Morphological variation on tomato leaves due to different nitrogen content

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ABSTRACT: Elliptic Fourier Analysis (EFA) is a method used to quantify shape differences. It mathematically describes the entire shape of an object by transforming the contour into Fourier coefficients, used as variables for statistical analysis, and involving the fitting of some type of curve to the object outline. Generally, the shape of agricultural products such as fruit, vegetables, grain and in addition other organs of plant is one of the most important factors for their classification and grading in relation to commercial quality and organoleptic properties. The aim of this study is to quantify the morphological variation of the shape of tomato leaves in response to their different nitrogen (N) content using the EFA coefficients, the fractal geometry and the perimeter ratio in combination with the Partial Least Squares Discriminant Analysis (PLS-DA). The analyses were realized on a tomato crop where each sample was chemically analyzed at the laboratory to establish the N content. The leaves (168) were divided into 3 groups following different N concentrations. Results suggest no relation between leaves lengths and N concentration is present following the Kruskal-Wallis performed with a p=0.735. The PLS-DA performing on the EFA coefficients, fractal index and perimeter ratio shows a high sensitivity, sensibility, and reduced mean classification error (82.3%, 81.07% and 18.3% respectively). The percentages of the correct classification in the model resulted to be 69.29% while the independent test equal to 56.1%. This study demonstrated the relation between leaf shape and N content (expressed in 3 concentration groups).

Keywords: Tomato leaf, elliptic Fourier analysis, fractal index, perimeter ratio, partial least squares discriminant analysis.

1. INTRODUCTION

Digital image analysis on shape has been used to evaluate various plant organs, suggesting various methods to describe shape quantitatively (Yoshioka et al., 2004). As reviewed by Costa et al. (2011), many different approaches were used in literature to describe shape variation. Among these, the method based on Elliptic Fourier Analysis (EFA) is one of the most commonly used (Cannon & Manos, 2001; Menesatti el al., 2008; Costa et al., 2009; Antonucci et al., 2010; Costa et al., 2010).

This method mathematically describes the entire shape of an object by transforming the contour into Fourier coefficients, used as variables for statistical analysis, and involving the fitting of some type of curve to the object outline (Rohlf and Bookstein, 1990). The EFA decomposes a curve into a set of harmonically related ellipses (Crampton, 1995; Lestrel, 1997; Loy et al., 2000;

Jensen et al., 2002; Costa et al., 2010). Examples of Fourier processing for morphological discrimination among different species and cultivars based on different plant organs are abundant (Iwata et al., 1998; Ohsawa et al., 1998; Iwata and Ukai, 2002; Morimoto et al., 2005; Neto et al., 2006, Antonucci et al., 2010). The different morphological kind of approaches within the fruit and vegetables context was recently reviewed by Costa et al. (2011). In this context, for the first time, our study tried to apply a complex morphometric approach to relate it with environmental parameters (in this case the nitrogen utilization as fertilizer in agricultural practices), making this analysis objectively quantifiable.

Leaf shape is a key feature for plant species identification by the specialists (Jensen et al., 2002; Neto et al., 2006). Kincaid and Schneider (1983) first used normalized Fourier coefficients and a dissection index (roundness) to approximate leaf shape. Guyer et al. (1986) studied four classical shape features regarding leaves from eight species of plants grown in containers. Guyer et al. (1993) investigated 17 quantitative shape features to classify 8 different plant species. Petry and Kuhbauch (1989) found that leaf shape parameters with five canonical indices were statistically different for several weed species. Franz et al. (1991) identified whole and occluded leaves using a leaf shape curvature and Fourier-Mellin correlation method at two stages of growth. Moreover, a quantitative comparison of leaves morphology of *Acer* spp. was conducted by Jensen et al. (2002) to evaluate taxonomic relationships utilizing traditional morphometrics, outlines analysis and geometric morphometrics.

Another process used to analyze leaf shapes is the fractal geometry. Fractal index is just an index which quantifies the shape complexity using a single number (Matabos et al., 2011). Since Mandelbrot (1982) introduced the concept of fractal, a geometric which exhibits its structure at all spatial scales, fractal geometry is becoming increasingly popular among ecologists (Phillips, 1985; Pennycuick and Kline, 1986; Krummel et al., 1987), largely because ecological patterns cannot be neatly described by Euclidean forms such as lines, planes, spheres, cubes, etc. Plant ecologists have generally been aware that vegetation varies over a wide range of spatial scales and have developed methods for studying the scale of vegetation variation (Greig-Smith, 1961; Kershaw, 1963). Specifically, the outline of a leaf is a fractal because it is irregular at all scales, i.e. it does not become linear upon repeated magnification (Palmer, 1988).

