resistant group with DI<2.5 (BPGV4533, BPGV1773, BPGV1726, BPGV1747, UNICT3944, UNICT4448 and UNICT4633). A group of 14 accessions were considered moderately resistant, presenting $2.5 \leq DI < 4.5$ (BPGV5435, BPGV2884, BPGV6980, $HDF_1$3, BPGV3853, BPGV3826, BPGV7452, $HDF_1$1, $HDF_1$2, UNICT3404, UNICT3513, UNICT3169, UNICT418, and UNICT3108). A last group of eight accessions included the most susceptible lines, registering $DI \geq 4.5$ (UNICT914, UNICT3605, UNICT4447, UNICT4642, UNICT3406, UNICT4636, UNICT3270, and the susceptible control (Table 5).

The accessions can be distributed in three resistance groups (resistant, 7 accessions; moderately resistant, 14 acc.; susceptible, 8 acc.) (Table 5).

All accessions showed high within accession variability to DM response, especially those classified in resistant and moderately resistant groups (Table 5). Some accessions are segregating for resistance; it would be interesting to proceed to a genetic study of DM resistance in these accessions. Regarding the six wild type accessions from the Italian collection, *B. villosa* (UNICT3944) was the most resistant (DI=1.4); Sicilian *B. rupestris* (UNICT3404) and *B. incana* (UNICT3513) showed an intermediate level of resistance (DI=3.1 and 3.4); Calabrian *B. rupestris* accessions (UNICT3406 and UNICT3270) were very susceptible (DI=5.6 and 5.8); and *B. macrocarpa* (UNICT2987) did not germinate (Table 5). Within the four tested hybrids, $HDF_1$3 (cabbage), $HDF_1$1 (broccoli), and $HDF_1$2 (savoy cabbage) had an intermediate level of resistance (DI=2.9, 3.3, and 4.4 respectively) and UNICT4642 (cabbage) was susceptible, registering DI=5.5.

Comparing DM resistance and the subtaxa of the accessions, it was found that the five wild types fell in the three groups, *B. villosa* in the resistant group, one *B. rupestris* and the *B. incana* accessions in the intermediate group, and two accessions of *B. rupestris* in the susceptible group. The four *acephala*, six *costata* and two *sabauda* accessions fell in the resistant and intermediate groups. Four *capitata* and five *botrytis* accessions were classified in the intermediate and susceptible groups (Table 5). The accession from *italica* subtaxa was classified as intermediate and the *gongylodes* accession was classified as susceptible (Table 5).

DISCUSSION AND RECOMMENDATIONS

The full list of accessions recommended for inclusion in the AEGIS European Collection is given in Table 6, tabulated according to the following criteria:

- The MAAs chosen to be proposed for AEGIS for **biomorphological traits** are BPGV01747, BPGV01773, BPGV02884, BPGV03826, BPGV03853, BPGV04553, BPGV06980, UNICT498, UNICT636, UNICT3108, UNICT3270, UNICT3404, UNICT3406, UNICT3513, UNICT3716, UNICT3726, UNICT3944, UNICT4447, UNICT4448, UNICT4633, UNICT4636 and UNICT4780 in relation to their different morphotypes.
- The accessions of interest as MAAs for AEGIS for **biochemical compounds** are BPGV1747, BPVG2884, BPGV3826, BPGV3853, BPGV4533, BPGV6980, BPGV7452, $HDF_1$2, UNICT418, UNICT636, UNICT914, UNICT3169, UNICT3404, UNICT3605, UNICT3944, UNICT4633, UNICT4636 and UNICT4640 in relation to their different ASA, PHP, GLS profiles and DPPH antioxidant capacity as evidenced in Figs. 2, 3, 4, 5 and 6.
- Regarding the **GLS-ELONG SSR primer products** the accessions of interest are BPVG2884, UNICT636, UNICT649, UNICT 4780, UNICT4781 and UNICT3944.
- Finally, the **most interesting DM-resistant accessions** are the seven accessions identified as resistant (BPGV1726, BPGV1747, BPGV1773, BPGV4533, UNICT3944, UNICT4448 and UNICT4633; they could become good germplasm sources to be included in a DM resistance breeding programme in the near future.

REFERENCES

Branca F, Bas N, Artemyeva A, De Haro A, Maggioni L. 2013. In Progress Activities of the *Brassica* Working Group of the European Cooperative Programme for Plant Genetic Resources (ECPGR). Proceedings of the VIth International Symposium on Brassicas and XVIIIth Crucifer Genetics Workshop, 12-16 November 2012, Catania, Italy (F. Branca and A. Tribulato, eds.). Acta Horticulturae 1005:149-156

Li G, Quiros C. 2001. Sequence-related amplified polymorphism (SRAP), a new marker system based on a simple PCR reaction: its application to mapping and gene tagging in *Brassica*. Theoretical and Applied Genetics 103:455.

Li G, Quiros CF. 2002. Genetic analysis, expression and molecular characterization of BoGSL-ELONG, a major gene involved in the aliphatic glucosinolate pathway of *Brassica* species. Genetics 162(4):1937-1943.

