



Physio-morphological traits and drought stress responses in three wild Mediterranean taxa of Brassicaceae

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Abstract

Crop wild relatives (CWRs) have extremely relevant roles in biodiversity conservation, in investigating phylogeny and improving abiotic stress tolerance of crop plants. We screened the variability in leaf functional traits of three CWRs of kale crops (*Brassica oleracea*) from Sicily, Italy, grown in pots under well-watered and drought conditions. Our aim was to highlight traits in the different genotypes of endemic Sicilian threatened taxa. We measured several structural/anatomical traits (stomatal size, density and stomatal pore index—SPI, leaf mass per area—LMA) and leaf functional traits (stomatal conductance— g_s , leaf water potential— Ψ_L , leaf temperature (T_L), leaf relative water content—RWC) at pre-dawn and midday of leaves of three wild taxa: *B. macrocarpa*, *B. rupestris* subsp. *rupestris* and *B. villosa* subsp. *bivoniana*. Pressure–volume curves were constructed to obtain leaf water potential at turgor loss point (Ψ_{tlp}), osmotic potential at full rehydration ($\Psi_{\pi 100}$), relative water content at turgor loss point (RWC_{tlp}), elastic bulk modulus (ϵ_{max}) and leaf area specific capacitance at full turgor (C_{it}^*). Several significant differences were found among the taxa: under water deficit, *B. macrocarpa* had the less negative Ψ_{tlp} and showed the smallest $\Delta\Psi_L$ between pre-dawn and midday. *B. villosa* subsp. *bivoniana* showed the highest SPI and had significantly higher g_s under water availability, while under drought it had the most negative Ψ_L . Each of the taxa investigated possessed traits that confer particular stress tolerance, offer competitive advantage in their natural environment and may be exploited for crop improvement.

Keywords Crop wild relatives · Sicily · Leaf water potential · Stomatal conductance · LMA · SPI

Introduction

The genus *Brassica* L. (Brassicaceae) comprises a large number of extremely variable taxa, both from the morphological and genetic point of view, many of which are crops widely used for human nutrition as health-promoting vegetables, condiments and edible oils.

Selection of traits included in the so-called domestication syndrome (Hammer 1984) have often led to a decrease in

the fitness of a cultivated species in natural environments or under changing climatic conditions (Gepts 2004). Furthermore, following the ‘Green Revolution’ traditional cultivars and local varieties have been substituted with uniform, widespread cultivars, reducing the genetic diversity of modern crops and making them more susceptible to abiotic stresses, particularly drought, which affect plant growth and development causing large yield losses (Fita et al. 2015).

Crop wild relatives (CWRs) have been playing increasingly relevant roles for the study of plant genomes and the genetic improvement of the cultivated relatives. This aspect is present in the Convention on Biological Diversity (CBD) and is highlighted in the Global Strategy for Plant Conservation 2011–2020. Objective II, target nine, states that “70% of the genetic diversity of crops including their wild relatives and other socio-economically valuable plant species conserved [...]” (<https://www.cbd.int/gspc/objectives.shtml>). Research on CWRs has contributed to the investigation on both the evolutionary and phylogenetic relationships, and the inheritance of genes of several crop plants (Kole 2011).

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Several donor genes from CWRs have already been used to improve domesticated relatives, increasing the yields and nutritional quality of crops, providing cultivars with resistance against pests and diseases, and improving tolerance to abiotic stresses (Hopkins and Maxted 2010; Kole 2011).

Brassica species are moderately salt-sensitive (François 1994; Hayat et al. 2007) and this genus shows a certain variability with regard to drought and salt tolerance (Ashraf and Sharif 1997; Beltrao et al. 2000; Maggio et al. 2005). Wild species, particularly those living in harsh environments such as cliffs and coastal areas, are well adapted to tolerate abiotic stress and the genetic diversity of wild relatives may be an useful source of genes to improve drought and salt tolerance in cultivated *Brassica* (Siddiqui et al. 2008; Zhang et al. 2014). Sicily is one of the main centres of diversification of wild species belonging to the same cytodeme of *Brassica oleracea* L. (*Brassica* sect. *Brassica*, $2n=18$), that comprises several crop species, such as cabbage, cauliflower, broccoli, Brussels sprouts and kale. The Sicilian wild taxa can hybridize with cultivated forms so they represent a useful genetic resource for the improvement of cultivated varieties (Snogerup 1990; Von Bothmer et al. 1995; Geraci et al. 2001, 2004; Branca and Cartea 2011). Indeed, Sicilian *Brassica* wild species have been used for their resistance/tolerance to biotic and abiotic stress and to obtain new cultivars with high levels of glucosinolates (Faulkner et al. 1998; Mithen et al. 2003; Branca et al. 2012).

Identifying the CWRs' functional traits that confer tolerance to abiotic stress is an important step for the selection of genotypes that may be used for crop improvement. Plant functional traits (e.g. leaf stomatal conductance, leaf mass per area, leaf water potential) are related to plants' ecological strategies and determine how plants respond to environmental factors such as drought or salinity (Kattge et al. 2011; Pérez-Harguindeguy et al. 2013; Heschel et al. 2017). Assessment of fundamental plant functional traits is being increasingly used as a potential tool to detect and predict responses to drought, for example in the case of forest mortality (Greenwood et al. 2017; O'Brien et al. 2017). Leaf water relation traits are particularly important since water is the main factor limiting plant growth and crop productivity (Schulze et al. 1987; Zaghoud et al. 2013). Among these, turgor loss point, i.e. the leaf water potential when turgor reaches zero, is a key functional trait that quantifies plant drought tolerance and is associated with stomatal closure, wilting and loss of hydraulic conductance (Bartlett et al. 2014), which in turn influence plant productivity.

