UNIVERSITÀ
DEGLI STUDI
DI PADOVA

ALMA MATER STUDIORUM
UNIVERSITÀ DI BOLOGNA

UNIVERSITÀ DEGLI STUDI DI PADOVA

Department of Biomedical Science

Interuniversity Bachelor's degree programme
in Biology of Human and Environmental Health

Final dissertation

EFFECTS OF CLIMATE CHANGE OVER PLANT PHYSIOLOGY

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ACADEMIC YEAR 2024/2025

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ABSTRACT

Climate change is a dynamic and multifaced system of alterations in environmental conditions, that has evoked variations in temperature, CO_2 concentrations, intensity of rainfall and atmospheric conditions. This has exposed plants to harsh and extreme climatic conditions, that adversely affect the morphological, developmental, cellular and molecular processes in plants. In this study, to observe a possible effect of climate change over plant physiology, stomata structures have been taken into consideration, because of their crucial role of regulation of both CO_2 uptake, for photosynthesis, and evaporative loss of water. Two species have been examined, *Quercus robur* and *Fagus sylvatica*. Past leaves samples of these trees, taken from the "Erbario di Antonio Bertoloni", have been compared to leaves samples of extant trees, by considering two main aspects: the number of stomata situated on leaf samples and superficial area of each stoma. The statistical analysis has been accomplished also by using an algorithm of machine learning. The results obtained differ between the two species: while in *Fagus sylvatica* there is a clear reduction in stomatal density and size, consistent with physiological strategies that plants might be adopting in response to recent climate change, the contrasting results for *Quercus robur* highlight the complexity of these possible plant variations.

INTRODUCTION

1.1 Plant physiological changes due to climate change

Climate change is a natural process, when it happens gradually over millions of years. It is indeed considered “any systematic change in the long-term statistics of climate elements (such as temperature, pressure, or winds) sustained over several decades or longer; it may be due to natural external forcings, natural internal processes of the climate system, or anthropogenic forcing, as the American Meteorological Society stated in its glossary. Nowadays, the real problem is the climate change that is ongoing at a high speed and that is drastically altering the Earth. It began during the Industrial Revolution of the 19th century, leading to a persistent increase in harmful greenhouse gases. Therefore, it can be noted that this climate change is mainly a result of anthropogenic activities, at least in terms of its unprecedented speed, as FCCC (Framework Convention on Climate Change) states. This gives humans both responsibility and the opportunity to do something about it.

Climate change has caused many changes in Earth's living conditions, such as variations in temperatures, CO_2 levels, rainfall intensity, and atmospheric conditions (Chaudhry et al, 2022).

Plants are especially sensitive to these kinds of changes because of their long lifespan, which prevents them from quickly adapting to rapid environmental shifts (Lidner et al. 2010). They need to cope with these different kinds of stresses (such as drought, heat, salinity, ozone, nutrient deficiency, UV, and high light intensity) both individually and, more broadly, in combination with one another (Sato et al., 2024). Stress interactions can either be additive or antagonistic; they typically cause a much greater negative effect on plants, although sometimes combined effects can be beneficial.

Anyway, these variations in Earth's conditions frequently damage plants in their morphological, developmental, cellular, and molecular processes (Chaudhry et al, 2022). The two most significant and prevalent stresses are drought and heat. Alterations in CO_2 concentrations are also critical, but they can be seen as the root of climate change itself, as they mainly drive the greenhouse effect and rising temperatures, along with all the known consequences.

Drought stress results from a decrease in soil water content, leading to lower water availability and dehydration in cells (Sato et al., 2024). This can trigger both morphological and physiological responses. Primarily, because of the reduction in turgor pressure, cell growth slows down or stops, affecting various aspects of the plant, such as a decrease in overall plant height, stem diameter, number of leaves, leaf longevity, and leaf area (Anjum S. A. et al, 2011). The decline in leaf area is

particularly significant because it directly reduces the photosynthesis process. Additionally, another morphological response is a decrease in photosynthesis caused by impaired leaf gas exchange, which involves irregular stomatal closure due to low soil water content (there is always a strong correlation between leaf water potential and stomatal conductance). Stomatal closure results in a reduction of net photosynthesis by limiting CO_2 intake. This effect is further worsened by drought's direct impact on the photosynthesis machinery, which disrupts key components such as the thylakoid electron transport chain, the carbon reduction cycle, stomatal regulation of CO_2, and leads to increased carbohydrate accumulation, lipid peroxidation, and water balance disturbances. A decline in photosynthetic activity under drought conditions is also due to "non-stomatal" mechanisms, which involve changes in chloroplasts and disruptions in the processes of assimilate accumulation, transport, and distribution. Notably, water stress causes a significant loss of chlorophyll content, further impairing photosynthesis and decreasing primary production. Other common drought-induced responses in plants include the generation of reactive oxygen species (ROS), inhibition of long-distance transport, and destabilization of membrane structures and proteins, all of which contribute to cellular damage (Sato et al., 2024).

The other major stress related to climate change is heat (Ahuja et al., 2010). Each plant has its own specific temperature range that allows optimal growth and development. As environmental conditions are changing, in particular toward extremely elevated temperatures, plants may undergo significant adjustments in their physiological and molecular mechanisms to try to adapt, maintain homeostasis, and survive. These adjustments can include changes in the organization of cellular structures (such as organelles, the cytoskeleton, and membrane functions), a decrease in protein synthesis, and an increase in the transcription and translation of heat shock proteins and in the production of phytohormones and antioxidants.

1.2 Stomata's role in plant physiology

Stomata are specialized pores found in many plants, especially on the underside of leaves (Urry et al., Campbell Biology in Focus). They play a crucial role in both gas exchange, involving CO_2, the source of glucose in photosynthesis, and O_2, a byproduct of the process, as well as water exchange, since they are the primary pathways through which water evaporates from the plant. Each stomatal pore is surrounded by a pair of guard cells that control its size by changing shape. These guard cells, by narrowing or widening the stomata, help regulate water and CO_2 concentrations inside the leaves. Three main stimuli are involved in opening and closing: light, which causes guard cells to accumulate K^+ through illumination of receptors in the plasma membrane, making them turgid; depletion of CO_2; and an internal "clock" that ensures stomata follow their daily rhythm of opening and closing. The actual mechanism of changing the size of the stomatal opening relies on the guard cells' ability to adjust their turgor pressure: when they become more turgid, they bow outward, enlarging the pore. In terms of water evaporation, guard cells absorb water from neighboring cells, becoming more turgid. Yet it can also happen through reversible absorption and loss of potassium ions: when K^+ accumulates from surrounding epidermal cells, this flow is coupled with the activity of proton pumps that actively transport H^+ out of the guard cells; this creates a voltage that causes water to enter the guard cells via osmosis, making them more turgid.

1.3 Relationships between stomata and climate change

Stomatal density of a plant leaf is a developmentally plastic feature that is influenced by both genetic and environmental factors (Urry et al., Campbell Biology in Focus). For example, it has been stated that CO_2 concentrations and light exposure significantly affect the number and size of stomata on a leaf. As a matter of fact, an effective stomatal response to environmental changes is crucial for maintaining the necessary water balance inside the plant, from the roots to the leaves (Hetherington et al., 2003). This characteristic has been of great importance in studying the effects of climate change: the correlation between rising atmospheric CO_2 levels and changes in stomatal density has identified the latter as a trait of vital ecophysiological importance under changing conditions (Woodward, 1987). Indeed, a common response of plants to climate change, particularly to increased CO_2 levels, is a reduction in stomatal density.