ANNEXES

Table 1. Final list of the accessions studied

	ACCESSION CODE	Type (*)	Species	Variety	COMMON NAME	PROVENANCE
1	BPGV01726	LR	*B. oleracea*	*costata*	Couve tronchuda	Braga
2	BPGV01747	LR	*B. oleracea*	*costata*	Couve penca Gondomar	Gondomar
3	BPGV01773	LR	*B. oleracea*	*costata*	Couve penca asa de cantaro	S. Cosme - Gondomar
4	BPGV02884	LR	*B. oleracea*	*acephala*	Couve de todos os dias	Curral ds Freiras
5	BPGV03826	LR	*B. oleracea*	*costata*	Penca	Chaves
6	BPGV03853	LR	*B. oleracea*	*capitata*	Couve Bacalan	Mirandela
7	BPGV04533	LR	*B. oleracea*	*acephala*	Couve porqueira	Mortàgua
8	BPGV05435	LR	*B. oleracea*	*costata*	Couve penca	Freixo de Espada à Cinta
9	BPGV06980	LR	*B. oleracea*	*costata*	Couve penca	Mascarenhos- Mirandela
10	BPGV07452	LR	*B. oleracea*	*acephala*	Couve galega	Montalegre
11	$HDF_1$1	H F1	*B. oleracea*	*italica*	Naxos F1	
12	$HDF_1$2	H F1	*B. oleracea*	*sabauda*	Savonastar F1	
13	$HDF_1$3	H F1	*B. oleracea*	*capitata*	Capehorn F1	
14	UNICT418	LR	*B. oleracea*	*botrytis*		Lamezia
15	UNICT498	LR	*B. oleracea*	*botrytis*		Catania
16	UNICT636	LR	*B. oleracea*	*italica*		Palermo
17	UNICT649	H F1	*B. oleracea*	*italica*	RS89006 F1	Royal Sluis
18	UNICT914	LR	*B. oleracea*	*botrytis*		Adrano
19	UNICT3108	LR	*B. oleracea*	*botrytis*		Catania
20	UNICT3169	LR	*B. oleracea*	*botrytis*		Catania
21	UNICT3270	W	*B. rupestris*			Stilo
22	UNICT3404	W	*B. rupestris*			Caltavuturo
23	UNICT3406	W	*B. rupestris*			Pazzano
24	UNICT3513	W	*B. incana*			Agnone Bagni
25	UNICT3605	LR	*B. oleracea*	*botrytis*		Catania
26	UNICT 3716	W	*B. rupestris*			Caltavuturo
27	UNICT3726	LR	*B. oleracea*	*acephala*		Caltavuturo
28	UNICT3944	W	*B. villosa*			Marianopoli
29	UNICT4447	LR	*B. oleracea*	*gongylodes*		Milazzo
30	UNICT4448	LR	*B. oleracea*	*acephala*		Capizzi
31	UNICT4633	LR	*B. oleracea*	*sabauda*		Maniace
32	UNICT4636	LR	*B. oleracea*	*capitata*		Siracusa
33	UNICT4638	H F1	*B. oleracea*	*italica*	Forester F1	ISI Sementi
34	UNICT4640	H F1	*B. oleracea*	*botrytis*	Clima F1	ISI Sementi
35	UNICT4642	H F1	*B. oleracea*	*capitata*	Velvet F1	ISI Sementi
36	UNICT4779	LR	*B. oleracea*	*italica*	cavolo broccolo calabrese tardivo	Randazzo
37	UNICT4780	LR	*B. oleracea*	*italica*	cavolo broccolo calabrese natalino	Acireale
38	UNICT4781	LR	*B. oleracea*	*italica*	cavolo broccolo calabrese tardivo	Catania
39	UNICT4787	LR	*B. oleracea*	*italica*	cavolo broccolo calabrese tardivo	Messina

(*) Type: *Brassica* Wild species (W); Landrace *B. oleracea* (LR); Hybrid F1 (H F1)

Table 2. IBPGR and UPOV descriptors utilized[3]

	Descriptor		Units of measure
Plant			
VSL	Vegetative stem length	IBPGR 4.2.56	cm
VSW	Vegetative stem width	IBPGR 4.2.55	mm
PH	Plant height	IBPGR 4.2.3	cm
PD	Plant diameter	IBPGR 4.2.4	cm
NL	Average leaf per plant main stem	IBPGR 4.2.11	Number
ALS	Average leaf scars	IBPGR 4.2.10	Number
HH	Heading habit	IBPGR 4.2.34	0=nonheading; 5=semiheading; 7=heading
IA	Inflorescence appearance	IBPGR 4.3.2	Days from the transplanting
Leaf			
LW	Leaf blade width	IBPGR 4.2.13	cm
LL	Leaf blade length	IBPGR 4.2.12	cm
LA	Leaf angle	IBPGR 4.2.15	Angle
LC	Leaf colour	IBPGR 4.2.24	L*,a*,b*
LS	Leaf blade shape	IBPGR 4.2.16	from 1 to 7
LAT	Leaf lamina attitude	IBPGR 4.2.23	3=convex; 5=straight ; 7= Concave
LBL	Leaf blade blistering	IBPGR 4.2.21	0=none; 3=low; 5=intermediate; 7=high
LLB	Leaf lobes	IBPGR 4.2.18	1=absent ; 9=present
LAN	Leaf anthocyanin coloration	UPOV_5	1= absent/ 9=present
LD	Leaf blade density of curling	UPOV_14	0=absent; 3=low; 5=intermediate; 7=high
Petiole			
PL	Petiole length	IBPGR 4.2.28	cm
PW	Petiole width	IBPGR 4.2.29	mm
PE	Petiole enlargement	IBPGR 4.2.27	3=narrow; 5=intermediate; 7=enlarged
PC	Petiole midvein colour	IBPGR 4.2.33	1=white;2=light green; 3=green; 4=purple; 5=red; 6=other

[3] IBPGR 1990. Descriptors for *Brassica* and *Raphanus.* International Board for Plant Genetic Resources, Rome, Italy. (http://www.bioversityinternational.org/fileadmin/user_upload/online_library/publications/pdfs/339.pdf)
CPVO-OCVV. 2011. Protocol for distinctness, uniformity and stability tests. *Brassica oleracea* L. var. *sabellica* L. Curly Kale. UPOV Code: BRASS_OLE_GAS. CPVO-TP/090/1 Final. Community Plant Variety Office. Office Communautaire des Variétés Végétales. (http://www.cpvo.europa.eu/documents/TP/veg/TP_BRASSICA_SABELLICA_090-1.pdf).

Table 3. SSR Primers utilized by Di3A UNICT of Catania

Primer mix code	Primer code	Gene	Primer Forward	Primer Reverse
P11	CY713F ; CY2000R	BoCYP79F1	TAGGACAAGCGGAGAAAGAT	TTCAGTTTCGACCAGAGAAA
P12	GT435F; GT1028R	BoS-GT	CACCGTTTGCTCTGTTCTAC	AAAAACCACTGACTTGCTCA
P13	IPM2; IPM9	GLS-ELONG	GTGACGGTGAACAATCTCC	GTAGTATTCTCAAAATCTTGT
P14	ODD-12; ODD-48	GLS-ALK	TTCCATCATTTACTTTCTCAG	TTGAATATCCAGTGTAAGGTT
P15	PM87; PM132	BoGSL-PROa	AGAAGGGTGGTGATTGTTG	ACGCATTGTCAGAATGATCT
P16	OH621F; OH1274R	BoGS-OH	GGTACGAACAAGGCTTCTCT	CGGAGTTGAAGAGGAAAACT
P17	PL132; PL581	BoGSL-PROb	ACGCATTGTCAGAATGATCT	GTCATAACAATGTGCCGAGT
P18	LS360F; LS2230R	BoCS-lyase	CGGCAAAAGCAATTCTTAC	CCACTATCCCGACACTATCA

Table 4. Genes involved in PCR analysis and primer sequences utilized by BPGV-INIAV of Braga

Gene	Primer forward	Primer reverse
BoGSL ELONG a	GTGACGGTGAACAATCTCC	GTAGTATTCTCAAAATCTTGT
BoGSL ALK	TTCCATCATTTACTTTCTCAG	TTGAATATCCAGTGTAAGGTT
Na10 F06	CTCTTCGGTTCGATCCTCG	TTTTTAACAGGAACGGTGGC
Na14E02	ACTGGCTACATGAGTTTCAGTG	GAGGGAAGACAACTGGTCTCA
BRMS 020	AACAAGAGAAGGAGAGCCACCG	CGCTTATAAAATGGCAGTCGCA
BRMS 030	TCAGCCTACCAACGAGTCATAA	AAGGTCTCATACGATGGGAGTG
FITO017	TTTTTGTATCCTCCATCATTTTTG	TGATATGTTTGACAATTTCCCC
OL12F02A	GGCCCATTGATATGGAGATG	CATTTCTCAATGATGAATAGT
BOGMS1570	TCAAGCCAACGCTACTACA	TGATGGGTGAACAACTAAACT
BOGMS1467	ATGGCTTTGTTCTTCTTTCTT	GACTTCAGCACGCCTTTC

Figure 1. Classification of the studied accessions on the basis of the biomorphological descriptors.