Generally, the shape of agricultural products such as fruit, vegetables, grain and in addition of other plant organs is one of the most important factors for their classification and grading in relation to commercial quality and organoleptic properties (Morimoto et al., 2000). In this scenario, the aim of this study is to quantify the morphological variation of the shape of tomato leaves in response to their different nitrogen content using two methods of image analysis, the EFA and the fractal geometry in combination with complex multivariate techniques: the Partial Least Squares Discriminant Analysis (PLS-DA). As reported by Chapin et al. (1988), the variation of the tomato leaf elongation rate declined in response to decrease in N. Nitrogen stress (reduced rates of cell division, cell expansion, photosynthesis, leaf production and tillering) caused a rapid decline in the leaf tissue aspect particularly in tomato which had smaller tissue nitrogen reserves. Organic N mobilized from old leaves provided an additional N source to support continued growth of N-stressed plants.

2. MATERIALS AND METHODS

2.1 Data collection

The analyses were realized on tomatoes farmed in the experimental Station of the Department of Agricultural and Environmental Sciences of the University of Perugia, in Papiano (Tiber Valley, Perugia province, Central Italy, 43°N, elev. 165 m). Each leaf (168) was randomly sampled from 20 plants (8-9 leaves from each plant). After the image acquisition, they were chemically analyzed at the laboratory of the University of Perugia to establish the N content. The tomato crop analyzed in this work resulted in the vegetative growth phase basing on the critical N curve presented by Tei et al. (2002). The leaves were divided into 3 groups following different N concentrations: lower than 3.6% (32 leaves); ranging between 3.6 and 4.3% (99 leaves); above 4.3% (37 leaves) (Fig. 1). This grouping has been used for a non-parametric ANOVA (Kruskal-Wallis test) comparison and for the multivariate statistical analysis (see below). The mean coefficient of variation of the N leaf content was evaluated through a non-destructive system (the chlorophyll meter readings, SPAD-502, Minolta).