The first publications by I. Woodward, published in 1986 and 1987, described the reduction of stomatal density with increasing CO_2 as a reasonable consequence of anthropogenic influences, without completely understanding the ecological basis of this concept. In his subsequent studies, I. Woodward hypothesized that the efficiency of changes in stomatal density could improve leaf water use. After these initial insights, Woodward had to address a major challenge: even though the common physiological response is a decrease in the number of stomata, the range of species-specific reactions is quite broad. *Arabidopsis thaliana* has been used as a model plant because of its behavior that resembles many interspecific observations (Woodward et al., 2002). Yet this was only the first of a long series of controversies and obstacles in the study of the effects of climate change on stomata physiology. Indeed, Woodward, as many other scientists after him (Yan et al., 2017a), realized that several factors could affect this aspect of plant physiology, such as environmental conditions, like soil water availability (and therefore drought), irradiance, high temperature; species-specific features, like genetic factors that can be responsible for differences in stomatal formation or stomatal responses; or even altitude, since it implies a change in CO_2 concentration.

Currently, the signaling pathways through which environmental signals regulate stomatal development are still being thoroughly studied, as very few concepts about these processes are known. It was recently demonstrated that only mature leaves can regulate stomatal development in response to both CO_2 concentration and light exposure, while developing leaves do not exhibit this tendency (Lake et al., 2001). Another research has also identified a specific gene, the HIC gene,

characteristic of *Arabidopsis thaliana*, that encodes a synthase involved in controlling stomatal development under elevated CO_2 levels (Gray et al., 2000).

THESIS OBJECTIVES

The main goal of this project is to understand how climate change has affected and continues to affect plant physiology. To investigate this, stomata were examined. These plant structures are a critical part of plant homeostasis and health, playing a vital role in regulating both gas exchanges needed for photosynthesis and water evaporation. Therefore, they should be primarily involved in any physiological changes in plants caused by various stresses from climate change, such as increased CO2 levels, drought, elevated temperatures, and so on.

Since the aim was to detect alterations in plant functioning before and during the effects of climate change, a comparison among past and current leaf samples has been carried out, focusing on two main aspects: the number of stomata on the leaf samples and the surface area of each stomata. For this reason, the entire project used materials from “Erbario di Antonio Bertoloni”, located in Bologna’s botanical garden, which collects a large number of leaf samples from the 19th and 20th centuries, as well as from current plants collected in the exact location as the ancient ones.

METHODS AND MATERIALS

3.1 Study area

The whole project was based on samples from the "Erbario di Antonio Bertoloni," a collection of leaves and plant sections from the 19th and 20th centuries made by the botanist himself and other coworkers. Slide preparation, for both past and current leaf samples, was performed in the florist laboratory at the University of Bologna's botanical garden.

A map was created showing the locations of all plants used by Bertoloni's collections for the project (Fig. 1). To make the comparison as accurate and realistic as possible, the current samples were gathered in the same locations as the past ones, ensuring similar growth and development conditions for the plant specimens.

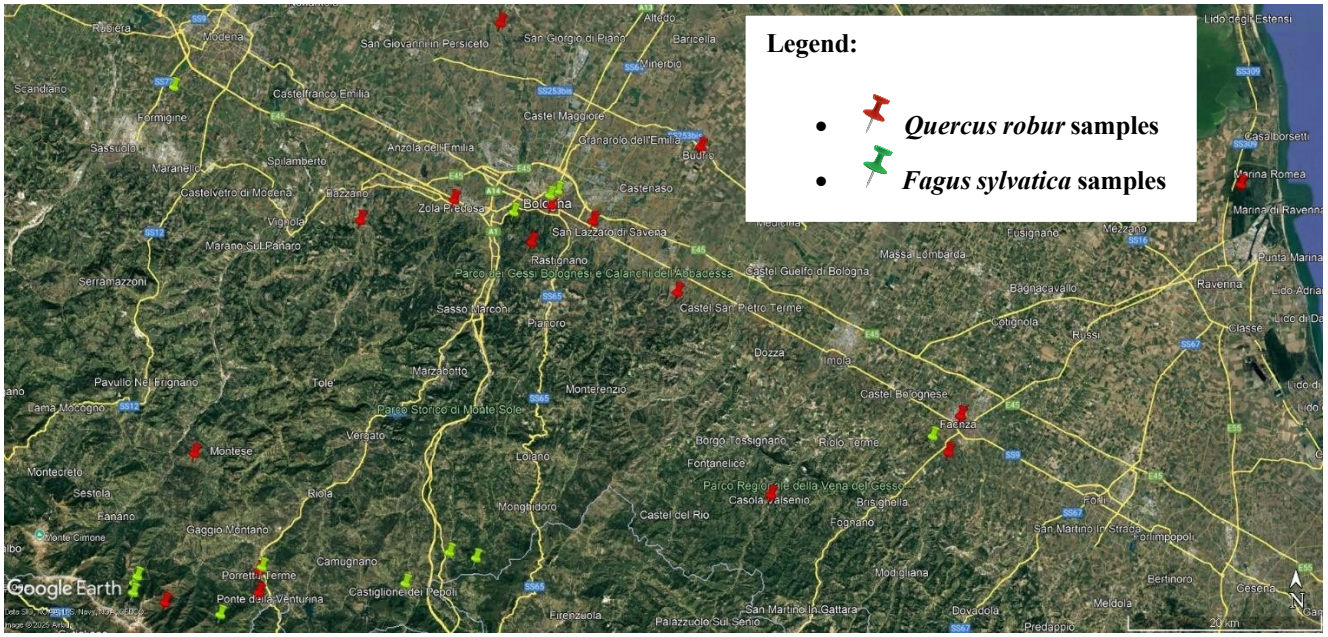

Figure 1 – *Map of the locations of the samples from Alberto Bertoloni's Herbarium*

3.2 Samples collection

For the first part of the comparison, concerning the conditions of leaves and plant physiology before the impacts of climate change, Antonio Bertoloni's collections were analyzed. Among all possible leaf samples that could have been studied, two specific species were selected: *Quercus robur* and *Fagus sylvatica* (Table 1 and Table 2). They were chosen because of the large number of samples available in the herbarium and because of the ease of finding current plants with the same conditions and in similar locations nowadays.

Regarding the opposite of the analogy, about plant physiology after the effects of climate change, a selection of locations where the "past samples" had been taken was documented. In these spots, new leaf samples were collected from individuals of the same species found as close to the original site as possible. Moreover, trees with trunks measuring between 5 and 30 centimeters were preferably identified to try to maintain a comparability with old sample. Afterwards, these fresh leaves were pressed and partially dried to facilitate easier examination during microscope analysis.

For each sample, five different slides were prepared from five separate, non-matching points on the leaf. This approach increased the amount of data for the study, enhancing the statistical significance of the project.

