Suitability of a European nuclear collection of *Brassica oleracea* L. landraces to grow at high temperatures

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ABSTRACT

Statistical models predict that global warming will have a negative impact in crop yields in the next decades. Especially vulnerable are winter crops such as kales or cabbages (*Brassica oleracea* L.). We evaluated the impact of high temperatures in morphological and biochemical traits of a *B. oleracea* core collection during early development. When grown at 30 ºC, plants showed a reduction on chlorophyll content, early vigor and biomass compared to values observed on plants grown at 20 ºC. Likewise, the total content of glucosinolates shows a reduction at high temperatures. The *alboglabra* group showed the best general performance at 30 ºC for both morphological traits and glucosinolate content. Based on a cluster analysis, we selected four populations (MBG0072, MBG0464, MBG0535 and HRIGRU5555) as the most promising to be used in further breeding programs for heat tolerance.

INTRODUCTION

The Brassicaceae family includes more than 300 genera, most of them encompassing economically important species. This is the case of the *Brassica* genus which includes species that are used as vegetables, forage and mustard or oil production. *Brassica oleracea* L. is the most diverse species within this genus. Originally domesticated in the Atlantic coast of Europe, nowadays cultivars of this species are spread worldwide and grown under a wide range of climate conditions. The introgression of genes from wild allies has probably contributed to the high variability and adaptability of this crop (*1*). Human selection has lead to the development of diverse cultivars within this species in which different organs are used for human or livestock consumption. For example, the engrossed hypocotyls of kohlrabi (*gongylodes* group), the flower heads of cauliflowers (*botrytis* group) and broccolis (*italica* group) or the fresh leaves of kales (*acephala* group) and cabbages (*capitata* group) are used as vegetables.

The major abiotic stresses that cultivars of *B. oleracea* have to cope with during their life cycle are extreme temperatures. Although, either low or high temperatures could reduce crop establishment, yield and quality (*2*), increasing air temperatures have raised as the major challenge for *B. oleracea* cultivation in the near future (*3*). Different climate projections for the next decades were used to predict the effect of global warming in cauliflower (*B. oleracea botrytis* group) cultivation. Whichever scenario is considered, models predict that increasing air temperatures will advance the end of juvenility and delay curd initiation in cauliflower (*4*), resulting in a reduction of the curd diameter (*5*). Climate models have been revised in the last decade resulting in two different scenarios for the next century (*6*). The low scenario predicts an increase of 1.8 ºC by the end of the century whereas the estimate for the high scenario is an increase of

4 ºC. Even in the case of the low scenario, the increase of air temperature would likely have a negative impact in yield, especially for winter crops, such as *B. oleracea* (*7, 8*).

The effect of temperatures on curd initiation has been extensively studied in broccoli (*B. oleracea italica* group) and cauliflower (*B. oleracea botrytis* group) (*4, 9*). However, little attention has been paid to the effect of temperature on vegetative development. In general terms, higher growth rates could be observed at higher temperatures until reaching a maximum threshold, above which growth is drastically repressed. Different studies performed in cauliflower established the threshold for leaf growth at 30 ºC (*10, 11*). This threshold correlates with that established for photosynthesis rates in other *Brassica* species. According to Paul *et al* (*12*) maximum photosynthesis rates are reached at 22 ºC in *Brassica napus* whereas plants cultivated at 30 ºC showed a significant reduction of the photosynthesis rate, thus reducing the amount of photosynthates available for growth. In *B. oleracea*, Diaz et al (*13*) reported a reduction in the capacity of the photosynthetic electron transport at high temperatures, though high temperatures were combined with high light intensities, and the single effect of the high temperature could not be ascertained. So to our knowledge, not information is available about the impact of high temperatures during the early development in *B. oleracea* crops. In a similar way, little is known about the effect of high temperatures in the chemical composition of these crops. In *Brassica,* the most important metabolites are glucosinolates, since they have a role in the plant defense against biotic and abiotic stresses, as well as anticancer and other health properties (*14, 15*). Several studies showed the effect of climate conditions in the accumulation of glucosinolates in different *B. oleracea* cultivars (*16, 17*). These works are, however, performed in the field where the individual effect of temperature is difficult to establish. Just recently, Justen et al (*18*) reported that high temperatures induced the accumulation

of the total and individual content of glucosinolates in the shoot and roots under controlled conditions, although this study was performed in *B. rapa*.

