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ORIGINAL ARTICLE

Assessment of a non-destructive method to estimate the leaf area of *Armoracia rusticana*

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Abstract
The aim of the study was to analyze horseradish growth for developing a mathematical model to estimate the leaf area based on linear measurements of the leaf surface. Leaf area (LA), number, and morphometric characteristics of the leaves including lamina length (L) and width (W) were evaluated on two horseradish accessions (Cor and Mon) throughout a 2 year growing cycle. In both accessions, increased values of LA and leaf number were found by comparing the second with the first-growing season. Leaf development occurs along with variations in size and not in shape during the plant growth. The leaves are elliptical in shape but tend to be wider and bigger in Cor accession and tapered and similar to narrow ellipses in Mon showing different length/width relationship. Consequently, several regression models relating to the LA and L, W, L^2, and W^2 individually or in combination were fitted for each accession based on a set of 1000 leaves. The horseradish LA can be predicted based on either length or width alone. However, the regression linear model $\mathrm{LA}=a\mathrm{LW}+b$ ($\mathrm{LA}=0.71\mathrm{LW}-0.27$ and $\mathrm{LA}=0.76\mathrm{LW}-3.22$ for Cor and Mon, respectively) provided the best LA estimation ($R^2>0.95$). The validation of this latter model showed high correlation between LA measured and LA predicted in both accessions ($R^2=0.98$). Considering the type of foliage of horseradish, the proposed model can be used to estimate the leaf area throughout the entire crop cycle.

Keywords *Brassicaceae* · Horseradish · Plant growth · Leaf morphology · Plant phenotyping

Introduction

Horseradish (*Armoracia rusticana* P. Gaertner, B. Meyer & Scherbius, member of *Brassicaceae* family) is a perennial herb that grows to a height of up to 120 cm. The species thrives during the spring and summer producing a copious foliage consisting of numerous leaves that dry completely during autumn and winter; the root then enters dormancy until the following spring when the formation of new little leaves begins and a new growing season starts with the rise of temperatures (Agneta et al. 2013; Nguyen et al. 2013; Shehata et al. 2009). The leaves are wrapped in a rosette, long-petioled, elliptic-ovate, cordate at the base, unfairly crenate, and can grow to a length of 30 up to 100 cm. The root system is characterized by a long, white, cylindrical, or tapering main root with several thin lateral roots (Courter and Rhodes 1969; Shehata et al. 2009). Native of the temperate regions of Eastern Europe and Western parts of Russia, the species has become naturalized in many parts of the world where it can be found growing wild or cultivated either as annual or perennial crop (Wedelsbäck Bladh and Olsson 2011). Horseradish is commercially propagated from root cuttings and the productivity of perennial plantation can last until 20 years (Shehata et al. 2009). The species has been known since ancient times as a traditional medicinal herb, natural preservative, and dish condiment, and is popular today nearly worldwide for the aromatic and spicy white root, used freshly grated or transformed into sauce (Agneta et al. 2013). The species has recently gained an increasing scientific attention due to the abundance of several bioactive compounds, which, besides their relevance for human health benefits, are promising candidate for innovative applications in different fields (e.g., cancer-protecting components, natural antibacterial, and fungicide) (Nguyen et al. 2013; Herz