Table 1 – *Selection of* Quercus robur *samples*

QUERCUS ROBUR SAMPLES						
BRAHMS	**NAMESTRING**	**DATA**	**MAJOR**	**MINOR**	**LOCNOTES**	**CURRENT SAMPLES**
6202	Bertoloni, G	08/1836	Emilia Romagna	Bologna	Parte meridionale del monte di Paderno; Bologna	YES
16790	?	06/1848	Emilia Romagna	Bologna	Sul Monte della Croce della Porretta subito sopra le Donzelle tra i dirupi di macigno.Bologna	NO
16813	?	10/1866	Emilia Romagna	Ravenna	Coltivata presso Faenza,Ravenna	YES
16806	?	06/1872	Emilia Romagna	Ravenna	Nei Sabbioni in Sarna presso Faenza,Ravenna	YES
6135	Bertoloni, G	10/1874	Emilia Romagna	Bologna	Nel podere dell'autore detto Vigna Nuova, Zola Predosa; Bologna	YES
16410	Bertoloni, J	10/1874	Emilia Romagna	Bologna	Bologna	YES
16724	?	11/1883	Emilia Romagna	Ravenna	Nei Sabbioni presso Faenza,Ravenna	YES
16799	Matteis	04/1885	Emilia Romagna	Bologna	Lungo il torrente Aposa nei luoghi boschivi.BO	YES
16794	Marta Grandi	09/1936	Emilia Romagna	Bologna	Varignana,Castel San Pietro,Bologna	YES
16796	Anna Fiori	07/1936	Emilia Romagna	Modena	Casinalbo,Modena	NO
16798	Anna Fiori	07/1937	Emilia Romagna	Bologna	Borgo Capanne,BO	NO
38084	Russo	05/1985	Emilia Romagna	Bologna	Parco dei Cedri, Bologna	YES
16454	Andina, G; Tonioli, M	05/1995	Emilia Romagna	Bologna	Monte Freddo(Monte Veglio)BO.CTR : Vignola	YES
16455	Galloni, M	10/1995	Emilia Romagna	Bologna	Scarpata di Canale in localitÓ Bagno di Piano.CTR:SanGiovanni in Persiceto,BO	NO
16456	Andina, G; Galloni, M; L.Gavioli.	07/1995	Emilia Romagna	Bologna	Lungo il sentiero Cai nr.119 dal rifugio Sega Vecchia al Corno alle Scale,BO CTR: Porretta Terme I	NO
16457	Galloni, M; Lupi, C	10/1995	Emilia Romagna	Bologna	Scarpata ferroviaria tra Castenaso e Budrio BO. CTR: Budrio	YES
52619	Cristina Buoso	09/1996	Emilia Romagna	Ravenna	Casola Valsenio, parco comunale lungo il corso del fiume Senio (Ravenna)	NO
49393	Bassi A.	??/1999	Emilia Romagna	Ravenna	Pineta San Vitale; Ravenna	NO
52400	Cristina Buoso	07/2000	Emilia Romagna	Ravenna	Casola Valsenio, parco comunale lungo il corso del fiume Senio (Ravenna)	NO

Table 2 – *Selection of* Fagus sylvatica *samples*

FAGUS SYLVATICA SAMPLES						
BRAHMS	**COLLECTOR**	**DATE**	**MAJOR**	**MINOR**	**LOCNOTES**	**CURRENT SAMPLES**
c6621b99-5f14-4097-b3fb-5761845a1e05	Managlia, A	09/1999	Emilia Romagna	Bologna	Pian del Voglio	NO
9f4c5299-4c1c-4e1c-8c7e-8a0f7c0ef63b	Savi	12/1904			Localita' non indicata	NO
37373b48-d8fa-4eb5-83f0-6b7eb4fc2d15	Anna Fiori		Emilia Romagna	Bologna	BO	NO
1e5a6a32-22ea-4dc4-8941-22ecf41dc679	Bertoloni	06/1848	Emilia Romagna	Bologna	Lungo il Rio Maggiore della Porretta ,BO	NO
1f907773-0e0d-4ca1-ba93-f78234c2b2e5	Galloni, M	06/1995	Emilia Romagna	Bologna	Val Serena,Pian del Voglio, Bologna	NO
29e6cbe6-9df5-4735-807e-a1fe9d6fed45	Bertoloni, J	07/1833	Emilia Romagna	Bologna	nell' Appennino Bolognese nel Bosco di Madonna dell' Acero, BO	YES
2f99374d-1ca0-4566-92c7-816dd2d90482	Anna Fiori	08/1942	Emilia Romagna	Modena	Casinalbo,Modena	NO
d6d80f77-7912-4730-ab1f-45b08c6b7eab	Anna Fiori	04/1936	Emilia Romagna	Bologna	BO	NO
d7e91ea2-3612-4fcf-aa12-f023181a8b60	Anna Fiori	05/1937	Emilia Romagna	Bologna	BO	NO
09375b5e-60ef-45ca-8345-6a50dfab303b	Bertoloni, G	06/874	Emilia Romagna	Bologna	Appennino bolognese	YES
cd8ce1b0-6548-4a72-9606-2a87f8307f53	Bertoloni, G	07/1837	Emilia Romagna	Bologna	Luoghi ombrosi settentrionali del Monte Mariano; Bologna	NO
1370e37a-ba52-4f47-8190-737bb8b4d97a	Alberto Fiori		Emilia Romagna	Bologna	BO	NO
0d7a17a0-3bb7-4c0c-9bf5-fe249bb04425	Bertoloni, G	07/1831	Emilia Romagna	Bologna	Appennino bolognese, a Castiglione dei Pepoli	NO
28b34afd-6937-43ef-9500-fd14662376bb	Bertoloni, G	08/1838	Emilia Romagna	Bologna	Bosco della Vergine dell'Acero; Bologna	YES
55c9e73f-1399-4b66-967b-bc4f608676df	Bertoloni, G	08/1871	Emilia Romagna	Bologna	Parte superiore della selva dell'Acero; Bologna	YES
831c9951-c488-483f-8e15-411dd0be7ef9	Bertoloni, G	06/1874	Emilia Romagna	Bologna	Bosco della Madonna dell'Acero; Bologna	YES
8395d69f-44bb-40fc-9e8b-386c97def7dc	Bertoloni, G	06/1874	Emilia Romagna	Bologna	Lungo la strada tra Ca' d'Iberna e l'Acero; Bologna	YES
897964c6-2c87-4306-a163-aedc04d0962f	Bertoloni, G	06/1874	Emilia Romagna	Bologna	Faggeto della Vergine dell'Acero; appennino bolognese	YES
e9787b89-162d-4b33-a50a-683cf6eab246	Bertoloni, G	06/1874	Emilia Romagna	Bologna	Selva dell'Acero, tra Ca' d'Iberna e il Santuario; Bologna	YES
c5940fd2-6aa5-41a6-a4b2-fd7a4a81d8fc	Bertoloni, G	07/ 1833	Emilia Romagna	Bologna	Bosco della Vergine dell'Acero; Bologna	YES
f96d6f50-f0e3-4c16-9eac-666cc8fefcee	?	??/1828	Emilia Romagna	Bologna	O.b. Bologna (dall'O.b. di Lucca)	NO
6902b2d7-92bb-42b6-a258-1461a5f30da6	?	06/1848	Emilia Romagna	Bologna	Tra il Monte Granaglione e il Monte Cavallo,BO	NO
8b742b13-1d41-4fc2-87c9-ac939e19a475	A.Bertoloni nip.		Emilia Romagna	Bologna	Bologna	NO
c2f81b3e-8321-4dff-8d04-de8259e04963	S. Sansanelli, L. Magagnoli	07/2013	Emilia Romagna	Bologna	Orto Botanico Bologna	NO