Therefore, our objectives were to evaluate the impact of high temperatures in a *B. oleracea* core collection (115 local populations) during early development, to identify those *B. oleracea* groups with better performance under high temperatures and to select those populations more suitable to be used as base germplasm in future breeding programs for heat tolerance.

MATERIAL AND METHODS

Plant material and growth conditions

A collection of 115 populations (Table S1) of *B. oleracea* were evaluated in a growth chamber. Two criteria were used to select these populations. On one hand, 111 populations represent all the *B. oleracea* groups present in the ECPGR *Brassica* database (http://documents.plant.wur.nl/cgn/pgr/brasedb/) and, in the other hand, all the major areas of cultivation of *B. oleracea* in the world are represented in this core collection. Populations from five different continents were selected (Table S1). Four populations (MBG0062, MBG0072, MBG0464 and MBG0535) were obtained from the *Brassica* stock center of the Misión Biológica de Galicia (CSIC-Spain). Seeds were planted in multipot trays filled with sterilized peat (Gramoflor GmbH & Co. KG, Vechta, Germany) with one seed per cavity. Each experimental unit consisted on 12 plants representing one population. Experiment was arranged in a randomized complete block with three replicates. Seedlings were grown under fluorescent light (228 µmol m^{-2} s^{-1}) in a 14 h light/10 h dark light regime. A constant day/night temperature regime was set up at 30 °C. A control experiment was also performed with the 115 populations at 14 h light/10 h dark light regime and 20 °C, to have data for standard conditions.

Agronomic traits

Unless otherwise stated, data were recorded at the three leaves developmental stage. Seven traits related to agronomic performance and photosynthetic parameters were recorded: 1. Germination: Number of germinated plants five days after showing. 2.

Early vigor following a subjective scale (from 1: very poor to 5: excellent). 3. Fresh weight (g/plant): seedlings were harvested for each plot and immediately weighted. 4. Dry weight (g/plant): samples were dried in an oven (80 ºC) until constant weight. The total plot weight was normalized by the number of plants. 5. Percentage of dry weight, calculated as: [(dry weight / fresh weight)*100]. 6. Chlorophyll content index (CCI) recorded using a chlorophyll content meter (CCM-200, Opti-Sciences, Inc., USA). 7. Quantum yield of the photosystem II (ΦPSII) recorded on light adapted plants using a portable chlorophyll fluorometer (OS-30p, Opti-Sciences, Inc., USA).

Glucosinolates quantification

Total glucosinolates (GSL) were extracted and desulfated according to Kliebenstein et al. (*19*) with minor modifications. Chromatographic analyses were performed using an Atlantis® T3 C18 column (3 µm particle size, 2.1 x100 mm i.d.) protected with a C18 guard cartridge. The mobile phase was a mixture of (A) acetonitrile and (B) ultrapure water. The flow-rate was 0.8 mL min^{-1} in a linear gradient starting with 100 % B during 1.5 minutes, reaching 25% A after 11 minutes, 25 % A after 1.5 min followed by a linear gradient to reach 100 % B after one minute. Column was further stabilized for 3 minutes with 100% B. Compounds were detected at 229 nm in a Nexera LC-30AD UHPLC (Shimadzu) equipped with a Nexera SIL-30AC injector and SPD-M20A UV/VIS photodiode array detector. The oven temperature was set at 30 ºC. Glucosinolates were quantified at 229 nm using sinigrin (SIN, sinigrin monohydrate from Phytoplan, Diehm & Neuberger GmbH, Heidelberg, Germany) and glucobrassicin (GBS, glucobrassicin potassium salt monohydrate, from Phytoplan, Diehm & Neuberger GmbH, Heidelberg, Germany) as external standard and expressed in µmol g^{-}