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et al. 2017; Petrović et al. 2017). Indeed, horseradish tissues are rich in peroxidase, an enzyme commonly used as a component of clinical diagnostic kits in medical research and neuroanatomy and in targeted cancer therapy (Veitch 2004; Krainer and Glieder 2015). In addition, the species is also abundant in glucosinolates (De Maria et al. 2016; Rivelli et al. 2016a; Ciska et al. 2017), phenols and flavonoids (Calabrone et al. 2015), and vitamin C (Rivelli et al. 2017) detected in high concentrations both in roots, which is the portion of the plant traditionally consumed, and in leaves. Despite the cross-cutting interest towards horseradish, the studies available in the literature about plant growth and development are very limited, and refer almost exclusively to the roots. Still, very little is published about leaf development, leaf–root growth, and its relation to biosynthesis and accumulation of secondary metabolites in plant tissues. The foliar apparatus expressed as leaf area (LA) has an important role in physiological and agronomic research, and it is usually monitored for observing plant growth and development (Bréda 2003; Jonckheere et al. 2004). In the case of horseradish, its evaluation would be necessary for crop management aimed at productivity and standardization of root marketing yield. The leaf area can be evaluated either directly or by one of the indirect non-destructive methods (Jonckheere et al. 2004). The former ones require the collecting of leaves and entire shoots by destructive sampling for detection of the surface area using several specific devices under the laboratory conditions. They are the most accurate methods for LA measurements, but are labour and time-consuming, hardly applicable in specific growing environments (i.e., forest ecosystems) and not suitable for monitoring leaf area development, particularly on plants with a few leaves. Thus, several non-destructive methods based on the measurements of light transmission through canopies, gap fraction analysis, canopy image techniques, or allometric methods have been developed for a while. All of these methods have numerous advantages, but also each has specific problems and limitations as highlighted by several authors (Brèda 2003; Jonckheere et al. 2004; Yilmaz et al. 2008; Thimonier et al. 2010; Li et al. 2014). Therefore, the choice as to which method to use is influenced by many factors such as the growing environment, the required accuracy, the research scale, and the equipment cost (Jonckheere et al. 2004). Concerning this latter issue, most of the indirect methods use expensive and sophisticated equipment; however, the recent advances in current technology in combination with wireless sensor network could provide a more reliable and convenient way to monitor leaf area than the traditional field surveys (Confalonieri et al. 2013; Li et al. 2015). Other simpler and low-cost non-destructive methodology rely on the measurements of allometric parameters (e.g., leaf dimensions, leaf shape, dry matter, or diameter of the sapwood area at breast), which are used to carry out mathematical models based on the correlation between actual leaf area and allometric measures of the plants (Bréda 2003; Jonckheere et al. 2004). Allometric techniques for LA estimation have been proposed for several annual and perennial crops such as cucumber and tomato (Blanco and Folegatti 2003), capsicum (De Swart et al. 2004), sugar beet (Tsialtas and Maslaris 2007), faba bean (Peksen 2007) sunflower (Firouzabadi et al. 2015), and grape (Montero et al. 2000). In those studies, the mathematical models for estimating LA have been based on leaf length and/or width, and they were more or less complex depending on several factors, including species and genotypes, variation of leaf shape during the plant growth and within a single plant, type of canopy, and position of the leaf on the plant canopy. In this context, the aim of this study was to analyze the leaf growth of horseradish plant to develop a mathematical model to estimate the leaf area based on linear measurements of the leaf surface.

Materials and methods

Experimental set-up

A 2-year experiment was carried out in Potenza, Italy, (PZ, 40°38′N–15°48′E, 819 m a.s.l.) on two selected accessions of *A. rusticana*, collected from local nurseries from Corleto Perticara (PZ, 40°23′N–16°03′E, 749 a.s.l.) and Montemurro (PZ, 40°18′N; 15°59′E, 723 a.s.l.) municipalities, henceforth referred as Cor and Mon, respectively. Root cuttings (nearly 20 cm in length and 1.0 cm in diameter) were transplanted on March 31, 2014 in single rows 60 cm apart with 40 cm in row spacing. Irrigation, plant protection, and weed control were carried out according to local practices and weather conditions.