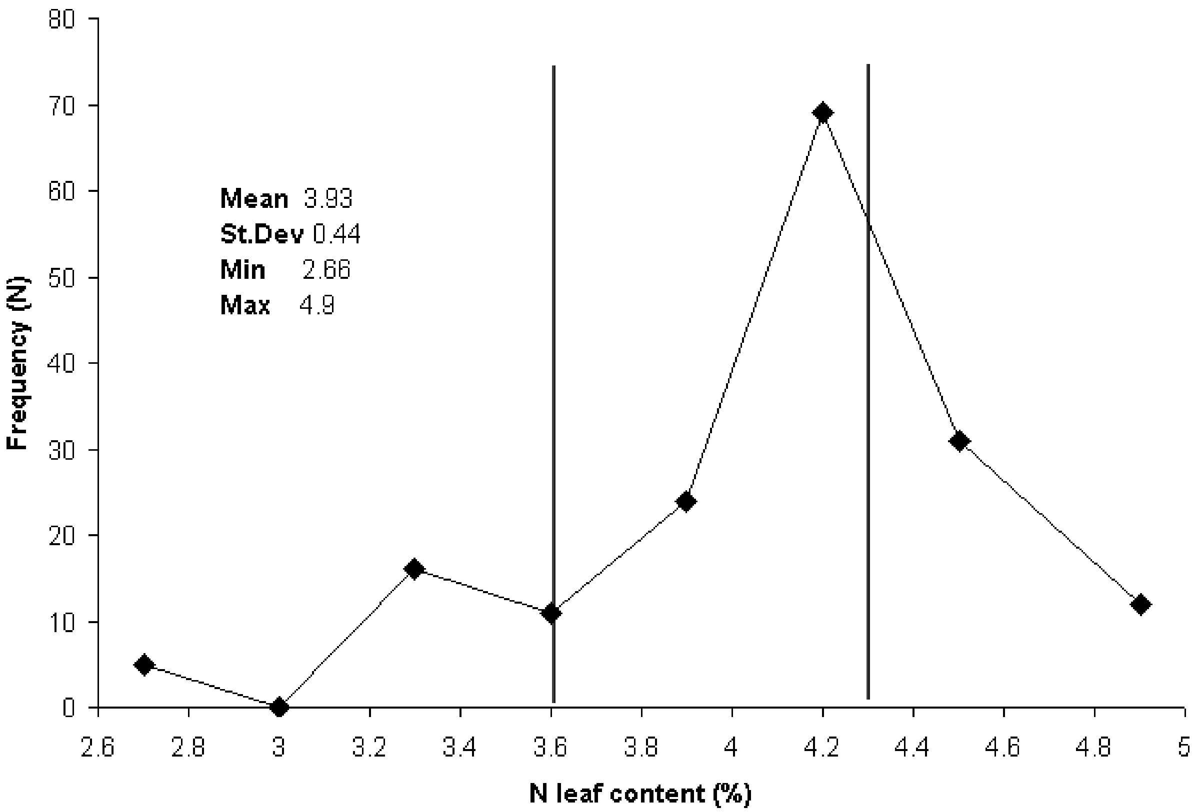

Figure 1. Nitrogen content frequency distribution for the total number of samples in relation to the three different groups with the relative threshold values (lower then 3.6%; ranging between 3.6 and 4.3%; above 4.3%).

2.2 Digital image processing

The leaves, randomly disposed on the plane, were acquired with a high resolution scanner (300 d.p.i. 24 bit colors) at the CRA-ING Laboratory. Color images were transformed into BW by means of the following procedure (Matlab 7.1): 1) extracting the blue channel; 2) applying a median filter (2 times); 3) thresholding using a fix background value of 0.2 (8 bit grayscale range: 0-1); 4) removing noise applying an area filtering that eliminates objects smaller than 5,000 pixels.

2.3 Elliptic Fourier Analysis

On the obtained images a total number of 400 points (x, y) equally spaced on the perimeter, describing the outline of the leaves, were acquired utilizing EFA (Fig. 2).