133 [1] dry weight (DW). Regressions were made at least with five data points, from 0.34 to
134 1.7mM for sinigrin and from 0.28 to 1.4 mM for glucobrassicin. The average regression
135 equation for sinigrin, and glucobrassicin were y = 148818x (R^2 = 0.99), y = 263822 x
136 (R^2 = 0.99) respectively.
137
138 *Anthocyanin analyses*
139
140 Total anthocyanins were extracted from lyophilized leaf tissue in 3 mL of MeOH:HCl
141 0.1 N (95:5 v/v) at 4 ºC for 3 h. Thereafter, samples were centrifuged at 4000 g at 4 ºC
142 for 5 min. The supernatant was recovered and evaporated to dryness under gentle
143 nitrogen flow. The residue was resuspended in 500 µL of acid water (pH 1.4 with HCl).
144 Pigments were quantified from the supernatant spectrophotometrically using a Spectra
145 MR™ Microplate Spectrophotometer. Quantification was performed as described in
146 Sims and Gamon (*20*).
147
148 *Statistical analysis*
149
150 An analysis of variance was performed for each trait using the procedure GLM of SAS
151 (*21*) using populations, groups and replications as the classification variables.
152 Replications were considered as random effects and populations and groups as fixed.
153 Comparisons of means were made by using the Fishers' protected LSD at P=0.05. Since
154 no significant correlations were observed among traits, cluster analysis was performed
155 with all characters and computed using the DARwin 5.0.158 software (*22*). Similarity
156 and distance matrix were calculated by using the Manhattan´s distance with
157 standardized data. Groups were formed using the Ward's clustering method (*23*).

RESULTS AND DISCUSSION

Brassica oleracea is a highly diverse species which encompasses more than ten crops. This high diversity is reflected in the analysis of variance where significant differences were observed among 115 populations for all traits evaluated except for glucobrassicin, neoglucobrassicin and the total glucosinolate content (data not shown).

In general terms, seedlings grown at 30 °C showed reduced chlorophyll content, early vigor and biomass compared to values observed on seedlings grown at 20 °C (Table 1). Reduction on biomass content has been previously reported in other crops with increasing temperatures (*24*). This reduction has been extensively attributed to decreasing activity of the photosystem II (*25*). We did not observe a reduction on the quantum efficiency of the PSII (ΦPSII) in our genotypes, suggesting that at 30 °C *B. oleracea* genotypes are still in the optimal range for the photosynthesis-driven electronic transport. Diaz et al (*13*) reported that at 30 ºC, *B. oleracea* showed an inhibition of the PSII quantum yield and reduced capacity of photosynthetic electron transport. However, this later study was performed under high light intensity which has been previously reported to produce photoinhibition in *Brassica* crops (*26*). In contrast, our results agree with other studies previously reported in *Brassica* species. For example, Slauenwhite and Qaderi (*27*) show a reduction of the biomass content in a evaluation of four canola cultivars under heat conditions without further inhibition of the PSII activity. These authors postulated that this reduction of biomass could be due to the reallocation of photosynthates towards metabolic pathways that help plants to cope with temperature stress. One of these pathways leads to the biosynthesis of anthocyanins which enhance plant protection against high temperatures, probably through activation of antioxidative intrinsic mechanisms (*28*). In agreement with this,

183 we observed an increase of the anthocyanin content in leaves of plants grown at 30 °C
184 compared to that observed in plants at 20 °C.

185 Adaptation of *B. oleracea* cultivars to heat conditions has been studied specially
186 in broccoli (*B. oleracea italica* group). However, this group is moderately sensitive to
187 heat temperatures, and its optimal threshold of development ranged between 15 and 18
188 °C (*3*). Indeed, other *Brassica* groups have been used to introgress heat tolerance genes
189 into broccoli hybrids (*29*). In agreement with this, broccoli populations showed and
190 intermediate performance in our experiment at 30 °C, being the *alboglabra* group the
191 one that showed the best general performance at such temperature, since it was not
192 significantly different from the best group for any of the traits recorded (Table 2).

193 Glucosinolates are secondary metabolites of the *Brassicaceae* family that play a
194 role in the plant response to abiotic stresses (*30*). Concretely, glucosinolate content has
195 been associated to heat tolerance in *Arabidopsis thaliana* (*31*). Although, the levels of
196 glucosinolates are thought to increase with temperature in *Brassica* crops (*16, 18*), in
197 our experiment we observed a decrease in the total amount of glucosinolates along with
198 a decrease in the content of glucobrassicin, the most abundant glucosinolate, when
199 seedlings were cultivated at 30 ºC (Table 3). However, three glucosinolates
200 (glucoiberin, sinigrin and glucoraphanin), the main aliphatic glucosinolates reported in
201 *B. oleracea* crops showed an increase when seedlings were cultivated at 30 °C (Table
202 1).