Plant growth parameters measured during a 2-year growing cycle

Plants were sampled throughout a 2-year growing cycle (2014–2015 and 2015–2016) during the stage of vegetative development of the plants (S1) in July at the beginning of drying of leaves (S2) in early October 2014 and late October 2015 and at the stage of root dormant (S3) in January (during the traditional period of root harvesting for commercial purposes). At each data sampling, the height (from the soil line to the top of the highest leaf) of four plants for each accession was measured and based on the descriptor list of Petříková et al. (1998) and UPOV guidelines TG/191/2-2001 (UPOV The International Union for the Protection of New Varieties of Plants 2001) selected morphometric parameters were detected for each leaf: shape, leaf and petiole length, twisting of tip, leaf blade margin, and undulation of margin. Then, the foliage was cut, cleaned with tap water, and dried with paper towels. Thereafter, leaves were subdivided into green and dry (at S1 and S2 stages) or in dry leaves and new small ones (at S3 stage) and counted. Green leaves, without the petiole, were scanned by an LI-COR leaf area meter (Model LI-3100, inc., Lincoln, Nebraska) for measuring the actual leaf area of each leaf (LA) and of the entire plant (LA_p). Then, the length (*L*, from lamina tip to the point of petiole intersection along the midrib) and the mid-length width of the lamina (*W*) were measured both to the nearest 0.1 cm with a simple ruler.

Data of number of leaves and dry ones, and the measured leaf area were analyzed using a mixed linear model ANOVA in which "accession" (fixed effect, two levels: Mon and Cor) and "stage" (random effect, six levels: S1, S2 and S3 in 2014–2015 and 2015–2016) were the main effects. This analysis was used to test accession and stage differences, as well as their interaction. Furthermore, taking into account the relationships among *LA, L*, and *W* leaf traits, distinct patterns between accessions were performed through the analysis of co-variance (ANCOVA).

Leaf area model building

In addition to the four plants sampled at stages S1 and S2, the leaves on six additional plants were collected from the initial stage of the plant development to perform a fitting model for estimating horseradish leaf area. Length and width of the lamina (*L* and *W*), as well as the actual LA, were recorded on a set of 1000 leaves, as described above. To estimate LA, an extensive range of mathematical models was tested considering as the independent variables *L, W*, L^2 (leaf length square), W^2 (leaf width square) individually, or in combination among them. To this aim, linear ($y=a+bx$), polynomial ($y=a+bx+cx^2$), power ($y=ax^b$), and exponential ($y=ae^{bx}$) functions were tested with "*lm*" and "*nls*" functions, where "*y*" is the measured leaf area (cm^2), "*a*" line slope, "*b*" line intercept, "*c*" constant value, and "*x*" is the independent variable. The adequacy of the models was evaluated by either the percentage of variance explained by the model, expressed by the coefficient of determination (R^2), or the root-mean-square residual estimation errors (RMSE). Furthermore, the '*leave-one-out*' cross-validation method (LOOCV) was applied over the full set of data to compute the average mean-squared error (bias corrected), namely cross-validation error (CV*e*), using the '*cv.glm*' function of the '*boot*' package. LOOCV is a special case of K-fold cross validation where the number of folds is the same number of observations ($K=N$), i.e., each observation is considered as a validation set and the rest *N*-1 observations are a training set. Visual inspection of residual plots did not reveal any obvious deviations from homoscedasticity or normality. The best models were selected according to the combination of the highest R^2 and the lowest RMSE and CV*e*. All analyses were performed with R statistical environment *v*. 3.4.1 (R Core Team 2017).

Results and discussion

Horseradish is a perennial herb that produces a copious foliage throughout the spring and summer seasons, which dry completely during the autumn and winter when the root enters dormancy (Agneta et al. 2013; Nguyen et al. 2013; Shehata et al. 2009). Characteristics of the foliar apparatus, such as the plant green leaf area (LA_p) and the number of total and dry leaves of both horseradish accessions (Cor and Mon) collected at S1, S2 and S3 stages during the two growing seasons, are shown in Fig. 1. Although both accessions exhibited similar patterns in LA_p and number of leaves, Mon presented a significant higher number of total and dry leaves