Functional traits have been genetically correlated at the intraspecific level in *Brassica rapa* L. providing combinations of traits useful for crop improvement (Edwards et al. 2011).

The ecophysiology and functional traits of most of the wild Sicilian taxa relatives of cultivated *Brassica* have not

yet been characterized, so the aim of this study was to screen the variability in leaf functional traits of three taxa, *B. macrocarpa* Guss., *B. rupestris* Rafin. subsp. *rupestris* and *B. villosa* Biv. subsp. *bivoniana* (Mazzola et Raimondo) Raimondo et Mazzola, grown under the same environmental conditions, comparing well-watered and drought treatments.

Materials and methods

Plant material

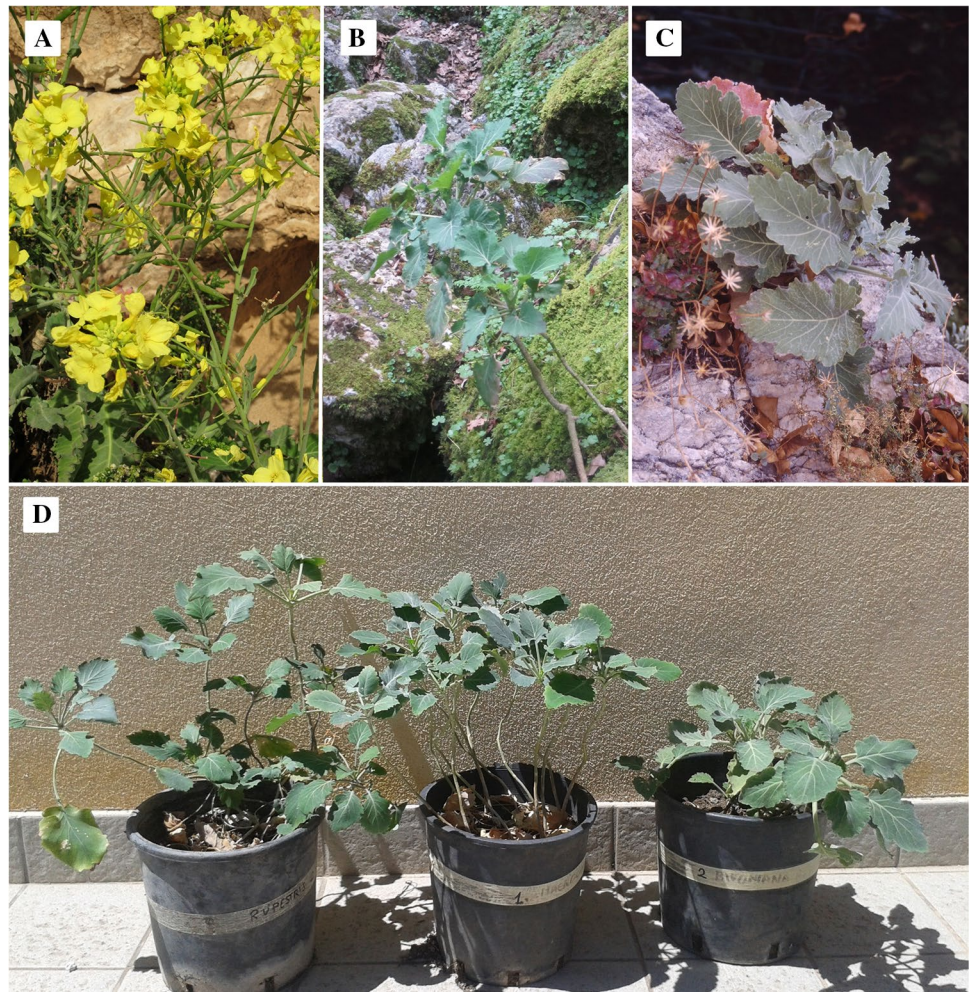
Three taxa with differences in leaf morphology, *Brassica macrocarpa*, *B. rupestris* subsp. *rupestris* and *B. villosa* subsp. *bivoniana*, were selected (Table A1). The habitats of all taxa occurring in Sicily and in the small surrounding islands consist of limestone cliffs, mostly facing north, from sea level to 1000–1200 m a.s.l.

Brassica macrocarpa is an endemic taxon restricted to two small islands, Favignana and Marettimo (Egadi archipelago), off the west coast of Sicily (Snogerup et al. 1990; Raimondo et al. 1991). The species is characterized by wide siliqua, with thick valves, stout and conical beak, yellow flowers, glabrous and petiolate leaves (Fig. 1a). It grows on limestone cliffs and rocky slopes from 0 to 300 m a.s.l., very close to the sea where it can be reached by seawater spray. This species is assessed as Critically Endangered because it has a highly fragmented habitat limited to two isolated subpopulations. The quality and the extension of the habitat is declining due to fires, grazing, reforestation and other anthropic activities (Geraci and Mazzola 2012; Branca and Tribulato 2013).

Brassica rupestris s.l. is endemic to Sicily and Calabria (Raimondo and Mazzola 1997). The plants have leaves with long petiole, hairs with bulbous base, light yellow flowers, long and thin siliqua (Fig. 1b). *Brassica rupestris* subsp. *rupestris* is the more widespread subspecies in north-west Sicily, except for one population found near Roccella Valdemone (eastern Sicily). Their populations are often threatened by human activities, grazing and fires, therefore, the taxon is included in the national and regional Red Lists as Low Risk (LR) and Near Threatened (NT) according to the IUCN categories (Conti et al. 1997; Scoppola and Spampinato 2005; Branca and Donnini 2013).