3.3 Microscope analysis

To study the number and size of stomata on leaves, photographs were taken through a microscope. Since all samples from Antonio Bertoloni's Herbarium are of great scientific and historical importance, they require careful handling, leading to the use of a special technique to prepare the microscope slides. Instead of exploiting the leaf itself, a reproduction of its surface was made using nail polish. A thin layer of this product was applied to a tiny area of the underside of the leaf and allowed to dry. Once ready, this layer was carefully peeled off and placed on a slide. Light passing through the glass and layers of nail polish made the stomata clearly visible. Photos of the slide images were then captured using the camera integrated into the microscope and enhanced with Zeiss Zen software, which improved the clarity and detail of the images (Fig. 2 and Fig. 3).

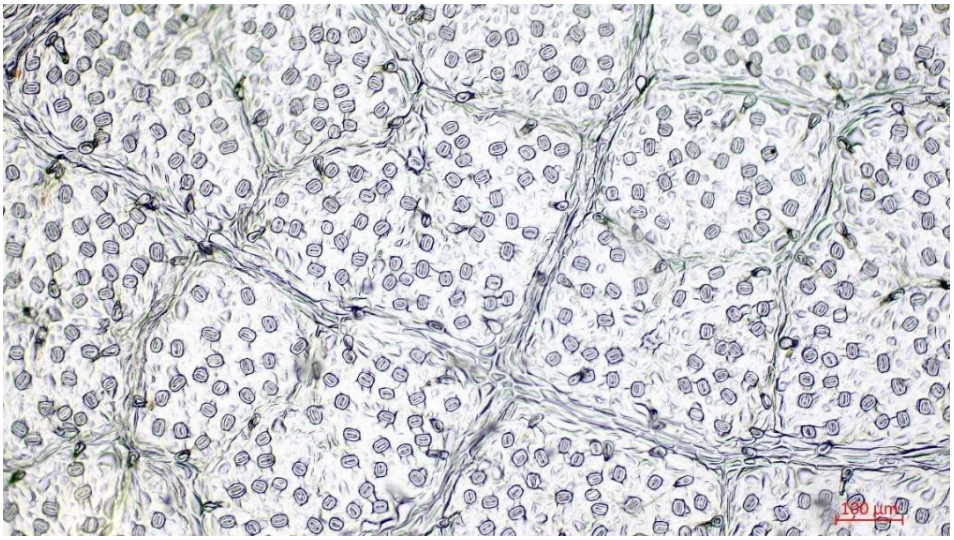

Figure 2 - *Micrography of a sample of* Quercus robur.

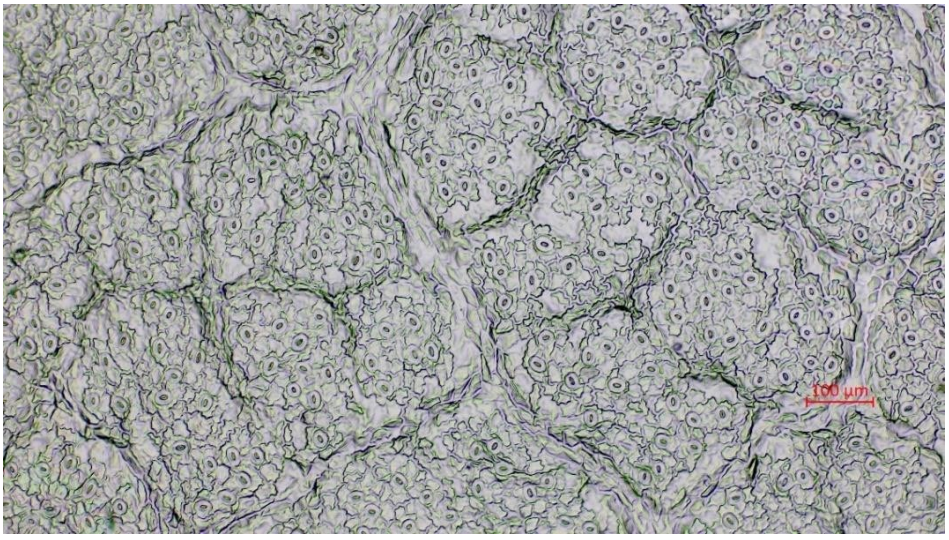

Figure 3 - *Micrography of a sample of* Fagus sylvatica.

3.4 Stomata counting

Once all the micrographs were prepared, a large amount of data was gathered. In the complex, 330 slides were obtained and divided into the following sections:

- 100 slides of *Quercus robur* prior to recent climate change;
- 60 slides of *Quercus robur* after recent climate change;
- 125 slides of *Fagus sylvatica* prior to recent climate change;
- 45 slides of *Fagus sylvatica* after recent climate change.

To achieve a statistically valid and adequate analysis of the number and size of stomata on each slide, a digital tool was needed. Therefore, two digital experimental procedures were used to count the stomata and measure their sizes, and a manual method was employed to verify their accuracy.

Because of this detailed and empirical process, only a subset of all the slides was used for the statistical analysis. Specifically, from each category (*Quercus robur* prior to recent climate change (QR), *Quercus robur* after recent climate change (OQ), *Fagus sylvatica* prior to recent climate change (FS), *Fagus sylvatica* after recent climate change (OF)), ten slides were examined for stomata count analysis, and five slides were studied for stomata size.

Once the data was collected through different counting processes, R Studio was used to create graphs and perform statistical analysis.

3.4.1 Manual counting

The manual counting involved an industrious but straightforward process. Stomata were counted, and their size was estimated by assuming the stoma has an elliptical shape and measuring the two axes of the ideal ellipse.

3.4.2 Digital counting through Python programming

A program was created using Python. Its main purpose is to place a red dot on each region of the slide image identified as a stoma, enabling later counting and analysis of these features.

First of all, once the image under examination is uploaded, the program converts it to grayscale to improve the effectiveness of segmentation and thresholding operations. The software also uses an algorithm to automatically find a threshold value that distinguishes the light background from the

dark structures and to eliminate small objects, which are considered just noise in the micrograph. Afterwards, the program begins calculating different geometric properties of the objects in the micrograph that are identified as possible stomata, such as area, eccentricity, barycenter, and so on. In particular, the software analyzes regions measuring between 60 and 700 pixels (the program can freely convert pixels to micrometers using the reference bar drawn in each image). The original micrograph is then modified by adding red dots at the barycenter of each structure identified as a stoma (Fig. 4). Finally, the new image is automatically saved, and the results, including the number and area of stomata, are compiled into a file (text) (Fig. 5).