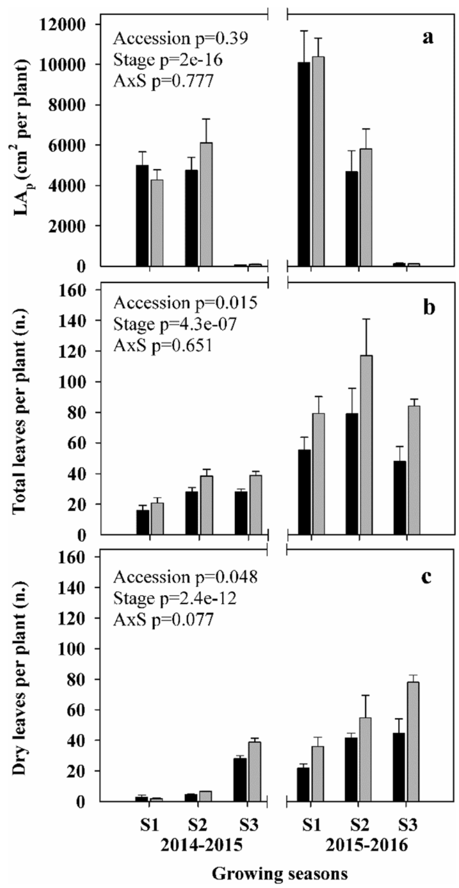

Fig. 1 Leaf area of the plant (**a**), total leaf number (**b**), and dry leaves (**c**) of horseradish accessions (Cor and Mon, black and grey bars, respectively) measured during the vegetative development (S1), beginning of drying of leaves (S2) and root dormancy (S3) stages during the growing seasons 2014–2015 and 2015–2016. Values are means ($n=4$) ± SE. Data were analyzed by factorial ANOVA; *A* accession, *S* stages, *AxS* interaction between accession and stage; *P* value (*p*) is given for each factor

during the stages analyzed (Fig. 1). We found significant statistical differences in LA_p among the stages analyzed in the 2 years. Particularly, during the first-growing season (2014–2015), the LA_p was on average 4600 cm^2 with about 20 leaves per plant (at S1 stage, after 4 months from transplanting). From this period forward, with autumn approaching and depending on the trend of climatic conditions (air temperature and rainfall), the plants continued to produce new leaves, and concomitantly, the oldest ones started to dry up (S2). At this stage, the LA_p was 5400 cm^2 with a number of dry leaves reaching 15% of the total. Later on, at the S3 stage, the foliage was completely dry, the root was dormant, and new young small leaves were already formed on the root crown with an LA_p of about 80 cm^2 (Fig. 1). During this period, horseradish roots are usually harvested for marketing as a fresh product (Walters and Wahle 2010). However, horseradish is a perennial species, and if roots are not harvested, they continue to grow increasing both in weight and diameter (Rivelli et al. 2016a, b). With the rise of temperatures, the new young small leaves begin to develop generating the new vegetation and a new growing season begins. At the vegetative re-growth stage (S1), the LA_p was much higher, up to 10,200 cm^2 and the number of the leaves was approximately 55 and 80 in Cor and Mon accessions, respectively (Fig. 1a, b); later, it declined by about 49% due to the higher number of dry leaves detected with the advancing of an early autumn season (at S2 stage). The doubling of the leaf surface from the first to the second growing season depended on the simultaneous enlargement of the root and the formation of multiple heads that are rounded knobs at or near the top of the root to which the leaves are attached.