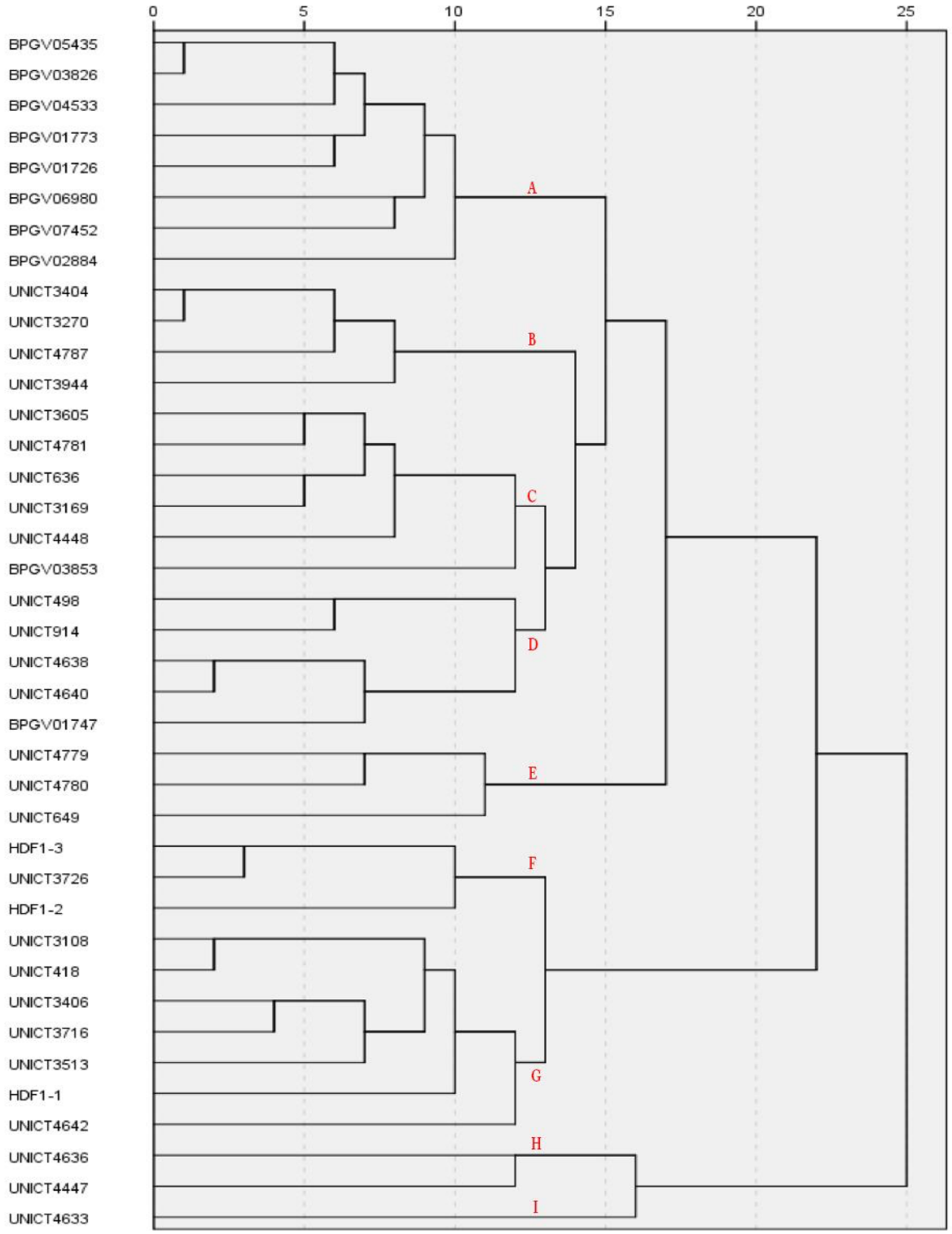
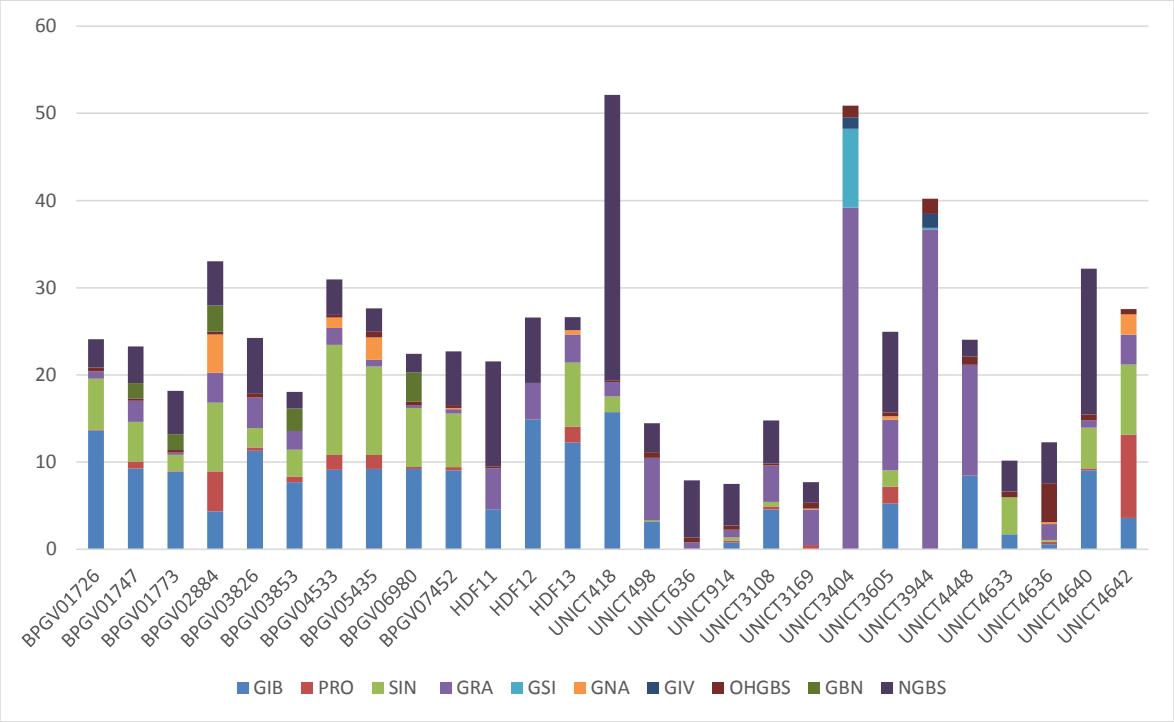