Brassica villosa comprises a group of intraspecific taxa endemic of central and west Sicily (Mazzola and Raimondo 1997; Raimondo and Geraci 2003) characterized by hairy leaves without wings at the base of the petiole, light yellow flowers (Fig. 1c). *Brassica villosa* subsp. *bivoniana* consists of several populations located in western Sicily. This taxon is also included in the national and regional Red Lists as LR or as NT in the IUCN list (Conti et al. 1997; Scoppola and Spampinato 2005; Branca and Donnini 2013).

Fig. 1 Wild specimens of: **a** *B. macrocarpa*, **b** *B. rupestris* subsp. *rupestris* and **c** *B. villosa* subsp. *bivoniana*. **d** Potted specimens of the three taxa used for the experimental treatment (*B. r.*, *B. m.*, *B. v.*, left to right)



Growth conditions

Seeds were collected from 3 to 5 wild specimens per taxon in June 2014. In October of the same year, seeds were sown in 5 L pots containing garden soil and grown in the open at the University of Palermo, Italy (Fig. 1d). Plants were watered to field capacity three times a week up to approximately 220 days from sowing, after which they were randomly divided in two treatment groups of nine plants per taxon. One group of plants continued to be irrigated regularly for measurements under watered conditions (*W*). To impose drought conditions (*D*), in the second group of plants irrigation was suspended for 5 days, after which measurements were taken on fully expanded, healthy leaves. Soil water potential was estimated from the pre-dawn leaf water potential measured as described below. All measurements were taken between the end of May and June 2015, on clear sunny days, approximately 230 days after sowing. Measurements were replicated in 3 days, on three plants per day. Average air temperature

and relative humidity on the days measurements were taken were 28.5 ± 0.4 °C and $68.3 \pm 8.4\%$, respectively.

Leaf surface anatomy

Stomatal size and density were measured from scanning electron microscope (SEM) micrographs, on ten replicates per taxon, collecting fully expanded, healthy leaves. Air-dried small portions of the leaves were placed on double-sided transparent tape on the surface of a polished aluminum stub. The samples were sputter coated with a 225 Å thick gold layer, and examined with a LEO 420 SEM Microscope (LEO Electron Microscopy Ltd.) using an acceleration voltage of 10–15 kV, at magnifications between 400 and 5500 \times .

Stomatal pore index (SPI) was calculated as the product of the stomatal density by the square of the mean stomatal pore length (Sack et al. 2003).

Functional trait measurements

Approximately 220 days from sowing, before imposing drought stress treatment, five fully expanded leaves per taxon were collected from three different plants grown under well-watered conditions and used to construct pressure–volume (PV) curves by the bench dehydration method (Tyree and Hammel 1972; Savi et al. 2017). From the analysis of the PV curves, we obtained the main water relation traits related to drought tolerance: leaf water potential at turgor loss point (Ψ_{tlp}), osmotic potential at full rehydration ($\Psi_{\pi 100}$), relative water content at turgor loss point (RWC_{tlp}), bulk modulus of elasticity (ϵ_{max}) and leaf area-specific capacitance at full turgor (C_{it}^*).

Daily changes in leaf water potential (Ψ_L) were measured with a pressure chamber (PMS 1505D, PMS Instrument Company, Albany, OR, USA) on a total of four fully expanded leaves collected from two plants per taxon per measurement time. The evening before measurements, plants were prepared for “pre-dawn” leaf water potential ($PD\Psi_L$) measurements, when values are assumed to be in equilibrium with soil water potential (Sellin 1999; Kangur et al. 2017). Selected leaves were wrapped in plastic film and aluminum foil and bagged plants were kept in the dark until measurement at 07:30 h, to avoid pre-dawn disequilibrium due to night time transpiration (Oddo et al. 2011). Preliminary measurements showed that Ψ_L did not change significantly in the time interval between “pre-dawn” and 08:00 h. After cutting off the leaves for estimation of $PD\Psi_L$, plants were placed in the open and allowed to reach steady state (30 min) before measuring, at 08:00 h, abaxial stomatal conductance to water vapour (g_s) and leaf temperature (T_L) with a porometer (SC-1 Leaf Porometer, Decagon, Pullman, WA) on five fully expanded leaves per plant. At 12:00 h g_s , T_L and Ψ_L measurements were repeated. Midday (MD) Ψ_L values of W and D plants were used to calculate $\Delta\Psi_{MD}$ and describe drought strategy according to Farrell et al. (2017). Immediately after measuring Ψ_L , two leaf discs per leaf were sampled with a cork borer (0.7 cm^2) and used to calculate relative water content (RWC). The leaf disc fresh weight (FW) was recorded with an analytical balance (AL

104, Mettler Toledo, Greifensee, Switzerland); leaf discs were floated on distilled water in plastic tubes for 20 h, after which turgid weight was recorded (TW). Dry weight (DW) was recorded after drying the samples at $70\text{ }^\circ\text{C}$ for 48 h. RWC was then calculated as:

$$RWC (\%) = 100 \times (FW - DW)/(TW - DW)$$

Leaf mass per unit area (LMA) was calculated for W plants at three different intervals during the experiment: 190, 210 and 230 days from sowing. On the first two sampling dates, leaf discs were cut out from fully expanded leaves with a cork borer, oven dried at $70\text{ }^\circ\text{C}$ for 48 h and DW was determined by dividing leaf DW over leaf disc area. For the 230 days data, LMA was calculated from the same leaf discs used for RWC determination.