Analysing the foliage characteristics of horseradish, a large variability among 30 accessions grown in a Mediterranean area was documented in an Italian study by Sarli et al. (2012) following the UPOV descriptor list for the conduct of tests for distinctness, uniformity, and stability of horseradish (UPOV guidelines 2001). The authors reported that about 65% of the analyzed accessions had medium-sized leaves (i.e., leaf dimension ranged from 35.1 to 55.0 cm in length and 10.1 to 20.0 cm in width) with slight differences in leaf shape from elliptic (56%) to narrow elliptic (28%) and to broad elliptic (16%) (Sarli et al. 2012). Differences among horseradish accessions were also found by Wedelsbäck Bladh (2014), who, by comparing a large collection of Nordic horseradish biodiversity (101 accessions) from different parts of Northern Europe (i.e., Denmark, Finland, Norway, and Sweden), reported several types of leaf shapes from narrow elliptic to ovate (although about 76 accessions had an elliptical-to-broad elliptical shape), with a leaf length varying from 16 to 100 cm, a petiole length from 10 to 58 cm, and width length from 8 to 29 cm. Following the above-mentioned UPOV guidelines (2001) and the descriptor list of Petříková et al. (1998), which referred to the fourth fully expanded leaf, we found that the leaf length varied from 26 in Cor to 33 cm in Mon with the petiole representing 17 and 21% of the total length, respectively; lamina length and width were 21.4 and 10 cm in Cor and 26 and 15 cm in Mon. Throughout the growing season, leaves of both accessions were elliptic in shape, with a medium undulation of margin and a weak twisting of the tip detected only on a few leaves. The leaf blade margin varied from entire (laminate) during the summer to lobed or parted (incised leaf) in a few leaves generally produced during the autumn, when plants approach dormancy (from S2 to S3 stages). By analysing all the leaves sampled during the two growing seasons, there was a close relationship between leaf width and length in both accessions (being $R^2 = 0.69$ and 0.67 for Cor and Mon, respectively), but the relationship was significantly different between accessions as highlighted by ANCOVA ($F_{(3,990)} = 718.6$, $p \leq 0.001$) as well as by different slopes and intercepts of the regression lines (Fig. 2). Those results indicate that horseradish leaves, in spite of an elliptic shape in both accessions, tend to be wider and bigger (broad elliptic) in Cor compared to Mon (narrow ellipses). Because of the different patterns observed in the length/width relationships, leaf area estimation models were fitted for each accession separately (Table 1). The models were performed using all of the 1000 leaves collected (including both accessions, stages, and years) whose length (*L*) spanning from 1.9 to 56.3 cm, width (*W*) from 0.2 to 24.3 cm, and LA from 0.4 to 960 cm^2. The equations relating the LA with the allometric parameters together with coefficient values, RMSE, CV*e*, and R^2 are reported in Table 1. For both accessions, all ten fitted models were highly statistically significant ($p \leq 0.001$). Overall the percentage of explained deviance (R^2) ranged from 79 to 96% indicating a close fit. It should be observed that the equations based on leaf *L* or *W* to estimate LA showed lower R^2 values compared to those that used combinations of *L* and *W* (including LW, L + W,

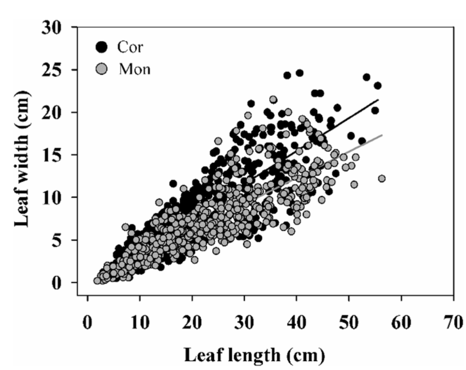

Fig. 2 Relationships between horseradish leaf width and length. The black and grey lines represent the linear regression for Cor ($y = 0.37x + 0.51$; $R^2 = 0.69$, $p \leq 0.001$) and Mon ($y = 0.28x + 0.66$; $R^2 = 0.67$, $p \leq 0.001$), respectively

Table 1 Equations and coefficient values (*a, b, c*) of regression models used to estimate leaf area (LA) of horseradish Corleto and Montemurro accessions based on leaf length and width (*L* and *W*, respectively)