203 Indolic glucosinolates were predominant, representing the 87.3 % of the total
204 glucosinolate content, followed by aliphatic (11 %) and aromatic (1.7 %) glucosinolates
205 (Figure 1). Indolic glucosinolates are especially abundant in the *sabauda* and *palmifolia*
206 groups, representing more than 90 % of the total content of glucosinolates. Previous
207 reports showed that aliphatic glucosinolates are predominant in *B. oleracea* varieties

208 evaluated in the field (*32, 33*). However, Velasco et al (*17*) demonstrated that the levels
209 of aliphatic glucosinolates increase with the plant' age, being the levels of indolic and
210 aliphatic glucosinolates equivalent in juvenile plants in the field. We performed our
211 analysis in an earlier plant developmental stage than any field experiment which could
212 explain the low levels of aliphatic glucosinolates observed and the predominance of
213 indolic glucosinolates.

214 No statistical differences were observed among the different varieties of *B.*
215 *oleracea* for the total content of glucosinolates (Table 3). Nevertheless, there were
216 significant differences for all individual glucosinolates except for the glucoiberin
217 content. Although there is not a clear pattern of glucosinolates accumulation, some
218 varieties outstand by the higher levels of sinigrin, glucoraphanin or glucobrassicin
219 which are the parent molecules of compounds with potential health promoting
220 properties (*34*). The *sabauda* group showed the highest levels of glucobrassicin which
221 represented more than 90 % of the total glucosinolates quantified in this group, whereas
222 three groups: *acephala medullosa*, *alboglabra* and *ramosa*, showed the highest levels of
223 sinigrin. The *gongylodes* group showed the highest levels of glucoraphanin even higher
224 than those observed in the *italica* group where this glucosinolate has been extensively
225 studied (*35*).

226 In the cluster analysis, the 115 *B. oleracea* populations were grouped in four
227 major clusters (Figure 2). There was not a clear pattern of distribution of the different
228 groups among the four clusters. The first criteria of separation was the biomass content
229 (fresh and dry weight). Clusters I and II included populations with low biomass which
230 are mainly from Europe. The cluster I is the largest, including 41 populations,
231 characterized by the low total glucosinolate content whereas the cluster II included 23
232 populations characterized by high levels of total glucosinolates, gluconasturtiin and

glucobrassicin. On the other hand, the clusters III and IV included varieties with higher biomass content. The cluster III included 38 populations, mainly from Europe, that showed high levels of total glucosinolates and glucoiberin content, whereas the cluster IV included 11 populations, mainly Asiatic, that showed higher levels of CCI.

Considering that *B. oleracea* is mainly used as vegetable, the most interesting populations are those with higher biomass production under heat stress, but also those with the higher content of health promoting compounds, such as glucosinolates (*36*). Populations included in the cluster III met both criteria. Among these populations, two cabbages and two kales showed the highest performance at 30 °C: MBG0072 (*B. oleracea capitata* group), MBG0464 (*B. oleracea acephala* group), and MBG0535 (*B. oleracea capitata* group) which are local landraces cultivated in the Northwest of Spain and HRIGRU5555 (*B. oleracea acephala* group) which was collected in the Netherlands (Table S1). Curiously, none of these populations were Chinese kales from the *alboglabra* group which, in general terms, showed the best performance at 30 °C. Besides the higher biomass and glucosinolate content, these varieties also outstand by the higher early vigor, CCI and percentage of dry weight. Concerning individual glucosinolates, these populations showed high glucoiberin and sinigrin contents.

In summary, *B. oleracea* shows a reduced biomass content during early development when cultivated at high temperature, probably due to the reallocation of photosynthates to metabolic pathway that protect the plant against high temperatures. We also observed a reduction on glucosinolate content in plants grown at 30 °C. Nevertheless, we identified five populations with higher performance at high temperatures that could be used as base germplasm in further studies.

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Table 1. Mean ± Standard Error of the 115 *Brassica oleracea* genotypes evaluated in a growth chamber at two different regimes of temperatures for agronomical, physiological and biochemical traits.