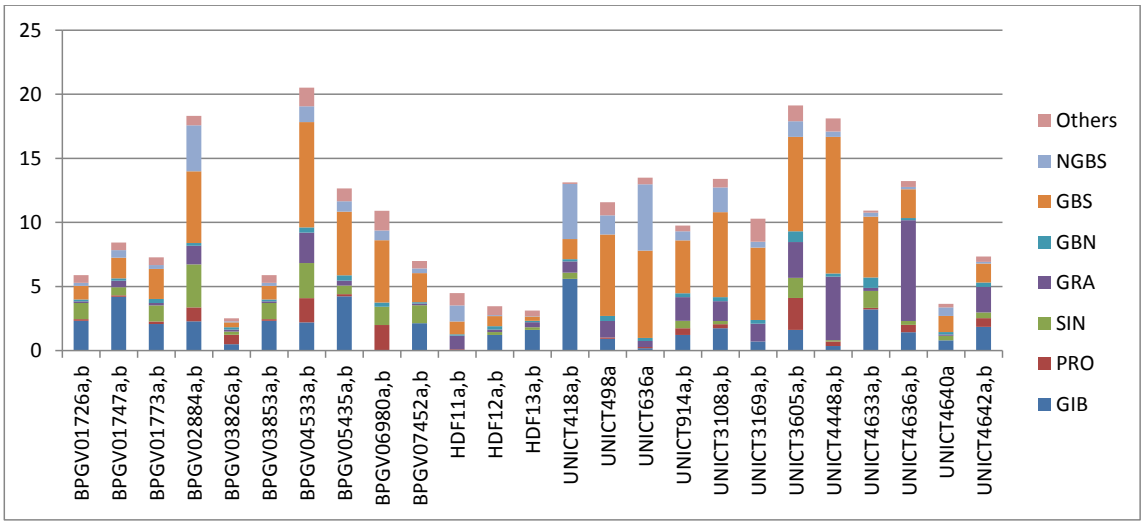
Figure 2. Glucosinolate profile of the freeze-dried leave samples of the accessions grown in Catania (µg/g dw).



Abbreviations:

GIB: glucoiberin
PRO: progoitrin
SIN: sinigrin
GRA: glucoraphanin
GSI: glucosinalbin
GNA: gluconapin
GIV: glucoiberverin
OHGBS: 4-hydroxyglucobrassicin
GBN: glucobrassicanapin
GBS: glucobrassicin
NGBS: neoglucobrassicin

Figure 3. Glucosinolate profile of the dry leaves of the accessions grown in Braga (µg/g dw).



Abbreviations:

GIB: glucoiberin
PRO: progoitrin
SIN: sinigrin
GRA: glucoraphanin
GBN: glucobrassicanin
GTL: glucotropaolin
GBS: glucobrassicin
NGBS: neoglucobrassicin

Figure 4. Ascorbic acid (ASA), Polyphenols (PHP) and Antioxidant capacity (DPPH) of the freeze-dried samples of Catania field collection (mg/100 g dw).

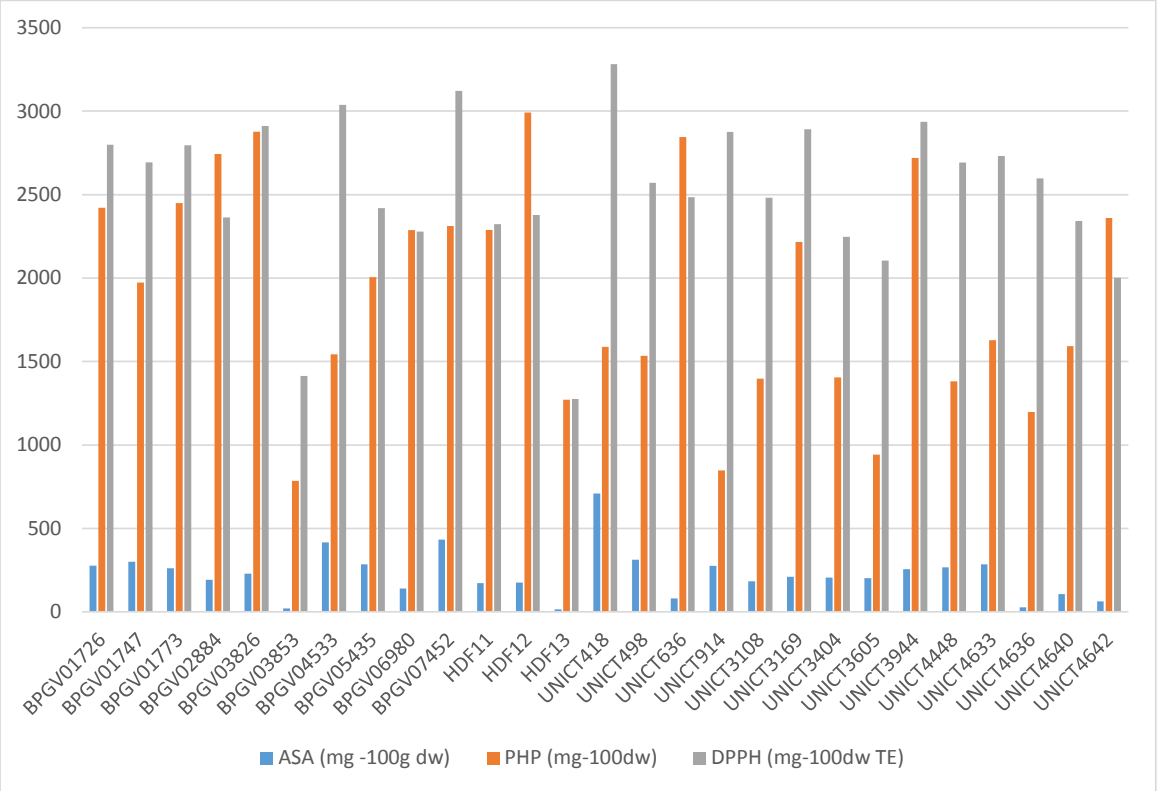


Figure 5. Polyphenol content of the dried leaf samples of the accessions grown in Braga and in Catania (mg/100 g dw).