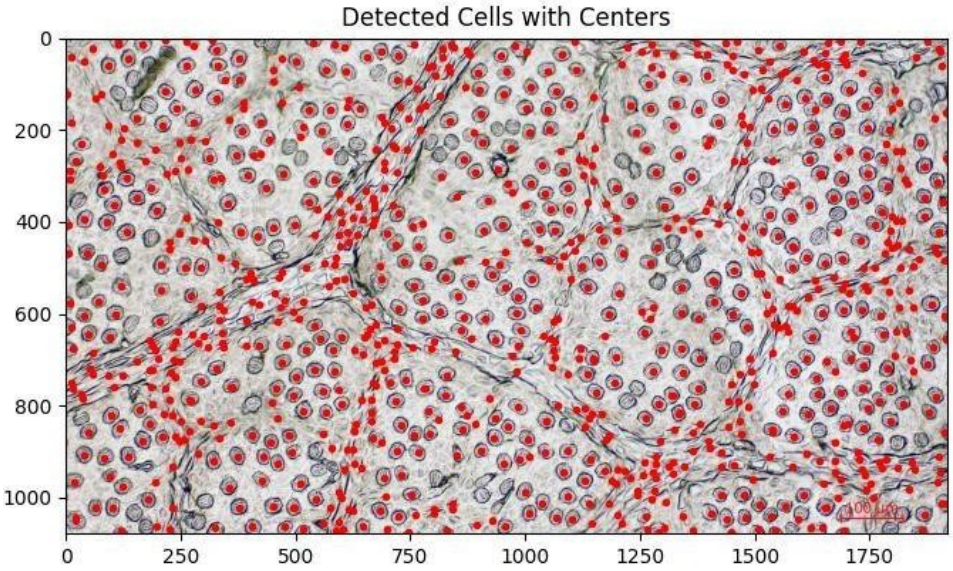

Figure 4- *Analysis of the micrography with the addition of red dots by the program.*

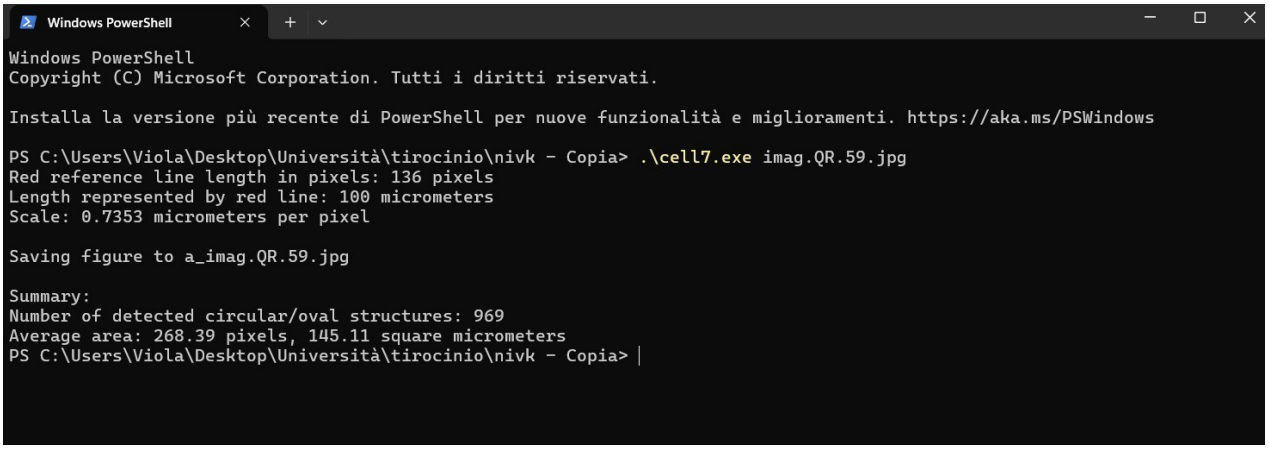

Figure 5 - *Example of how the program identifies the slide image to work with and reports the information.*

At first glance, it is clear that this program appears to overestimate the number of stomata. This likely occurs because some of the leaf vein structures share similarities with the empirical parameters used by the software to identify stomata. A more detailed analysis of this potential overestimation will be conducted in the results section.

3.4.3 AI counting

An algorithm based on machine learning is being developed. The focus is on achieving maximum accuracy in recognizing the stomata on leaf samples and confidently differentiating all other natural leaf features present in the micrograph. For now, there is a confidence estimation of 77%, but this algorithm is still under improvement.

The developed method for analyzing stomatal images combines digital image processing techniques with supervised machine learning models. Starting from microscopic images, a feature extraction process was carried out using the EBImage package in R. This step is essential because it converts complex, unstructured visual data into a set of understandable numerical variables, such as area, perimeter, eccentricity, orientation, and the ratio of the major to minor axes of the segmented objects. These features not only describe the shape of the stomata but also identify structural differences and shape variations that could significantly influence the final stomatal count.

Once this feature space was defined, a Random Forest model was selected, as it is especially suitable for small datasets with potentially correlated variables. Because of its ensemble approach based on decision trees, the Random Forest algorithm can handle noisy data and decrease overfitting risk, while also offering good interpretability of the results. To ensure a trustworthy evaluation of model performance, a repeated k-fold cross-validation strategy (with 100 repetitions) was used. This method provided more consistent error estimates (MAE and RMSE), offsetting variability caused by the random data splits.

The result is a robust and automated approach that not only reduces the subjectivity inherent in manual stomatal counting but also enables greater scalability in future analyses.

Using the AI algorithm, only the stomata number data can be obtained. The analysis of the stomata area with this method still needs to be developed.

RESULTS

4.1. Python program validity

The first analysis concerns the validity of the data from the Python program. A comparison between this data and manual counting was conducted (Fig. 6, Fig. 7, Fig. 8, Fig. 9, Fig. 10, Fig. 11, Fig. 12 and Fig. 13).

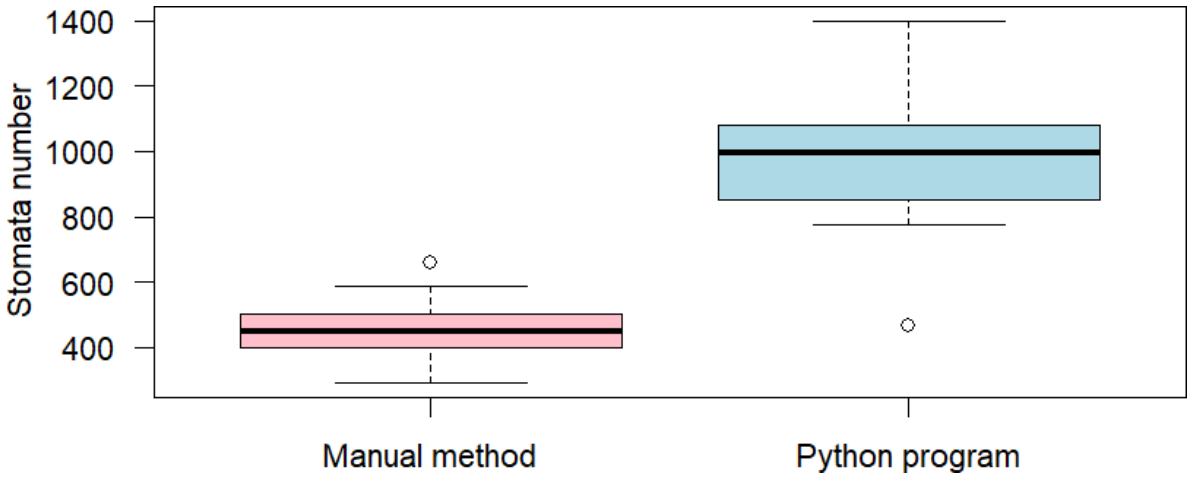

Fig. 6 – *Stomata number of samples of* Quercus robur *species prior to climate change, with a specific comparison between data collected through the manual method and the Python program approach.*