Trait	**Temperature**	
	20 ºC	**30 ºC**
ΦPSII	0.77 ± 0.001	0.77 ± 0.001
CCI	27.89 ± 0.74	24.83 ± 0.42
Germination (Nº)	9.46 ± 0.23	9.99 ± 0.11
Early vigor (1-5)	3.1 ± 0.07	2.9 ± 0.07
Fresh weight (g / plant)	1.9 ± 0.06	1.4 ± 0.04
Dry weight (g / plant)	0.30 ± 0.00	0.15 ± 0.00
Percentage of dry weight (%)	15.70 ± 0.23	11.42 ± 0.21
Anthocyanin content (µmol/g FW)	0.93 ± 0.14	1.19 ± 0.09
Total Glucosinolate content (µmol g^{-1} DW)	24.3 ± 1.40	19.7 ± 1.27
Glucoiberin	0.55 ± 0.07	1.00 ± 0.09
Progoitrin	0.16 ± 0.04	0.11 ± 0.02
Sinigrin	0.45 ± 0.05	0.65 ± 0.07
Glucoraphanin	0.31 ± 0.05	0.52 ± 0.07
Gluconasturtiin	0.75 ± 0.03	0.34 ± 0.02
Glucobrassicin	17.72 ± 1.21	12.28 ± 0.81
Neo-Glucobrassicin	4.34 ± 0.63	5.67 ± 0.79

Table 2. Comparison of means among the 12 *Brassica oleracea* groups evaluated in a growth chamber at 30 °C for agronomic, physiological and biochemical traits.

Groups	Number of populations	ΦPSII	CCI	Germination (Nº)	Early vigor (1-5)	Fresh weight (g / plant)	Dry weight (g / plant)	Percentage of dry weight (%)	Anthocyanin content (µmol/g FW)
acephala	14	0.769	23.1 cd	10.6 ac	3.3 a	1.5	0.16	11.9 bc	1.0
acephala medullosa	2	0.775	25.3 cd	10.2 ac	3.3 a	1.2	0.15	13.6 ab	0.8
acephala sabellica	2	0.765	15.1 e	11.0 ab	2.5 ab	1.5	0.16	11.6 bc	0.7
alboglabra	6	0.767	34.4 a	9.7 ac	2.8 ab	1.5	0.15	13.2 ab	0.4
botrytis	15	0.769	27.6 bc	9.4 c	2.4 ab	1.2	0.12	10.8 bc	1.2
capitata	35	0.768	23.9 cd	10.2 ac	3.0 a	1.5	0.15	10.7 bc	1.6
gemmifera	11	0.772	21.9 d	9.4 bc	2.7 ab	1.6	0.16	10.1 c	1.2
gongylodes	12	0.775	23.0 cd	10.3 ac	3.0 a	1.4	0.16	12.0 bc	0.8

italica	9	0.769	30.7 ab	9.4 c	3.1 a	1.3	0.16	12.9 bc	1.0
palmifolia	1	0.760	34.3 a	7.5 d	2.0 b	1.0	0.13	16.0 a	0.6
ramosa	5	0.771	23.9 cd	10.6 ac	3.2 a	1.3	0.15	12.7 bc	0.8
sabauda	3	0.779	15.7 e	11.3 a	2.8 ab	1.5	0.15	10.0 c	2.3
LSD		-	*4.8*	*1.6*	*0.9*	-	-	*3.0*	-