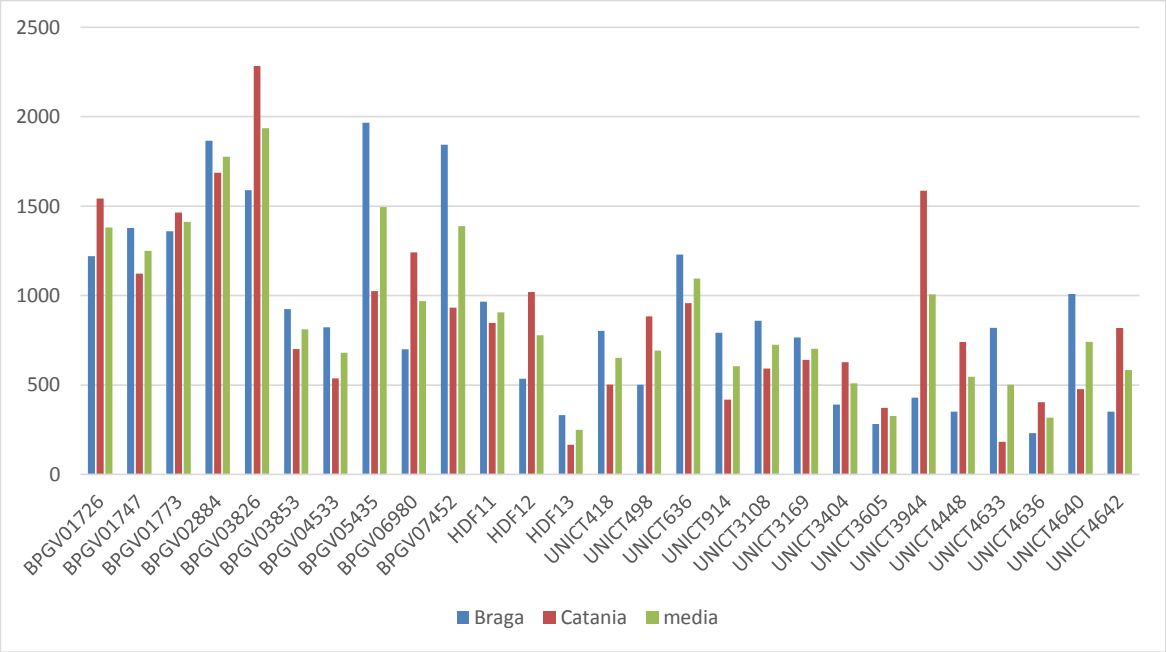


Figure 6. DPPH scavenging of the leaf dried samples of the accessions grown in Braga and in Catania (mg/100 dw TE).

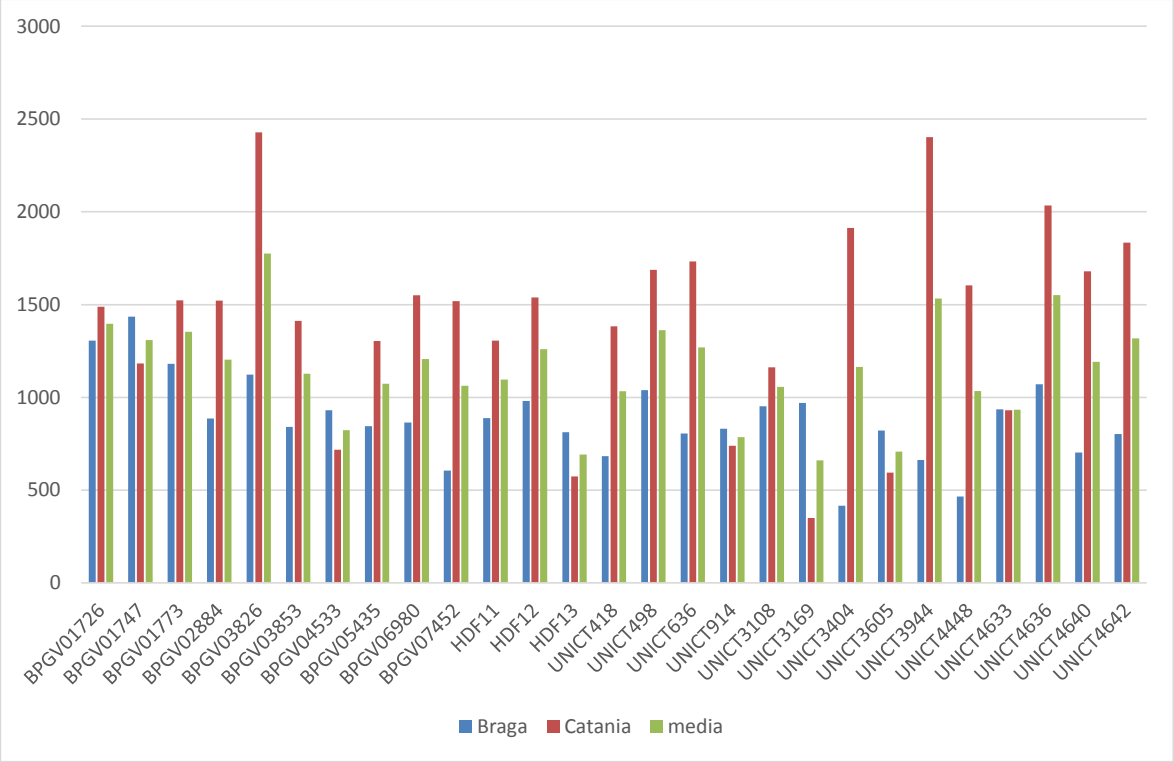


Figure 7. Sequence variations found by sequencing the 37 accession amplification products obtained with the p13 primers. Nucleotide positions are indicated for the 6 SNPs and the insA. The 30 bp cassette is indicated with the large line while the lacking tract by a dashed line.