Data analysis

Data are presented as mean values \pm standard deviation. After testing for equal variance and for normality (Shapiro–Wilk), one-way ANOVA was applied to test for significant differences among the means for stomatal traits and for PV parameters. Two-way ANOVA was applied to test the effects of factors species (S) and days from sowing (D) on LMA data. Differences in functional trait means (g_s , Ψ_L , RWC, T_{leaf}) were tested using a three-way ANOVA, with species (S), irrigation regime (I), time of day (T) and their interactions as fixed effects. Tukey’s post hoc test ($\alpha=0.05$) was used to compare means when ANOVA was significant. All analyses were carried out using the software package SigmaPlot 13 (Systat Software, Inc., San Jose, USA).

Results

Stomatal characteristics and SPI

All species showed stomata both on adaxial and abaxial sides, so the leaves may be considered amphistomatous. The values of stomatal density, length and SPI differed among the taxa (Table 1). *Brassica macrocarpa* and *B. rupestris*

Table 1 Stomatal characteristics of adaxial and abaxial leaf sides

Taxa	Stomatal density (mm^{-2})		Stomatal length (μm)	SPI ($\times 10^{-2}$)
	Adaxial (mean \pm SD)	Abaxial (mean \pm SD)	Adax + abax (mean \pm SD)	Total adax + abax (mean \pm SD)
<i>B. macrocarpa</i>	298.5 \pm 15.1a	304.4 \pm 16.3a	8.20 \pm 0.2a	4.05 \pm 0.21a
<i>B. rupestris</i> subsp. <i>rupestris</i>	295.5 \pm 10.9a	307.0 \pm 18.3a	8.69 \pm 0.7a	4.55 \pm 0.22b
<i>B. villosa</i> subsp. <i>bivoniana</i>	297.5 \pm 9.4a	602.0 \pm 12.9b	7.43 \pm 0.5b	4.97 \pm 0.12c

Different letters within the same column indicate significant differences (Tukey’s post hoc test, $P < 0.05$)

showed about the same stomatal density on both adaxial and abaxial sides, similar to that of the adaxial side of the leaves

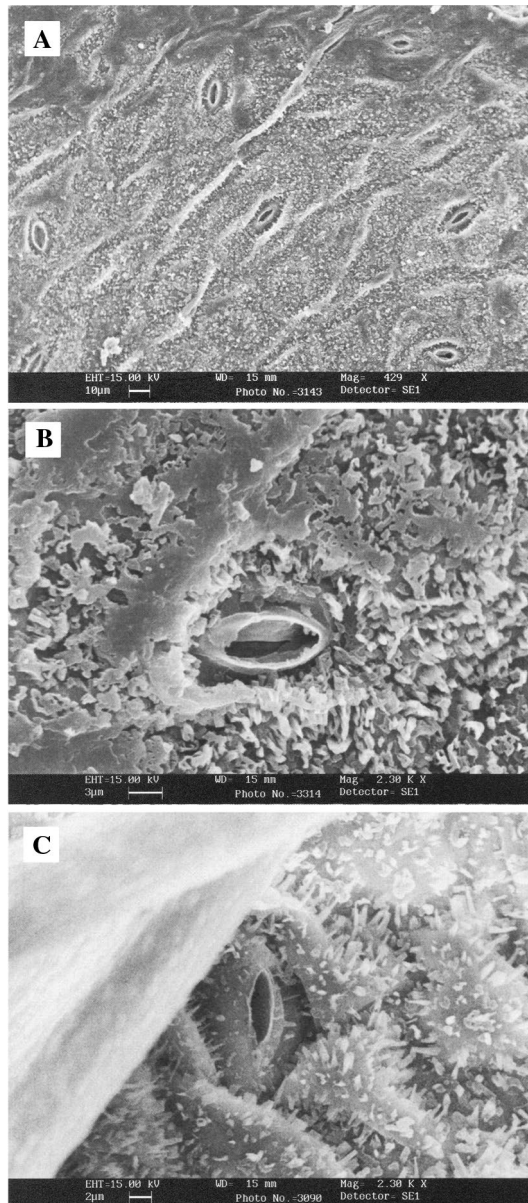


Fig. 2 Scanning electron micrographs of the adaxial leaf surface showing stomata of: **a** *B. macrocarpa*, **b** *B. villosa* subsp. *bivoniana* and **c** *B. rupestris* subsp. *rupestris*

of *B. villosa* subsp. *bivoniana*. On the abaxial side of the leaf of *B. villosa* subsp. *bivoniana*, instead, stomatal density was about twice that of the adaxial surface, significantly higher than in the other two taxa. Stomatal pore length was never significantly different between adaxial and abaxial sides, while differences between taxa were significant only for *B. villosa* subsp. *bivoniana*; the largest stomata were found in *B. rupestris*, followed by *B. macrocarpa*, while the smallest stomata were found in *B. villosa* subsp. *bivoniana* (Fig. 2).

Differences in SPI among the taxa were always significant. SPI was lowest in *B. macrocarpa*, while *B. rupestris* showed higher values than *B. macrocarpa* due to slightly longer stomata. *B. villosa* subsp. *bivoniana* had the highest total SPI value of the three taxa, due to the higher stomatal density of the abaxial side that compensated for the smaller size of stomata.

Functional traits

Data obtained from the analysis of PV curves (Table 2) showed that *B. rupestris* and *B. villosa* subsp. *bivoniana* had significantly more negative Ψ_{tlp} values than *B. macrocarpa*, with correspondingly more negative $\Psi_{\pi 100}$ values. Average C_{ft}^* was larger for *B. macrocarpa* than for the other two taxa, though this difference was not significant. The differences found in RWC_{tlp} and ϵ_{max} , were also non-significant among species.