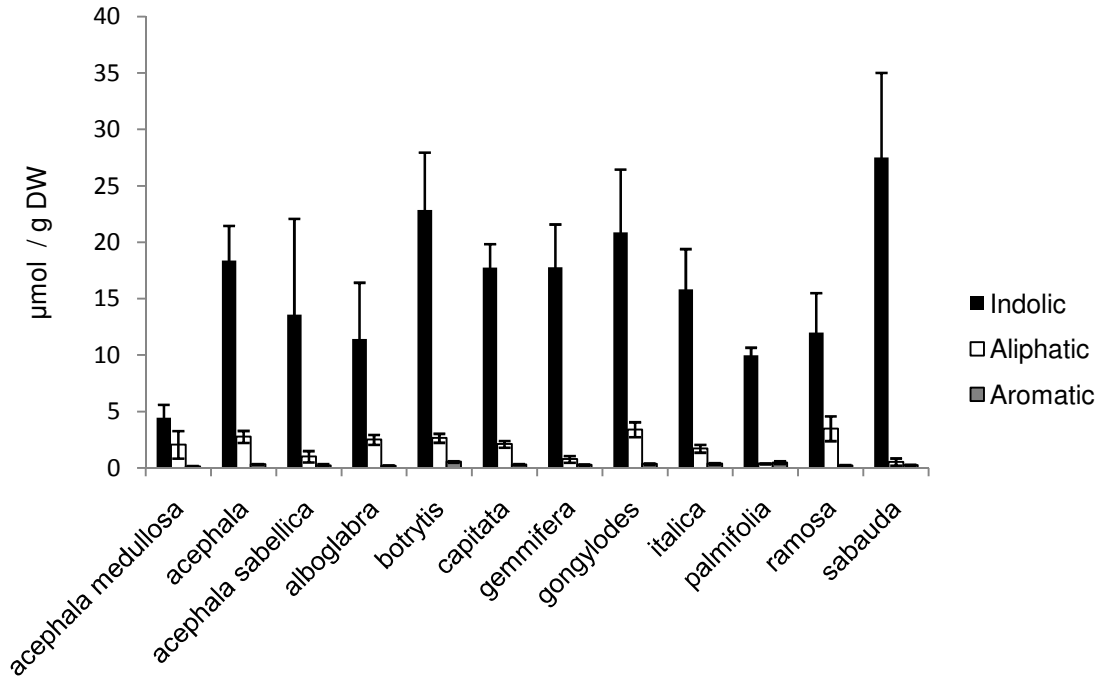

Figure 1. Quantification of aromatic, aliphatic and indolic glucosinolates in 12 groups of *Brassica oleracea* evaluated in growth chamber at 30 °C at seedling stage. Data are means of three biological replicates ± SE.

Table 3. Comparison of means among the 12 *Brassica oleracea* groups evaluated in a growth chamber at 30 °C for glucosinolates content. Glucosinolate content is expressed as μmol g^{-1} dry weight (DW).

Groups	Number of populations	Glucoiberin	Progoitrin	Sinigrin	Glucoraphanin	Gluconasturtiin	Glucobrassicin	Neo-Glucobrassicin	Total
acephala	14	1.34	0.14 b	0.86 ce	0.45 b	0.34 ac	14.57 b	3.81 ab	21.5
acephala medullosa	2	0.68	0.02 b	1.37 ac	0.00 b	0.15 c	3.77 cd	0.69 b	6.7
acephala sabellica	2	0.87	0.00 b	0.00 e	0.15 b	0.22 ac	8.88 bd	4.71 ab	14.8
alboglabra	6	0.33	0.08 b	1.65 ab	0.46 b	0.19 bc	3.04 d	8.40 ab	14.1
botrytis	15	1.58	0.00 b	0.99 bd	0.09 b	0.56 a	9.11 bd	13.75 a	26.1
capitata	35	1.08	0.09 b	0.39 de	0.54 b	0.31 ac	14.01 bc	3.74 ab	20.2
gemmifera	11	0.56	0.04 b	0.07 e	0.12 b	0.29 ac	16.07 ab	1.73 b	18.9
gongylodes	12	0.94	0.21 b	0.65 ce	1.62 a	0.36 ac	14.19 b	6.68 ab	24.6
italica	9	0.77	0.02 b	0.13 de	0.81 ab	0.39 ac	8.29 bd	7.53 ab	17.9
palmifolia	1	0.00	0.00 b	0.00 e	0.39 b	0.50 ab	8.52 bd	1.47 b	10.9
ramosa	5	0.55	0.70 a	1.95 a	0.31 b	0.23 ac	8.55 bd	3.46 ab	15.7

sabauda	3	0.50	0.00 b	0.05 e	0.00 b	0.25 ac	25.62 a	1.90 ab	28.3
LSD		-	*0.36*	*1.12*	*1.03*	*0.35*	*10.46*	*11.93*	-