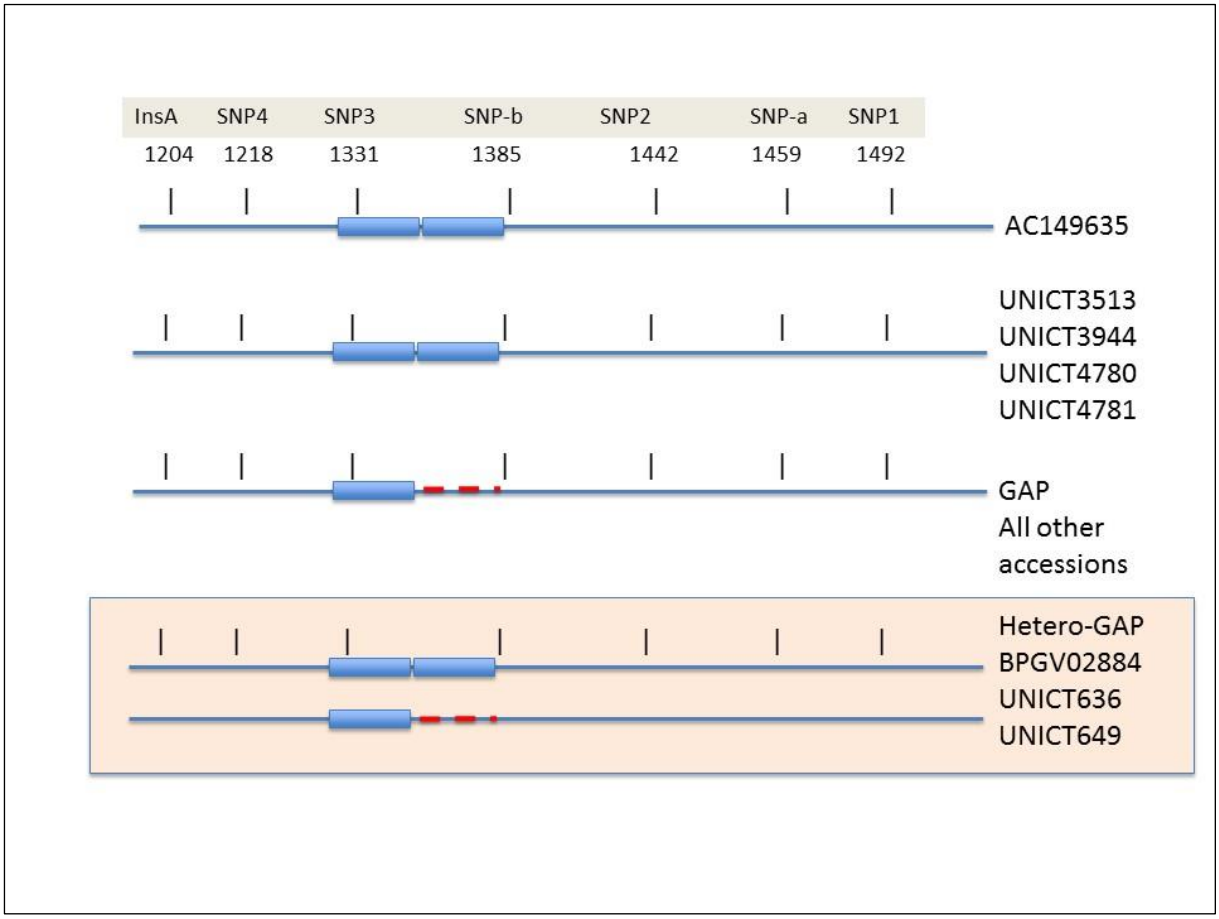


Figure 8. Alignment of the 5' region of *Brassica oleracea* 2-oxoglutarate-dependent dioxygenase gene (AY044425.1) and null allele (AY044424.1) coding sequences, where the reverse p14 primer anneals. Two amplification products are normally generated, 489 bp and 565 bp from the null and the coding allele, respectively.

Figure 9. Classification of the studied accessions on the basis of the SNPs, insertion and cassette deletion found sequencing the PCR product of BoGLS Elong SSR primer (P13).

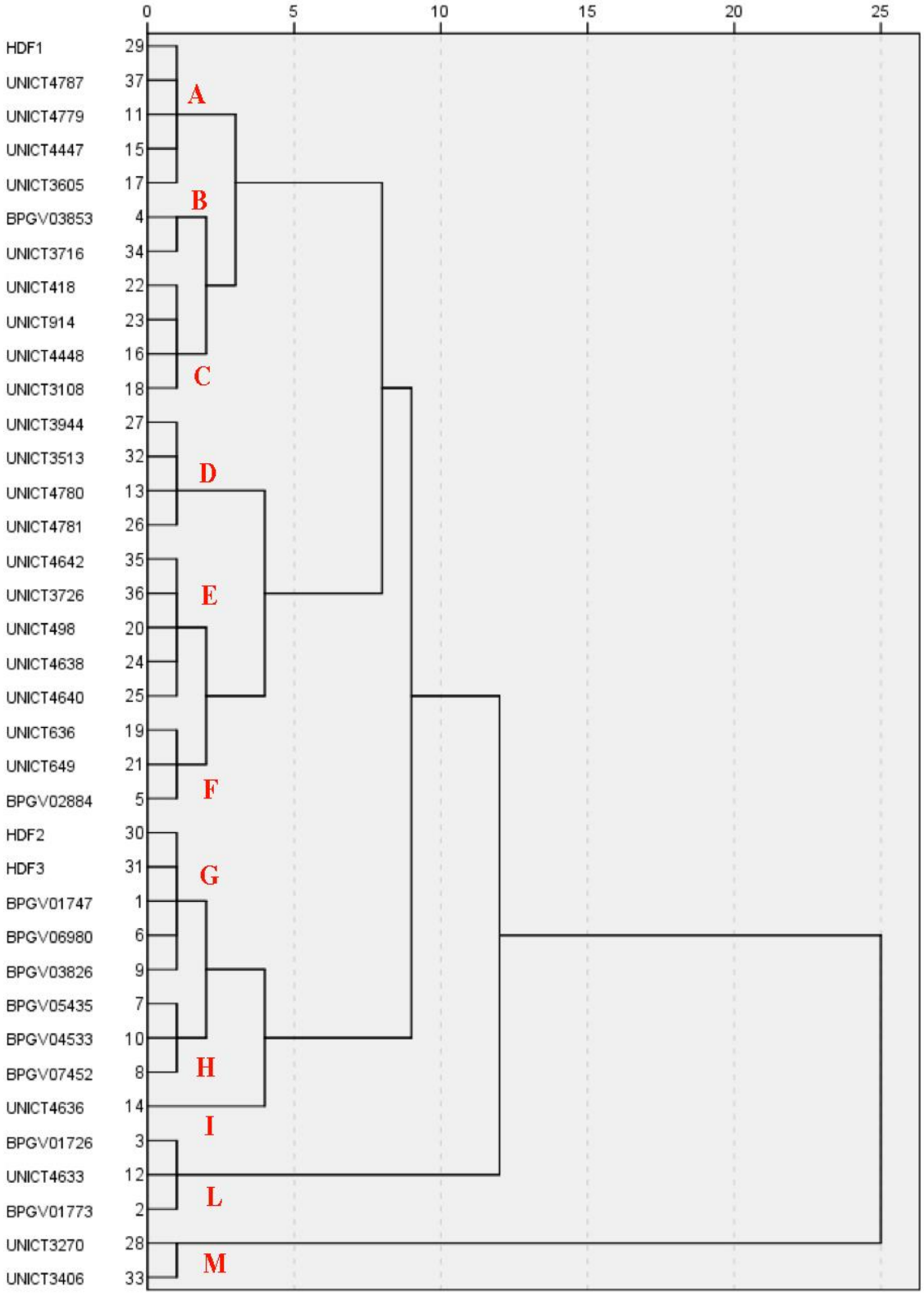


Figure 10. Phenogram obtained for 22 biomorphological descriptors scored in 27 accessions using average taxonomic distance coefficient and with cophonetic coefficient r = 0.90.