Irrigation regime, time of day and species all strongly affected leaf gas exchange (Fig. 3, Table 3). Under well-watered conditions, at 08:00 h g_s was similar in *B. macrocarpa* and *B. rupestris*, while it was significantly higher in *B. villosa* subsp. *bivoniana*. At 12:00 h, the latter taxon reached the highest levels of g_s (about $1000 \text{ mmol m}^{-2} \text{ s}^{-1}$) that were about twice those reached at the same time by the other two taxa. Five days after suspending irrigation, g_s dropped on average to $45 \text{ mmol m}^{-2} \text{ s}^{-1}$ in all three taxa, both in the early morning and at midday.

The interaction between time of day, irrigation regime and species on Ψ_L was significant (Table 3). Under well-watered conditions, Ψ_L at pre-dawn did not differ among taxa (Fig. 3); MD Ψ_L decreased significantly in all taxa, reaching about -1.5 MPa in *B. macrocarpa* and *B. villosa* subsp. *bivoniana*. In *B. macrocarpa*, this value was definitely below turgor loss point, while in *B. villosa* subsp.

Table 2 Functional traits of the investigated taxa derived from pressure–volume curve analysis

Species	Ψ_{tlp} (MPa)	$\Psi_{\pi 100}$ (MPa)	RWC_{tlp} (%)	ϵ_{max} (MPa)	C_{ft}^* ($\text{mol m}^{-2} \text{ MPa}^{-1}$)
<i>B. macrocarpa</i>	$-0.979 \pm 0.142\text{a}$	$-0.721 \pm 0.217\text{a}$	$92.06 \pm 3.81\text{a}$	$11.304 \pm 6.359\text{a}$	$1.324 \pm 0.53\text{a}$
<i>B. rupestris</i> ssp. <i>rupestris</i>	$-1.531 \pm 0.296\text{b}$	$-1.220 \pm 0.305\text{b}$	$91.44 \pm 2.18\text{a}$	$15.334 \pm 3.336\text{a}$	$0.869 \pm 0.23\text{a}$
<i>B. villosa</i> ssp. <i>bivoniana</i>	$-1.584 \pm 0.210\text{b}$	$-1.270 \pm 0.196\text{b}$	$87.68 \pm 2.14\text{a}$	$12.061 \pm 2.755\text{a}$	$0.989 \pm 0.33\text{a}$

Different letters within the same column indicate significant differences (Tukey's post hoc test, $P < 0.001$)

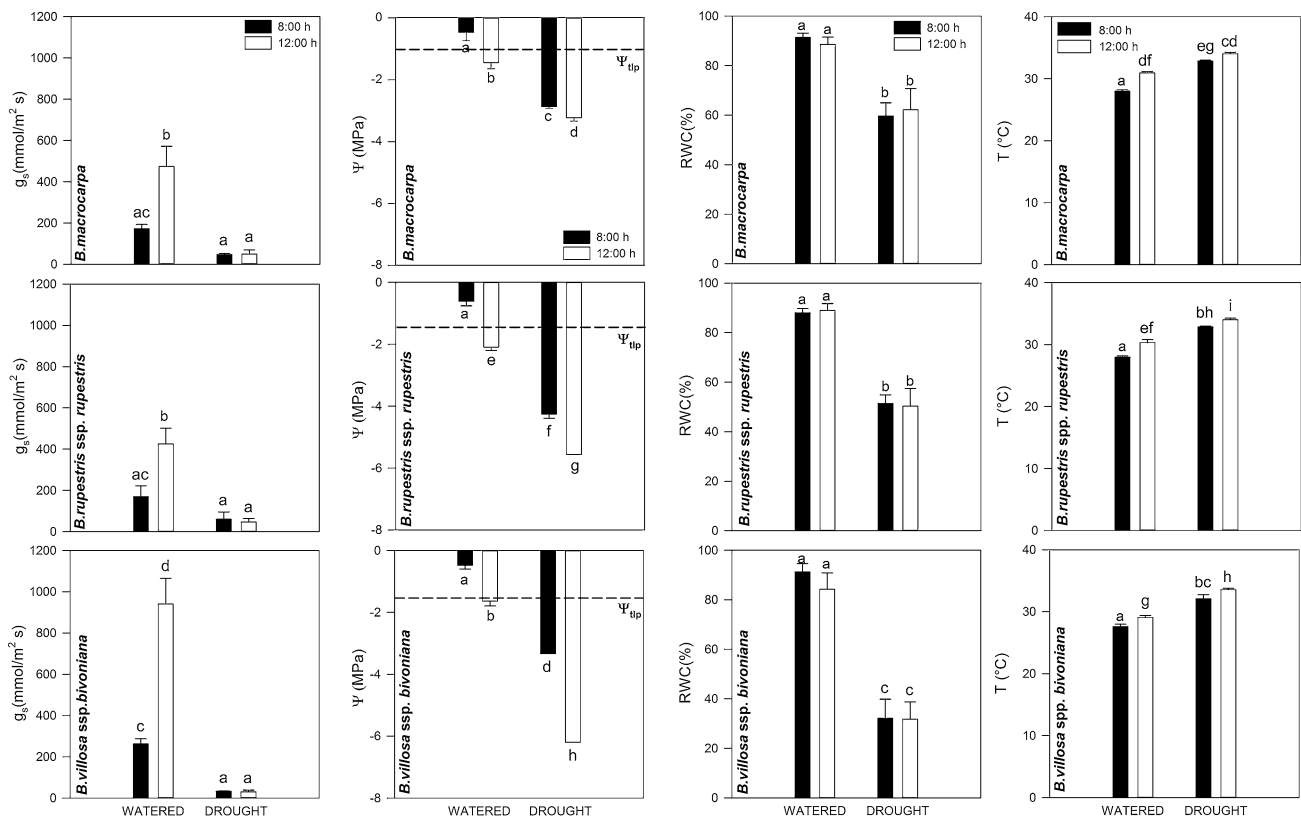


Fig. 3 Stomatal conductance (g_s), water potential (Ψ), relative water content (RWC) and temperature (T) of leaves of *B. macrocarpa*, *B. rupestris* subsp. *rupestris* and *B. villosa* subsp. *bioniana*, measured at 8:00 h and 12:00 h under watered or drought conditions. Accord-

ing to a three-way ANOVA with time of day, irrigation regime and species as factors, different letters indicate significant differences among bars within each vertical set of graphs (Tukey's post hoc test, $P < 0.05$)