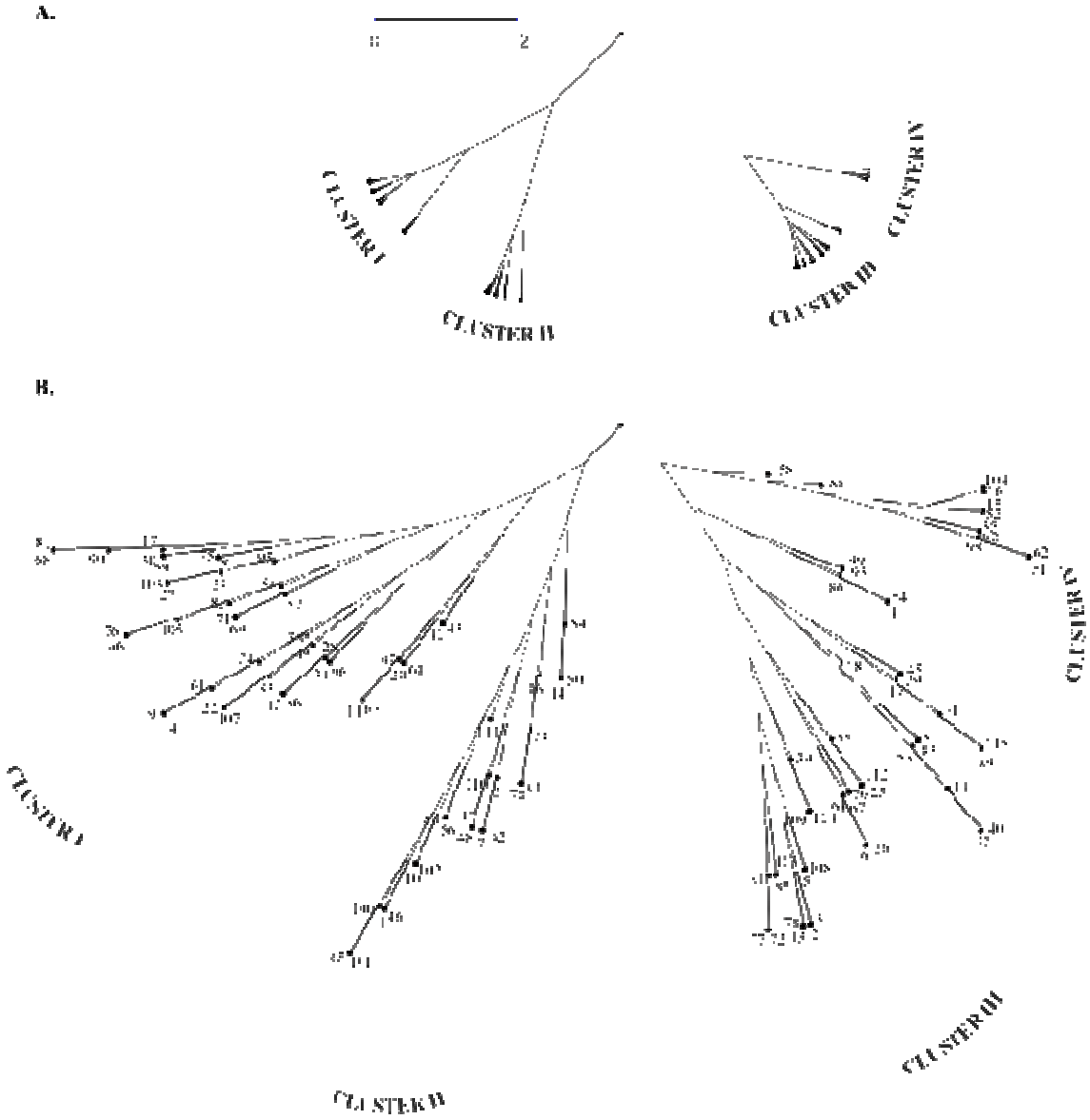

Figure 2. Dendrogram of 115 *B. oleracea* populations using morphological and biochemical data after evaluation in growth chamber at 30 °C. A. Dendrogram based on the Manhattan´s distance and the Ward's clustering method performed with the DARwin software. B. Detail of the former dendrogram setting the edge length to one to visualize all edges. Numbers correspond to *B. oleracea* populations (Table S1).