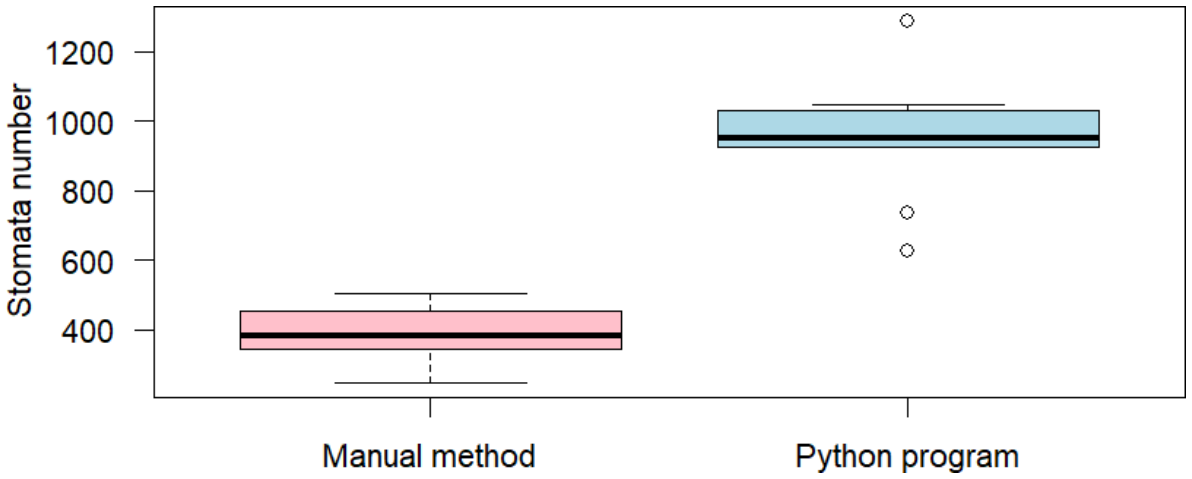

Fig. 7 – *Stomata number of samples of* Quercus robur *species after recent climate change, with a specific comparison between data collected through the manual method and the Python program approach.*

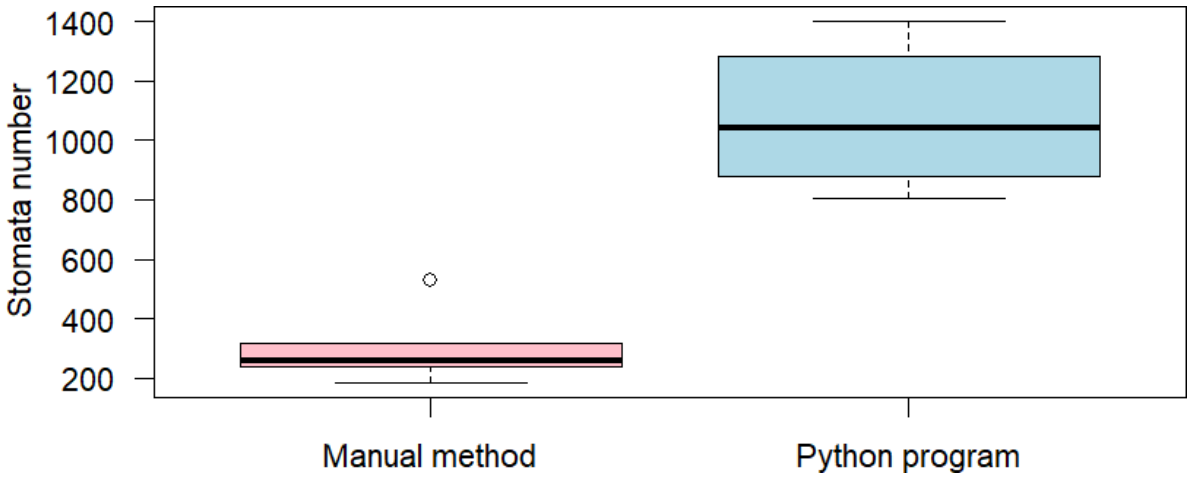

Fig. 8 – *Stomata number of samples of* Fagus sylvatica *species prior to climate change, with a specific comparison between data collected through the manual method and the Python program approach.*

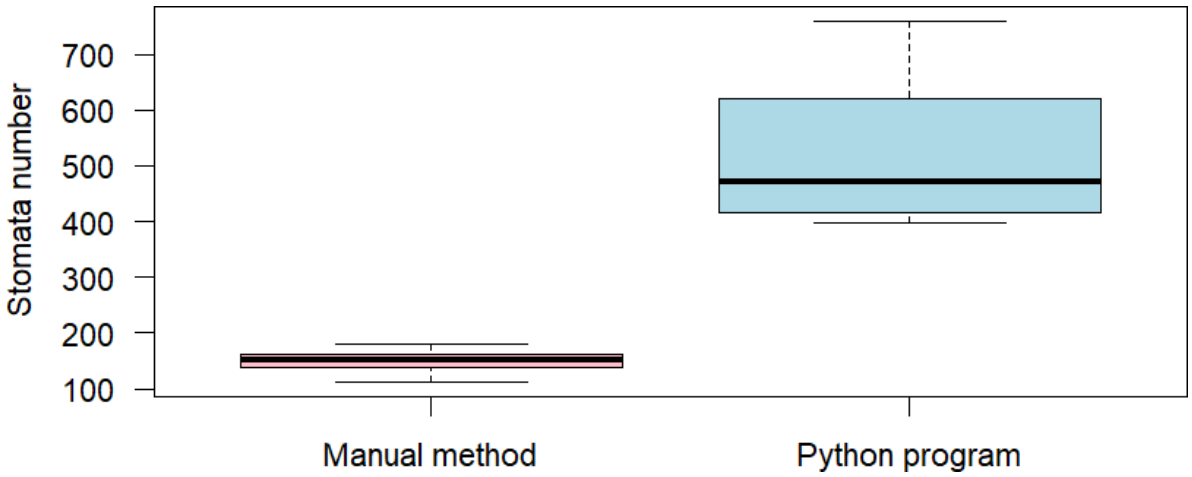

Fig. 9 – *Stomata number of samples of* Fagus sylvatica *species after recent climate change, with a specific comparison between data collected through the manual method and the Python program approach.*