Table 3 Results of two- or three-way ANOVA on plant functional traits (stomatal conductance, leaf water potential, relative water content and leaf mass per area) depending on interacting factors time of day (T), irrigation regime (I), species (S) and days from sowing (D)

Variable	g_s	Ψ_L	RWC	T_{leaf}	LMA
T	***	***	NS	***	
I	***	***	***	***	
S	***	***	***	***	NS
D					***
T×I	***	***	NS	NS	
T×S	**	***	NS	NS	
I×S	***	***	***	***	
D×S					**
T×I×S	**	***	NS	NS	

NS non-significant

*, ** and *** significant at $P < 0.05$, 0.01 and 0.001, respectively

bioniana Ψ_{tp} was barely reached. The drop in MD Ψ_L was greater for *B. rupestris*, and also in this case the turgor loss point threshold was passed. Under drought stress, none of the taxa managed to recover from water stress during

the night, and all values measured were far below turgor loss point. *Brassica macrocarpa* showed the less negative values, at both pre-dawn and midday, and the smallest difference between pre-dawn and midday values (12% reduction). *Brassica rupestris* showed the most negative PD Ψ_L value, which decreased a further 30% at midday. *Brassica villosa* subsp. *bioniana* reached extremely negative Ψ_L values at midday, -6.2 MPa. The $\Delta\Psi_{MD}$ values calculated as the difference between MD Ψ_L under drought and under well-watered conditions were -1.8 , -3.5 and -4.5 MPa for *B. macrocarpa*, *B. rupestris* and *B. villosa* subsp. *Bioniana*, respectively.

Under well-watered conditions, leaf relative water content was on average 90% at pre-dawn for all taxa. This value was maintained at midday for *B. macrocarpa* and *B. rupestris*, while it decreased slightly but not significantly in *B. villosa* subsp. *bioniana* (Fig. 3). Drought stress resulted in a significant decrease in RWC, down to 60% in *B. macrocarpa*, 50% in *B. rupestris* and 30% in *B. villosa* subsp. *bioniana*. No significant differences were found between pre-dawn and midday samples, and no significant interaction between time of day and irrigation regime or species (Table 3).

Watered plants at 08:00 h showed the lowest leaf temperatures, 27.8 °C on average with no significant differences at species level (Fig. 3). Watered plants of *B. villosa* subsp. *bivoniana* maintained significantly cooler leaves at midday (29.1 °C). Under drought stress, leaf temperatures increased significantly at 08:00 h in all taxa. Leaves of *B. macrocarpa* remained significantly cooler than those of the other two taxa (29.9 °C). At midday leaf temperatures increased in all plants, showing significant differences among all taxa, with *B. macrocarpa* reaching the lowest temperature (31.7 °C) compared to *B. villosa* subsp. *bivoniana* and *B. rupestris*, which reached the highest leaf temperature (34 °C). A significant interaction was found between irrigation regime and species (Table 3).

Leaf mass per area increased during the experiment (Fig. 4). Over a 6-week period, all three taxa reached a similar LMA value, 90–95 g m⁻², but with a different pattern. *Brassica macrocarpa* and *B. rupestris* showed an initial rapid increase in LMA, from 190 to 210 days from sowing, after which there was no significant increase. *Brassica villosa* subsp. *bivoniana* showed constant LMA during the same period, and a rapid increase in this trait between 210 and 230 days from sowing, when drought stress was imposed and measurements were taken. There was a significant interaction between days from sowing and species (Table 3).

Visual assessment of plant conditions after the 5-day drought period showed in plants of *B. macrocarpa* and *B. rupestris* moderate wilting of the leaves, and yellowing of 2–3 leaves per plant. In *B. villosa* subsp. *bivoniana*, leaves were very strongly wilted, but did not show yellowing. On rewatering, leaves of *B. villosa* subsp. *bivoniana* recovered

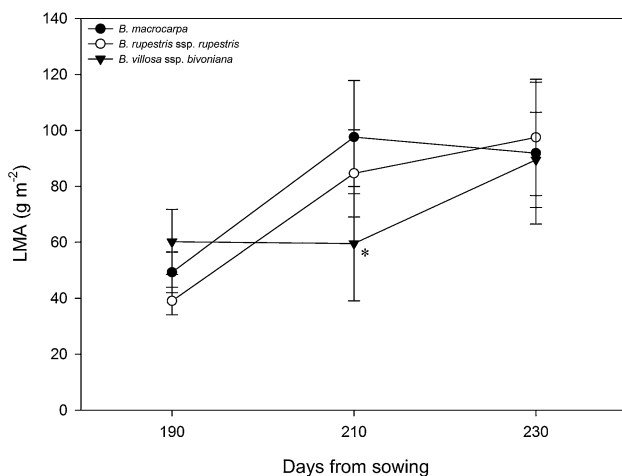


Fig. 4 Variation in leaf mass per area (LMA) during the study period in *B. macrocarpa*, *B. rupestris* subsp. *rupestris* and *B. villosa* subsp. *bivoniana*, measured under watered conditions. Asterisks indicate significant differences within sampling date (two-way ANOVA, Tukey's post hoc test, $P < 0.05$)

turgor rapidly, within 3–4 h, while the other two species recovered turgor more gradually, within 12–18 h.