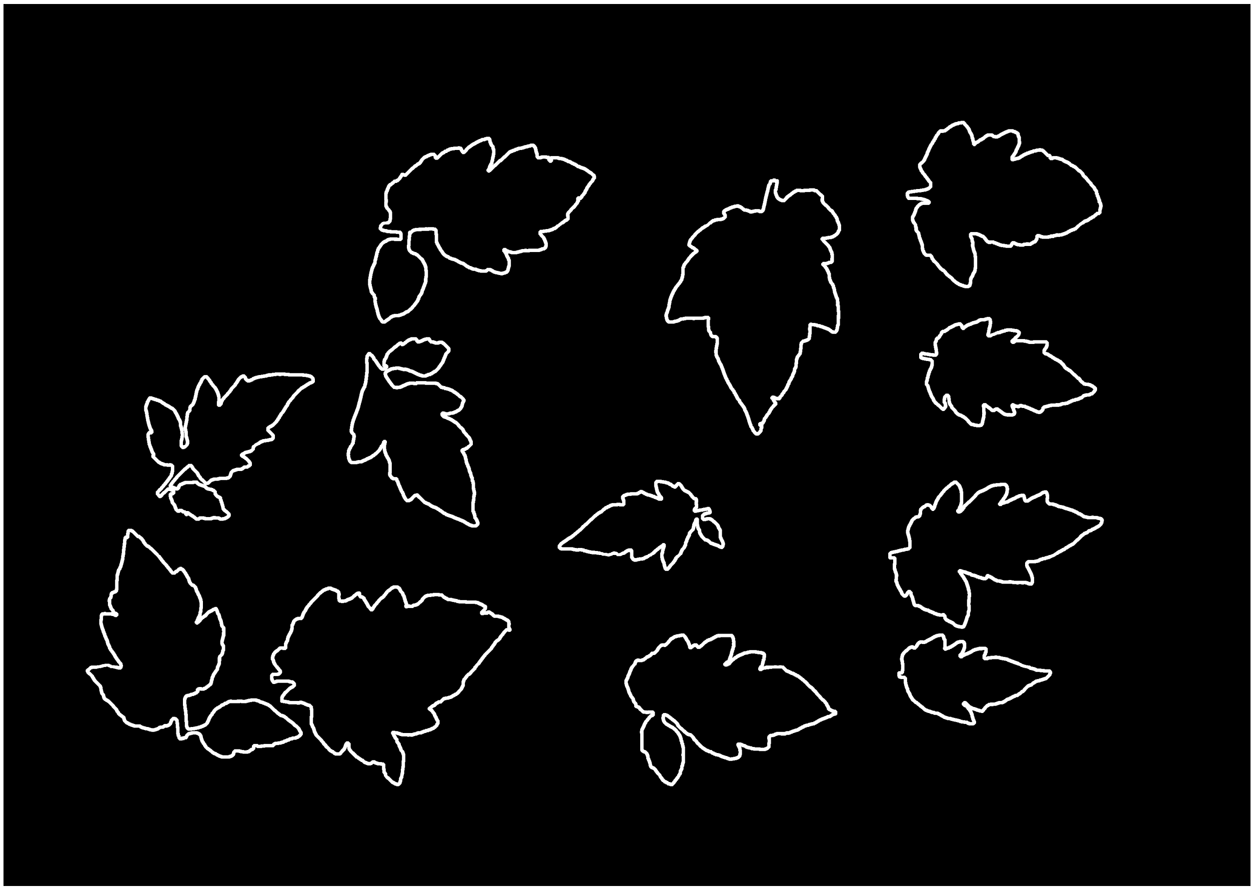

Figure 2. Example of an image where tomato leaf outlines were extracted after the digital image processing procedure.

All the procedure was fully written in Matlab 7.1. Leaf shape outline can be approximated by a polygon of x-y coordinates (Rohlf and Archie, 1984). EFA is based on the separate Fourier decompositions of the incremental changes of the x and y coordinates as functions of the cumulative chordal length of the outline polygon. It yields to the spectrum of the leaf shape closed contour in terms of harmonically related trigonometric curves. For each harmonic equation, two Fourier coefficients are computed for both the x and y projections, thus the total number of coefficients are 4n, where n is the number of harmonics fitted to the outline (Crampton, 1995). The total number of harmonics that can be computed for any outline is equal to half of the total number of outline coordinates (the “Nyquist frequency”). The Fourier series was truncated at the value of k at which the average cumulative power is 99.999% of the average total power (Menesatti et al., 2008). The number of harmonics utilized in this study is equal to 30 (120 coefficients). For any outline, the total power was calculated as the sum, from 1 to k, of individual harmonic powers where k is equal to the Nyquist frequency (Crampton, 1995). The harmonic coefficients describe the size, shape, and orientation of each harmonic ellipse and form one of the inputs to multivariate statistics.

2.4 Fractal index

Another input to multivariate statistics is the fractal index. An image is called fractal if it displays self-similarity: it can be split into parts, each of which is (at least approximately) a reduced-size copy of the whole. A possible characterization of a fractal set is provided by the box-counting method. The number N of boxes of size R needed to cover a fractal set follows a power-law:

$N = N0 * R^{(-DF)}$ (1)

with $DF \leq D$ and D is the dimension of the space, usually equal to 1, 2, 3. DF is known as the Minkowski-Bouligand dimension or simply box-counting dimension. In fractal geometry, the box-counting dimension is a way of determining the fractal dimension of a set in a Euclidean space, or more generally in a metric space (Moisy, 2008). To calculate this dimension for a fractal set, imagine this fractal lying on an evenly-spaced grid, and count how many boxes are required to cover the set. The box-counting dimension is calculated by observing how this number changes as we make the grid finer.
The calculation of fractal index was performed using a Matlab code (Moisy, 2008).

2.4 Perimeter ratio

Another variable used for the multivariate analysis is the perimeter ratio. It is calculated as the ratio between leaf perimeter and the diameter of a circle of equivalent area, being the ratio for a perfect circle equal to π.

2.5 Multivariate statistical analysis

The matrix (122 variables) composed by the 120 coefficients of the harmonic equations extracted by the EFA, the fractal index and the perimeter ratio were analysed via multivariate classification through the PLS-DA to predict the N content of each leaf. Calculation were performed in Matlab 7.1 and PLS Toolbox Eigenvector 4.0.
The samples from each group were subdivided in two subset: i) 75% of specimens for the class modelling and validation; ii) 25% of specimens for the independent test, optimally chosen with the Euclidean distances based on the algorithm of Kennard and Stone (1969) that selects objects without the a priori knowledge of a regression model (the hypothesis of a flat distribution of the data is preferable for a regression model). PLS-DA (Sabatier et al., 2003) is a partial least squares discriminant analysis regression where the response variable is categorical (Y-block; replaced by a set of dummy variables describing the 3 groups of nitrogen concentrations) expressing the class membership of the statistical units. For the methodology of this analysis see Costa et al. (2010) and Menesatti et al. (2008). This analysis also expresses the statistical parameters indicating the modelling efficiency in terms of sensitivity and specificity of the parameters. The sensitivity is the percentage of the samples of a category accepted by the class model. The specificity is the percentage of the samples of the categories different from the modelled one, rejected by the class model. Generally, the trend of the residual errors is decreasing in the calibration phase (Root Mean Square Error of Calibration; RMSEC) and increasing for the validation phase (Root Mean Square Error of Cross-Validation; RMSECV). This analysis was performed using Matlab on the shape variables (X-block; EFA coefficients, fractal index and perimeter ratio). The X-block values were pre-processed with a “Generalized Least Squares Weighting” (GLSW) Matlab procedure. This pre-processing method is used prior

in order to remove extraneous variance. The best performing models were selected among the ones with a number of LV ranging from 1 to 20.