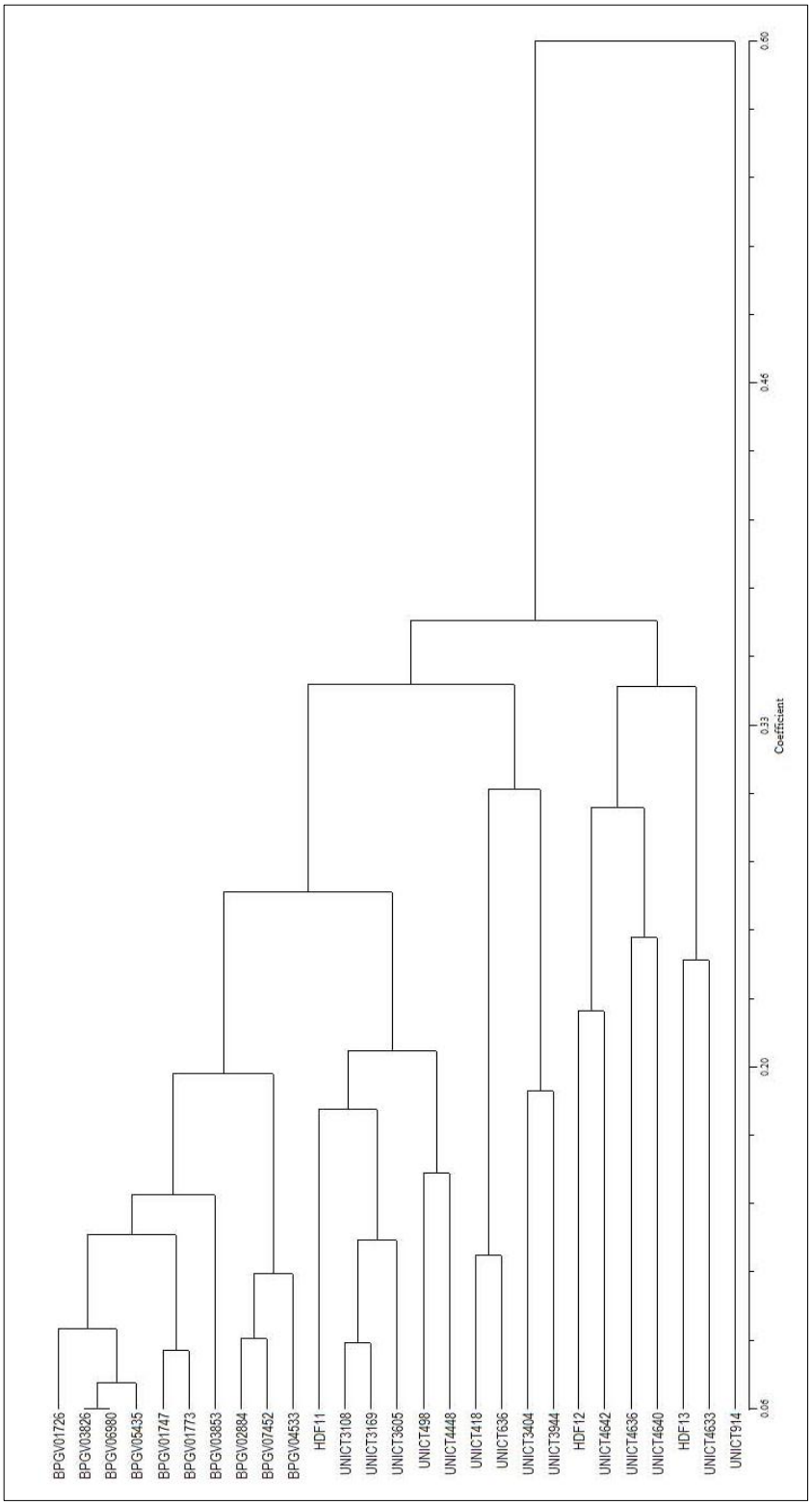


Figure 11. Dendrogram obtained for 10 SSR loci (172 alleles) scored in 33 accessions using Jaccard's coefficient and with cophonetic coefficient r = 0.64.