Discussion

The wild *Brassica* relatives occurring in Sicily generally grow on cliffs with little soil, a very arid habitat. For this reason, they are usually found on north-facing limestone cliffs, where exposure to direct sunlight is less and the evapotranspirational demand is lower. All taxa also rely on their root architecture and growth, with roots penetrating and extending deeply into rock cracks and crevices, and indeed rooting depth plays an important role in controlling the progression of drought responses (Nardini et al. 2016). Here, we present data on the morpho-physiological traits of the three taxa investigated in this study, under experimental conditions in which cultivation in pots limited the possibility to form an extensive root system. To the best of our knowledge these are the first reports for *B. villosa* subsp. *bivoniana* and *B. macrocarpa*, providing information that could be of great interest to aid in the selection of genotypes that may be used for crop improvement.

One of the adaptations crucial to drought resistance is the reduction of water loss through stomata. The ability to control stomatal conductance depends on stomatal density, stomatal size and the regulation of stomatal aperture (Pérez-López et al. 2012; Zaghoud et al. 2013), and stomatal traits can show substantial variability at the interspecific level, as reported for example for poplar (Pearce et al. 2006; Cirelli et al. 2016). The inverse relation commonly found between stomatal density and guard cell length (Wood 1934; Sack et al. 2003) was evident only for *B. villosa* subsp. *bivoniana*. The positive relation between SPI and guard cell length reported by several authors (e.g. Sack et al. 2003; Abrams and Kubiske 1990) was maintained for *B. macrocarpa* and *B. rupestris*. However, this relation was no longer valid for *B. villosa* subsp. *bivoniana*, where the significant increase in SPI was driven by stomatal density, which on the abaxial surface was twice that of the other taxa. The high SPI in *B. villosa* subsp. *bivoniana* was likely the factor driving its high g_s under well-watered conditions, as SPI has been shown to correlate tightly both with maximum stomatal conductance and leaf hydraulics in several temperate woody species (Sack et al. 2003, 2005). *Brassica villosa* subsp. *bivoniana* showed also the most rapid recovery of leaf turgor after rewatering, which could be related to a more efficient water transport due to a higher leaf lamina hydraulic conductance (Sack et al. 2003) and hypothetically a rapid recovery of leaf vein embolism (Trifilò et al. 2003).

Maintaining lower leaf temperature may positively affect carbon assimilation and decrease heat stress effects on cell metabolism (Yamori et al. 2014; Feller 2016).

Transpirational canopy cooling has been previously suggested as a tool to screen *Brassica* genotypes for drought tolerance (Singh et al. 1985). When water was available, *B. villosa* subsp. *bivoniana* maintained the lowest leaf temperatures, due both to high g_s allowing high evapotranspiration and to the presence of hairs that increased reflectance of the leaf surface (Holmes and Keiller 2002). The tomentose leaf surface could also be a main factor contributing to lowering cuticular transpiration when water supply was low. Under drought stress, instead, *B. macrocarpa* maintained the lowest leaf temperatures, possibly explained by a combination of leaf reflection due to higher presence of waxes on the epidermis that reduces also cuticular water loss (Holmes and Keiller 2002; Laila et al. 2017) and the higher water content, confirmed by higher RWC and less negative Ψ_L .

Several functional traits describing plant water relations have been proposed as predictors of drought tolerance, such as osmotic adjustment, osmotic potential at full turgor, MD Ψ_L , $\Delta\Psi_{MD}$, Ψ_{tip} and leaf RWC. Osmotic potential at full turgor can be a powerful indicator of drought tolerance, as it is the main driver of Ψ_{tip} (Bartlett et al. 2012; Mart et al. 2016) and more negative values generally indicate greater drought resistance. There is consistent evidence that solute accumulation maintains crop yield under water deficit conditions (Blum 2017) also in several genotypes of *Brassica napus* L. and *B. juncea* L. Czern. (Niknam et al. 2003; Ma and Turner 2006; Ma et al. 2006; Norouzi et al. 2008). Osmotic potential at full turgor was most negative in *B. villosa* subsp. *bivoniana*, significantly more negative than in *B. macrocarpa*. *Brassica villosa* subsp. *bivoniana* also showed the greatest overnight recovery in Ψ_L during drought, compared to the other two taxa, with a $\Delta\Psi_L$ of 2.9 MPa, while *B. rupestris* had a $\Delta\Psi_L$ of 1.3 MPa and *B. macrocarpa* of only 0.3 MPa. During the night, plants generally tend to equilibrate leaf water status with water available in the soil, and PD Ψ_L has been widely used as a proxy for soil water potential (e.g. Palmer et al. 2008; Petruzzellis et al. 2018). Though pre-dawn disequilibrium has sometimes been found under certain conditions (e.g. Donovan et al. 2001) this has most commonly occurred in field measurements, mainly due to night time transpiration and high levels of vapour pressure deficit (Kangur et al. 2017), which were avoided in our controlled experimental conditions.

During the day, leaf water potential declines below soil water potential, reaching minimum values around midday that can provide an index of drought tolerance for the species (Pérez-Harguindeguy et al. 2013). The hypothesis that the steeper midday decrease in Ψ_L measured under well-watered conditions in *B. rupestris* could be related to more rigid cell walls (highest average ϵ_{max} values), was not supported by statistical analysis. High C_{ft}^* can contribute to reduce the fluctuations in leaf water potential under changing water supply and transpirational demand (Sack

et al. 2003), however, differences among the taxa were not significant, so could not justify the less negative Ψ_L values shown by *B. macrocarpa* both under well-watered and drought conditions.