3. RESULTS

The Kruskal-Wallis ANOVA shows that the N concentration has no effect on the leaves length (average maximum length = 59.7 mm, p = 0.375).
The mean coefficient of variation of the N leaf content resulted equal to 8%.
PLS-DA was conducted to discriminate models based on the 3 groups associated to the different concentration of N. Table 1 reports the characteristics of the models performed on EFA coefficients, fractal index and perimeter ratio.

Table 1. Characteristics and principal results of the PLS-DA models performed on EFA coefficients, fractal index and perimeter ratio on the 3 different groups. N is the number of samples. n° units (Y-Block) is the number of unit to be discriminated by the PLS-DA. n° LV is the number of latent vectors. Random Probability (%) is the probability of random assignment of an individual into a unit.

N	168
n° units (Y-block)	3
n° LV	2
Mean Specificity (%)	81.07
Mean Sensitivity (%)	82.3
Mean Classification Error (%)	18.3
Mean RMSEC	0.49
Random Probability (%)	33.3
% Correct Classification Model	69.29
% Correct Classification Independent Test	56.1

The model based on 2 Latent Variables presents a high sensitivity, sensibility and a reduced mean classification error (82.3%, 81.07% and 18.3% respectively). The percentages of the correct classification in the model resulted to be 69.29% while in the independent test equal to 56.1%. These values, even if could appear low, are high with respect to the random probability percentage (33.3%).

4. DISCUSSION

In a study of Minkenberg & Ottcnheim (1990) the between-plant variation in leaf nitrogen of tomato plants was quantified. Generally, tomato plants in most western European glasshouses are grown on rock wool and individually watered and fertilized by drip-system irrigation to minimize water stress and nitrogen deficiency. Minkenberg and Ottcnheim (1990) found that differences in N content between plants in commercial glasshouses were considerable (range 1.6%). The total nitrogen found in leaves of the high middle region of the tomato plants was on average, 4.5% and varied between 3.8 and 5.4% during the season. With respect to variation with plant age or season, in the study of Minkenberg and Ottcnheim (1990) no trend was found in the nitrogen content of the leaves from the commercially grown glasshouse tomatoes, contrary to the generalization that nitrogen content of whole plants declines with age or time.
Generally, leaves with different nitrogen contents can be found on the same plant in relation with the growth phase and position (Tei et al., 2002). However, in this study in order to limit this

effect, only the well developed leaves were sampled. This effect was evaluated calculating the N leaf content mean coefficient of variation through a non-destructive system (the chlorophyll meter readings, SPAD-502, Minolta). This mean coefficient of variation resulted equal to 8%. Our results show that the shape of the leaves can not be used to assess the nitrogen content and that this does not affect the length of the leaves. However, we can affirm that N content affects the shape of the leaves. The last finding required the application of a refined method, such as EFA combined with complexity indexes (fractal index and perimeter ratio) combined with a modern multivariate modeling approach (PLS-DA). This approach demonstrated how the use of adequate tools for morphometric and data analysis, could provide remarkable results not reachable with traditional monovariate approaches. Similar results improvements were obtained in completely different morphometric research fields (Costa et al., 2008).

Although N content limits the growth of tomato plants, the mechanisms by which this occurs is not really clear (Chapin et al., 1988). Generally, limited supply of N reduces the rates of cell division, cell expansion, photosynthesis, leaf production and tillering (Chapin, 1980). These considerations, observing our results, lead to affirm that the reduction of cell division with N decreasing reflects to the whole leaf complex area and not to the maximum leaf length; this phenomenon is detectable only using complex shape analyses.

An automated method to identify different in nitrogen content by analyzing leaf shape could allow better knowledge of nutritional status of tomato plants, giving the possibility to obtain helpful details on wider areas. Moreover, increased information both in terms of space and in time (higher number of measurements and reduced time for the acquisition) represents an essential tool to enhance and stabilize crop quality, improve the nutrient use efficiency and develop program for optimizing application of nitrogen fertilizer (Havránková et al., 2007; Lan et al., 2009). Additionally, the accurate estimation of the nitrogen content in tomato leaves determined by EFA coefficient, fractal index and perimeter ratio by means of chemometric procedures as PLS-DA, can be used for image analysis in-field applications.

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