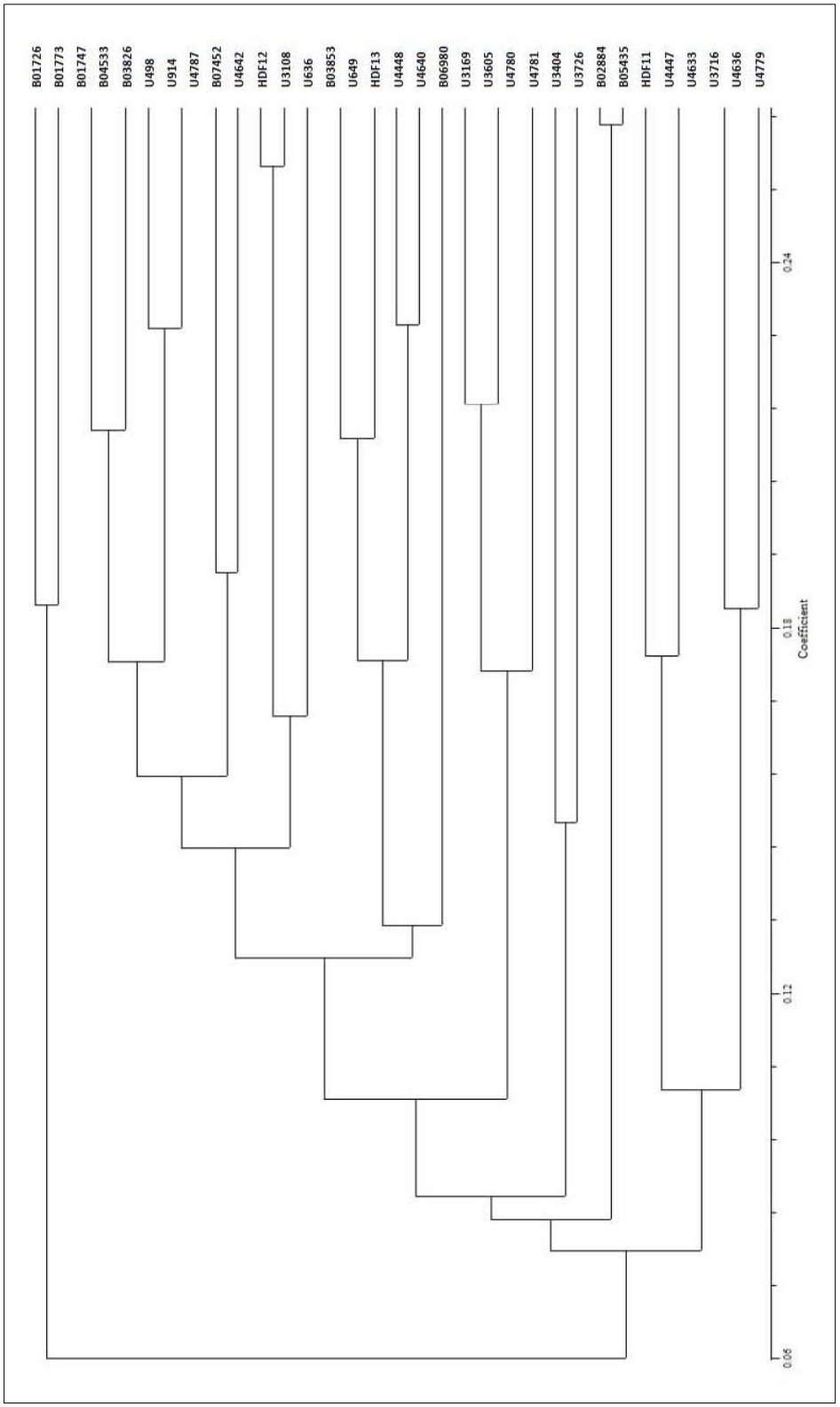


Table 5. Distribution of accessions in downy mildew resistance groups according to disease index (DI) value (LR= landrace; W= wild, H= hybrid F1).

Resistant DI<2.5	**Moderately resistant 2.5 ≤ DI<4.5**	**Susceptible DI ≥ 4.5**
BPGV4533 - *B. oleracea* (LR)	BPGV5435 - *B. oleracea* (LR)	UNICT914 - *B. oleracea* (LR)
BPGV1773 - *B. oleracea* (LR)	BPGV2884 - *B. oleracea* (LR)	UNICT3605 - *B. oleracea* (LR)
BPGV1726 - *B. oleracea* (LR)	BPGV6980 - *B. oleracea* (LR)	UNICT4447 - *B. oleracea* (LR)
BPGV1747 - *B. oleracea* (LR)	$HDF_1$3 - *B. oleracea* (H)	UNICT4642 - *B. oleracea* (H)
UNICT3944 - *B. villosa* (W)	BPGV3853 - *B. oleracea* (LR)	UNICT3406 - *B. rupestris* (W)
UNICT4448 - *B. oleracea* (LR)	BPGV3826 - *B. oleracea* (LR)	UNICT4636 - *B. oleracea* (LR)
UNICT4633 - *B. oleracea* (LR)	BPGV7452 - *B. oleracea* (LR)	UNICT3270 - *B. rupestris* (W)
	$HDF_1$1 - *B. oleracea* (H)	Susceptible control - *B. oleracea*
	$HDF_1$2 - *B. oleracea* (H)	
	UNICT3404 - *B. rupestris* (W)	
	UNICT3513 - *B. incana* (W)	
	UNICT3169 - *B. oleracea* (LR)	
	UNICT418 - *B. oleracea* (LR)	
	UNICT3108 - *B. oleracea* (LR)	

Table 6. List of accessions recommended for inclusion in the AEGIS European Collection

ACCESSION CODE	Type (*)	Species	Variety	Biomorpho-logical traits (Fig.1)	Biochemical traits (Figs. 2-4)	GLSs (Fig. 7)	DM-resistant (Table 5)
BPGV01726	LR	*B. oleracea*	*costata*				Resistant DI<2.5
BPGV01747	LR	*B. oleracea*	*costata*	Cluster D	GLS		Resistant DI<2.5
BPGV01773	LR	*B. oleracea*	*costata*	Cluster A			Resistant DI<2.5
BPGV02884	LR	*B. oleracea*	*acephala*	Cluster A	GLS-PHP	Hetero-GAP	
BPGV03826	LR	*B. oleracea*	*costata*	Cluster A	GLS-PHP		
BPGV03853	LR	*B. oleracea*	*capitata*	Cluster C	GLS PHP-DPPH-ASA		
BPGV04533	LR	*B. oleracea*	*acephala*	Cluster A	DPPH		Resistant DI<2.5
BPGV06980	LR	*B. oleracea*	*costata*	Cluster A	GLS		
BPGV07452	LR	*B. oleracea*	*acephala*		GLS-DPPH		
$HDF_1$2	H F1	*B. oleracea*	*sabauda*		PHP		
UNICT418	LR	*B. oleracea*	*botrytis*		GLS-DPPH-ASA		
UNICT498	LR	*B. oleracea*	*botrytis*	Cluster D			
UNICT636	LR	*B. oleracea*	*italica*	Cluster C	GLS-PHP	Hetero-GAP	
UNICT649	H F1	*B. oleracea*	*italica*			Hetero-GAP	
UNICT914	LR	*B. oleracea*	*botrytis*		PHP		
UNICT3108	LR	*B. oleracea*	*botrytis*	Cluster G			
UNICT3169	LR	*B. oleracea*	*botrytis*		GLS-		
UNICT3270	W	*B. rupestris*		Cluster B			
UNICT3404	W	*B. rupestris*		Cluster B	GLS		
UNICT3406	W	*B. rupestris*		Cluster G			
UNICT3513	W	*B. incana*		Cluster G		Express	
UNICT3605	LR	*B. oleracea*	*botrytis*		GLS		
UNICT3716	W	*B. rupestris*		Cluster G			
UNICT3726	LR	*B. oleracea*	*acephala*	Cluster F			
UNICT3944	W	*B. villosa*		Cluster B	GLS-PHP	Express	Resistant DI<2.5
UNICT4447	LR	*B. oleracea*	*gongylodes*	Cluster H			

Table 6 (cont.). List of accessions recommended for inclusion in the AEGIS European Collection

ACCESSION CODE	Type (*)	Species	Variety	Biomorpho-logical traits (Fig.1)	Biochemical traits (Figs. 2-4)	GLSs (Fig. 7)	DM-resistant (Table 5)
UNICT4448	LR	*B. oleracea*	*acephala*	Cluster C			Resistant DI<2.5
UNICT4633	LR	*B. oleracea*	*sabauda*	Cluster I	GLS		Resistant DI<2.5
UNICT4636	LR	*B. oleracea*	*capitata*	Cluster H	GLS-ASA		
UNICT4640	H F1	*B. oleracea*	*botrytis*		GLS		
UNICT4780	LR	*B. oleracea*	*italica*	Cluster E		Express	
UNICT4781	LR	*B. oleracea*	*italica*			Express	