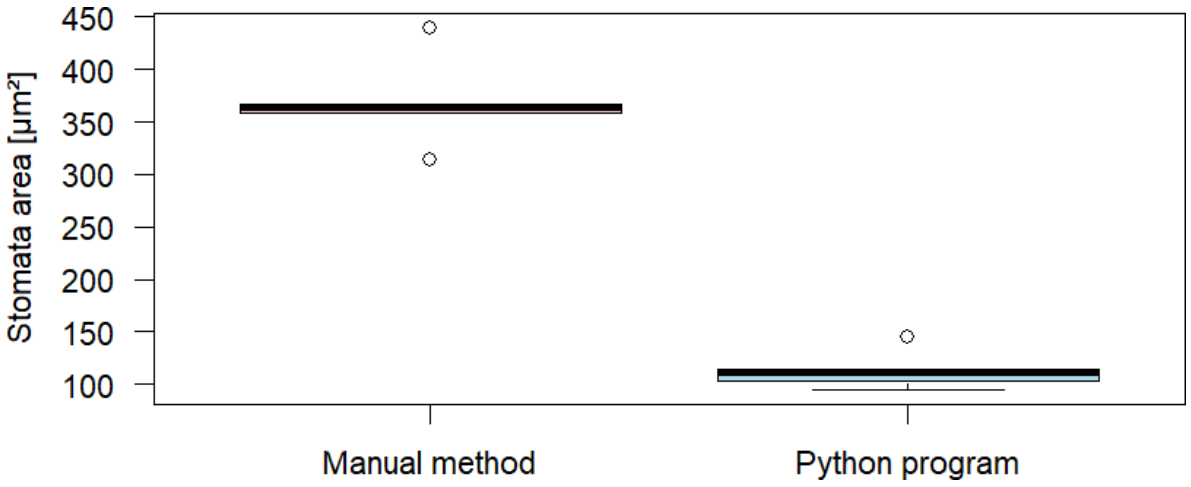

Fig. 10 – *Stomata area of samples of* Quercus robur *species prior to climate change, with a specific comparison between data collected through the manual method and the Python program approach.*

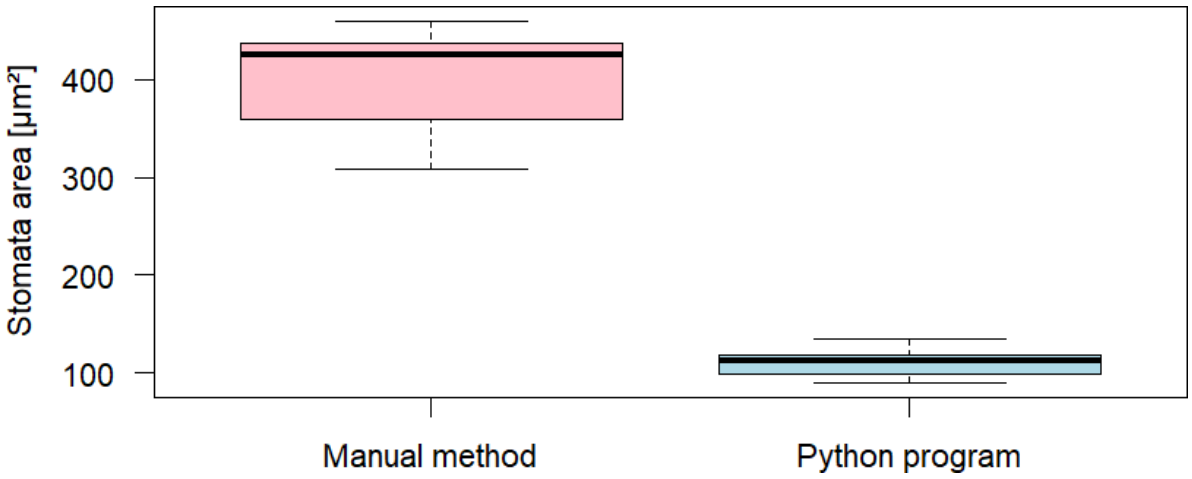

Fig. 11 – *Stomata area of samples of* Quercus robur *species after recent climate change, with a specific comparison between data collected through the manual method and the Python program approach.*

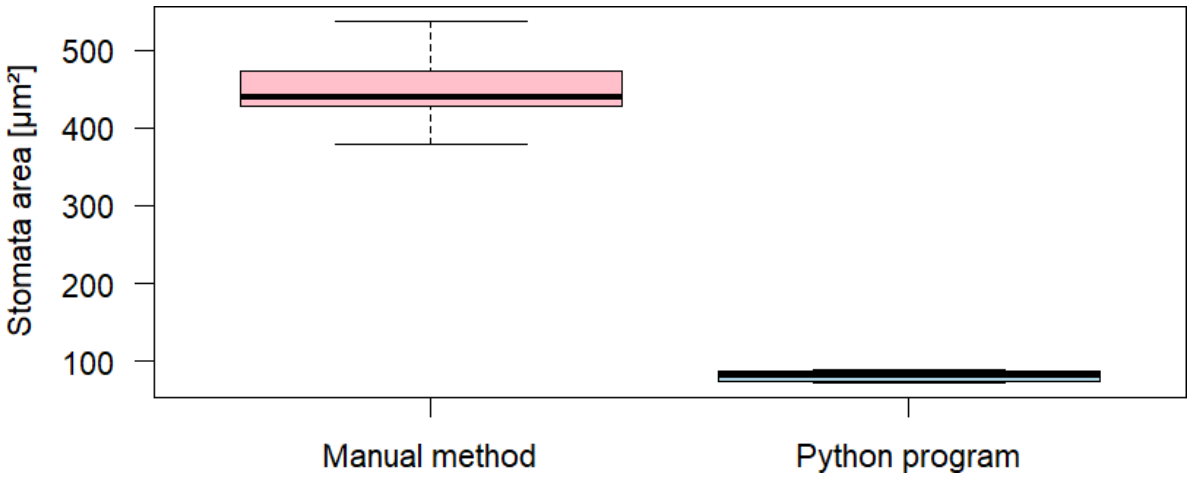

Fig. 12 – *Stomata area of samples of* Fagus sylvatica *species prior to climate change, with a specific comparison between data collected through the manual method and the Python program approach.*

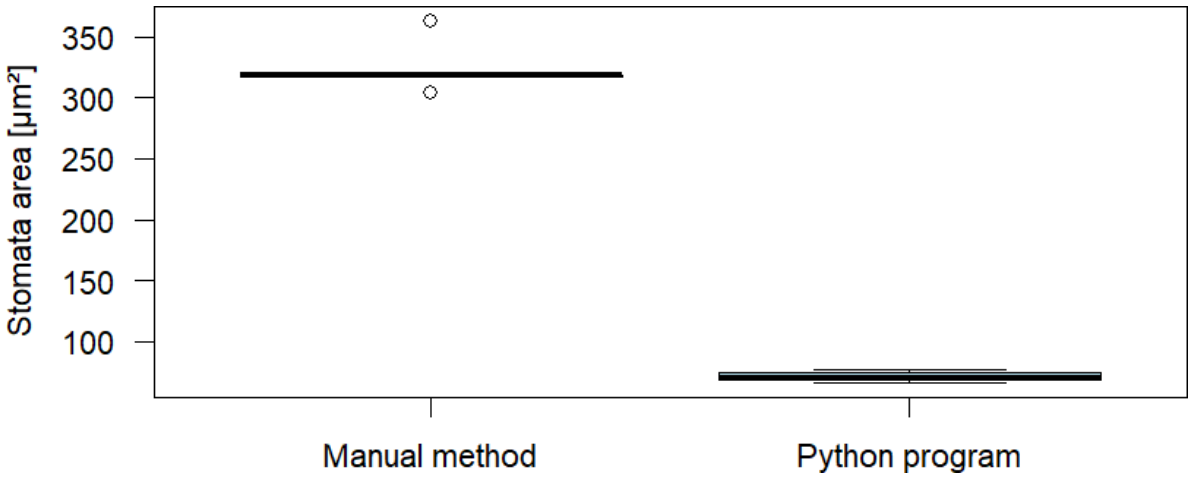

Fig. 13 – *Stomata area of samples of* Fagus sylvatica *species after recent climate change, with a specific comparison between data collected through the manual method and the Python program approach.*