No	Equation	Corleto						Montemurro					
		a	*b*	*c*	RMSE[a]	*CVe*[b]	R^2	*a*	*b*	*c*	RMSE	*CVe*	R^2
1	$LA = aW^2 + b$	1.48	24.55	–	59.6	3625.30	0.879	1.80	30.66	–	61.7	3914.63	0.822
2	$LA = aL^2 + b$	0.26	9.98	–	77.5	6077.31	0.795	0.22	7.09	–	63.6	4104.43	0.811
3	$LA = aLW + b$	0.71	− 0.27	–	37.4	1418.43	0.952	0.76	− 3.22	–	29.4	879.03	0.959
4	$LA = aLW^2 + bLW + c$	0.005	0.60	8.25	36.8	1382.72	0.954	0.003	0.71	0.25	29.3	884.35	0.960
5	$LA = aL^2W + bLW + c$	7.4e−04	0.68	1.89	37.4	1424.60	0.952	6.6e−04	0.73	− 1.64	29.4	885.89	0.960
6	$LA = aL^2W^2 + bLW + c$	8.13e−05	0.65	6.92	36.9	1386.40	0.954	7.9e−05	0.71	1.34	29.2	873.75	0.960
7	$LA = aLW^2 + b$	0.03	61.26	–	51.6	2718.54	0.909	0.04	54.04	–	46.9	2250.01	0.897
8	$LA = aL^2W + b$	0.01	53.87	–	51.9	2755.80	0.908	0.01	43.10	–	41.2	1726.27	0.921
9	$LA = a(L + W)^b$	0.09	2.09	–	49.6	4158.23	0.916	0.09	2.08	–	42.3	3010.82	0.916
10	$LA = a(L + W)^2 + b(L + W) + c$	0.16	− 1.45	18.47	49.6	2502.09	0.916	0.15	− 0.97	9.61	42.3	1823.88	0.916

RMSE Root-mean-square error, *CVe leave-one-out* cross-validation error, R^2 *r*-squared of the models are given

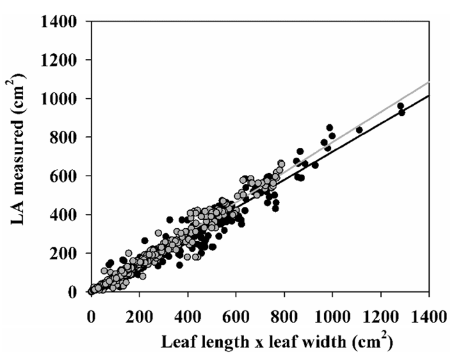

Fig. 3 Relationships between measured leaf area (LA) and leaf length per width. Black and grey circles and lines are referred to Cor ($LA = 0.71LW - 0.27$; $R^2 = 0.95$, $p \leq 0.001$) and Mon ($LA = 0.76LW - 3.22$; $R^2 = 0.96$, $p \leq 0.001$) accessions, respectively

L^2W, LW^2 or L^2W^2) (Table 1). It is interesting to note that, between the models using only one parameter to estimate LA, Eq. 1 based on width is more accurate in comparison to Eq. 2 based on length, because it has lower RMSE and CV*e* values (Table 1). Comparing the remaining models, the coefficients of determination were as high as 0.95 for equations from number 3 to 6, which also showed a good performance in the predictive accuracy as indicated by the lowest *leave-one-out* cross-validation errors. Based on the selection criteria above described, the model number 3 for both Cor ($LA = 0.71LW - 0.27$; $F_{(1,500)} = 9983$, $p \leq 0.001$) and Mon accessions ($LA = 0.76LW - 3.22$; $F_{(1,486)} = 11{,}511$, $p \leq 0.001$) can be considered the most efficient model to predict horseradish leaf area, because it met both statistical accuracy and sharpness (Table 1; Fig. 3). Many studies concerning different crops reported that the selection of simpler models against more accurate but complex ones is the best alternative for the LA estimation (Blanco and Folegatti 2003; Montero et al. 2000; Peksen 2007). Shabani and Sepaskhah (2017) highlighted that allometric methods based on simple equations are practicable mainly for species whose leaf dimension changes during the growing seasons when the leaf shape is invariant. In the same study on 16 arboreous species, it was shown that, in the case of leaves invariant in shape during growth, it is possible to estimate the area of large leaves based on a coefficient *k* determined only on the smallest leaf sampled in the initial stage of the plant growth (Shabani and Sepaskhah 2017). In horseradish, we found that, because the leaf shape remains invariant during plant growth and development (less or more elliptic depending on the accessions), although the leaf blade margin may vary throughout the seasons from entire to lobed or parted (laminate or incised leaves), the easy linear regression model can be used for predicting leaf area during the entire growing cycle and not limited to a specific stage of development.