One of the traits allowing the maintenance of cell turgor under water deficit is a low Ψ_{tip} . Since Ψ_{tip} integrates osmotic adjustment, ϵ and other individual water relations traits, it is one of the fundamental traits to select for in breeding programs aimed at improving drought tolerance, even though the determination of Ψ_{tip} by PV curves is time consuming (Mart et al. 2016). It must, however, be kept in mind that the use of Ψ_{tip} alone to predict drought resistance could be misleading if whole-plant drought strategies are not considered (Farrell et al. 2017). *Brassica rupestris* and *B. villosa* subsp. *bivoniana* showed significantly lower Ψ_{tip} than *B. macrocarpa*. Considering $\Delta\Psi_{MD}$ as a descriptor of drought strategy in terms of the degree of isohydry and anisohydry (Farrell et al. 2017), *B. macrocarpa* showed a greater degree of isohydry than the other two genotypes.

Leaf relative water content is another trait that describes plant water status and is well correlated with physiological responses to water supply (Slatyer 1962; Munné-Bosch and Peñuelas 2004). Taking the appropriate precautions in leaf sampling and measurements (Tanentzap et al. 2015), it is a helpful and often used tool for screening for drought tolerance in different genotypes (Rachmilevitch et al. 2006). Differences in RWC among the genotypes were not significant under well-watered conditions, but under drought stress *B. macrocarpa* had significantly higher RWC, even if midday g_s was extremely low and similar to that of *B. rupestris*.

Leaf mass per area—or its reciprocal, specific leaf area (SLA)—is still another of the key traits related to plant function and performance, and is implicated in responses to several environmental factors, most importantly light availability and drought (Valladares and Sanchez-Gomez 2006; Poorter et al. 2009). A lower LMA is often related to greater leaf water loss, while higher LMA is associated with adaptation and resistance to water stress (Poorter et al. 2009; Bussotti et al. 2015). The values of LMA of the cabbage wild relatives we examined were in the same range as that reported in another study on two subspecies of *B. rupestris* (Crescente and Gratani 2013), and for *B. oleracea*, 80 g/m² (Sage et al. 1989), while they were higher than those reported for *B. napus*, 30–50 g/m² (Shengxin et al. 2016). LMA may vary with environmental conditions and with plant age, reflecting variations in plant plasticity related to genotype (Jullien et al. 2009). The change in LMA with time for our taxa was similar to that reported by Biemond et al. (1995) for leaves of Brussels sprouts, which increased up to 90 days after planting and then remained stable for the rest of the experiment, and for leaves of turnip, and green cabbage (Li et al. 1999). The different LMA profile with time shown in *B. villosa* subsp. *bivoniana* could be related to

source/sink relationships within the plant, as suggested by Jullien et al. (2009) for oilseed rape.

Though it is time consuming to obtain some of these functional traits, combining information from more than one trait results in the most complete picture of the stress tolerance of a particular genotype, which is necessary when screening CWRs for breeding purposes.

Among allotetraploid hybrids of *Brassica U-Triangle* species, Baker et al. (2017) found significant effects of species for morphological, anatomical and physiological traits, though there was no significant correlations between these traits in the diploid parents. This evidence shows that novel trait correlations could arise in the polyploid hybrids. The breeding of the ancestors examined in this study with cultivated varieties of the same cytodeme could give rise to hybrids with greater resistance to drought or disease, higher productivity under stress, and these CWRs are, therefore, of great interest for artificial selection and crop improvement.

Conclusions

CWRs offer the opportunity of finding new sources of variation to face changing environmental conditions (Esquinas-Alcázar 2005). Each of the taxa examined possessed traits conferring particular stress tolerance. *Brassica rupestris* subsp. *rupestris* and *B. villosa* subsp. *bivoniana* suffered the most under the applied drought stress, however, all three species were able to recover. Their particular functional traits could be, therefore, exploited for crop improvement.

Brassica macrocarpa, which grows on cliffs overhanging the sea exposed to high salinity due to sea spray, among the taxa examined was the one subject to the harshest conditions in its natural habitat. Its functional traits, such as thick, leathery leaves with a thick wax layer, a high leaf succulence index (data not shown) and greater degree of isohydry under drought stress—compared to the other two taxa—are all traits that favour adaptation to drought and/or salt stress. For several of the functional traits (Ψ_{tp} , $\Delta\Psi_{\text{MD}}$, $\Psi_{\pi 100}$), *B. macrocarpa* was significantly different from the other two genotypes.

Brassica rupestris had the greatest capacity of lowering Ψ_{L} , and consequently the highest water extraction capacity when water was available. The slight opening of stomata in the early morning under drought, compared to the other two taxa, could avoid carbon starving under stress conditions. Crescente and Gratani (2013) comparing some functional traits of *B. rupestris* subsp. *rupestris* with those of *B. rupestris* subsp. *hispidula* found that the former was more competitive, as it was able to maintain higher photosynthetic rate, stomatal conductance and water use efficiency under water stress. Indeed, *B. rupestris* is the most widely distributed of

these taxa as for population numbers, arriving to Calabria, on the Italian mainland.

Brassica villosa subsp. *bivoniana* had very high g_s under water availability, maintained the lowest leaf temperatures and barely reached turgor loss point, all traits that correlate positively with net photosynthesis and, therefore, result in high productivity, a highly desirable trait for crop improvement.

Author contribution statement AG and EO conceived and designed the experiments. SI, AG and EO performed the experiments and analyzed the data. AG and EO wrote the manuscript.

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