The validation of selected models was performed by relating the predicted leaf area against measured leaf area: the strength of their relationship was emphasized by the high value of $R^2 = 0.97$ for both accessions (Fig. 4a). The intercept and regression coefficients between predicted and measured areas were similar between accessions indicating a good performance of the predictive model for both Cor and Mon. As expected, a strong relationship ($R^2 = 0.98$ for both Cor and Mon) was also found between the predicted leaf area for the whole plant, obtained by adding up the predicted leaf area of the single leaves for each plant, with the measured LA_p (Fig. 4b). Such results highlight that the selected equation $LA = a + bLW$ can easily be used also for estimating the entire leaf area of the horseradish plant during the whole growing season.

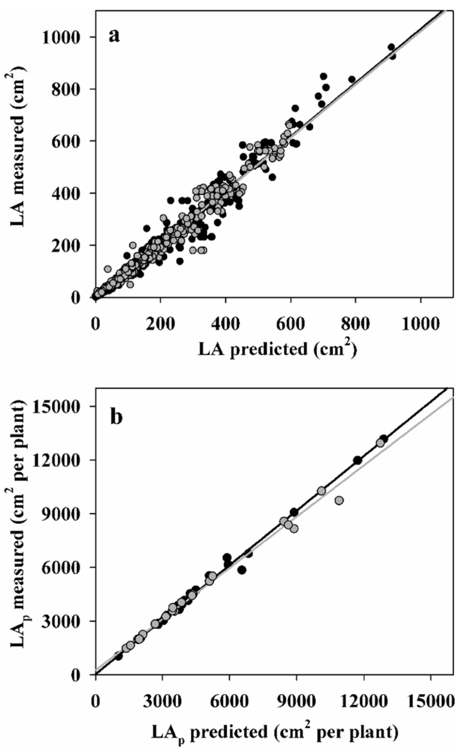

Fig. 4 Relationships between measured *vs* predicted single leaf area (LA) (**a**) and plant leaf area (LA_p) (**b**). In each panel, black and grey circles and lines are referred to Cor and Mon accessions, respectively. Linear regression lines: $R^2=0.97$, $p\leq0.001$, slope = 1.03 for both accessions (panel **a**); $R^2=0.98$, $p\leq0.001$, slopes = 1.02 and 0.94 for Cor and Mon, respectively (panel **b**)

Conclusion

The horseradish plant develops a thick foliage characterized by petioled leaves wrapped in a rosette, whose number and leaf area were massively higher in the second growing season with respect to first one (98 vs 33 and 10,000 vs 5500 cm^2). There were significant differences between accessions in leaf/width relationships, and leaf area estimation models were fitted for each accession separately. Results found indicated that horseradish is suitable to measure leaf area indirectly using simple allometric methods. The most accurate regression model was LA = 0.71LW − 0.27 and LA = 0.76LW − 3.22 for Cor and Mon, respectively. These models can easily estimate the leaf dimension with a simple ruler in the field without the use of any expensive instruments.

Author contribution statement SDM and ARR designed and performed the experiment, including data collection; AR and VT performed statistical analysis. All authors revised and approved the final manuscript.

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