In all eight figures shown above, both for analyzing the number and size of stomata, it is clear that the Phyton program is not as accurate as needed and expected. In fact, when it comes to the number of stomata on the leaf sample, there is significant overestimation by the software; conversely, for stomata size, an underestimation seems to occur.

Due to the uncertainty of this Python program, this method is not used in this particular study. The results will be based on data collected manually, including counting the stomata and measuring the axes of their elliptical shapes, as well as using the AI algorithm approach, specifically for the stomata number analysis.

4.2 Stomata number analysis.

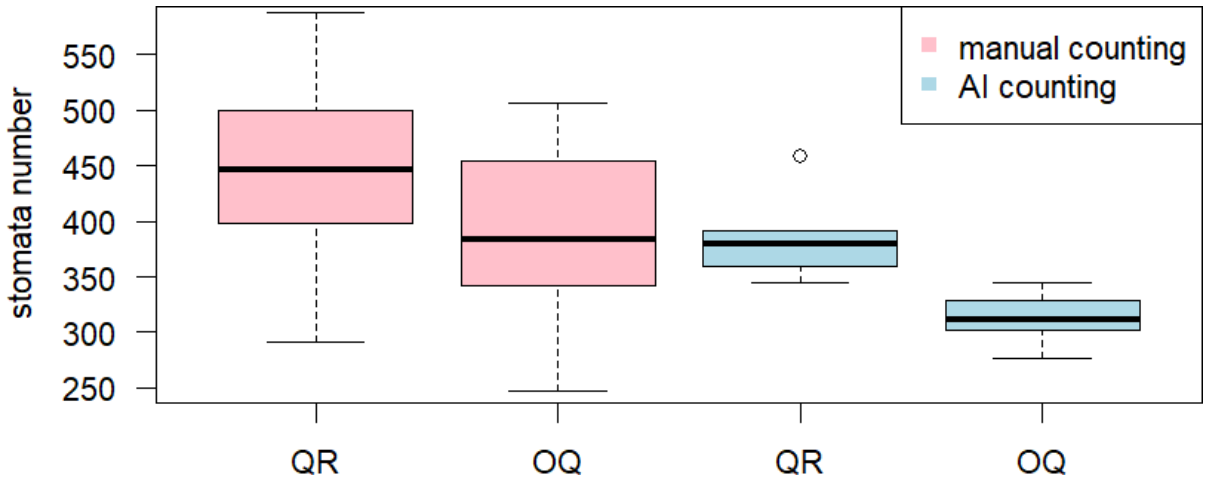

Figure 14 – *Stomata number of samples from the* Quercus robur *species, shown in four boxplots based on the counting method (manual or AI) and the collection period (prior to recent climate change (QR) or after recent climate change (OQ)).*

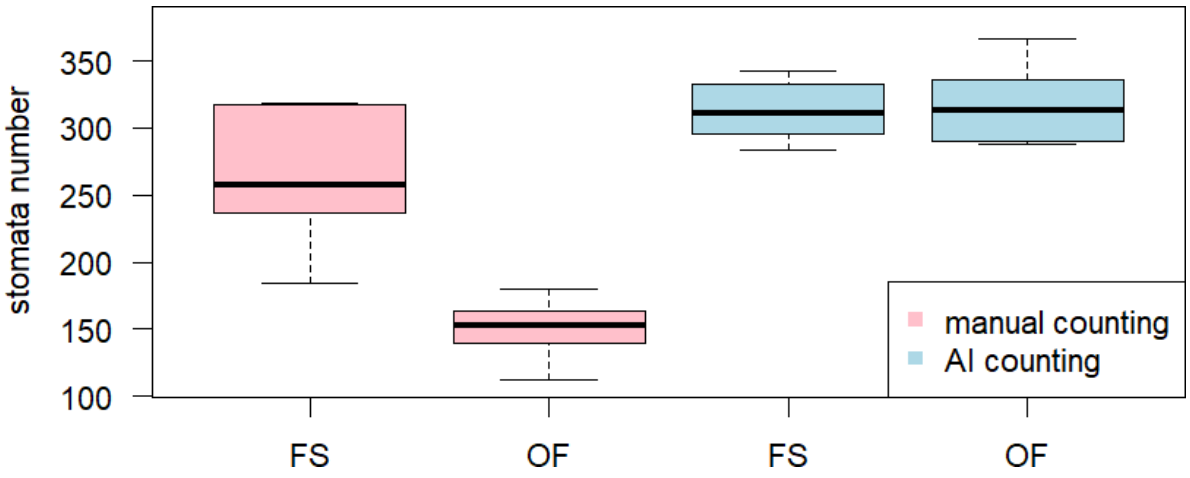

Figure 15 – *Stomata number of samples from the* Fagus sylvatica *species, shown in four boxplots based on the counting method (manual or AI) and the collection period (prior to recent climate change (FS) or after recent climate change (OF)).*

The graphs show differences in the number of stomata on the micrographs for both species (Fig. 14 and Fig. 15). However, to analyze the data more accurately statistically, Wilcoxon-Mann-Whitney tests are performed for each comparison.

The most confident value is definitely the one related to the comparison between manually collected data of *Fagus sylvatica*, where the p-value from the Wilcoxon-Mann-Whitney test is 1.083e-05, providing strong statistical evidence of the difference in stomata number of samples before and after Climate Change effects.

Another statistically significant difference in stomata counting is observed between the "past leaves" and the "current leaves" of *Quercus robur* analyzed with the AI algorithm, since its p-value is 0.000211. However, some uncertainty should be considered when interpreting this result, as the same *Quercus robur* values obtained through manual counting do not reach statistical significance in the Wilcoxon-Mann-Whitney test (p-value of 0.1735). Since the AI algorithm has a confidence level of 77% (as explained in 3.4.3), and manual counting can almost achieve full validity, the possibility of errors in the AI calculation must be taken into account.

The opposite situation occurs in samples of *Fagus sylvatica*. Contrary to the manual counting analysis, where the Wilcoxon-Mann-Whitney test confirms a statistically significant difference, the p-value for the comparison between data collected via the AI procedure is very high (0.8501), indicating an almost nonexistent difference between the two groups.

4.2 Stomata area analysis.

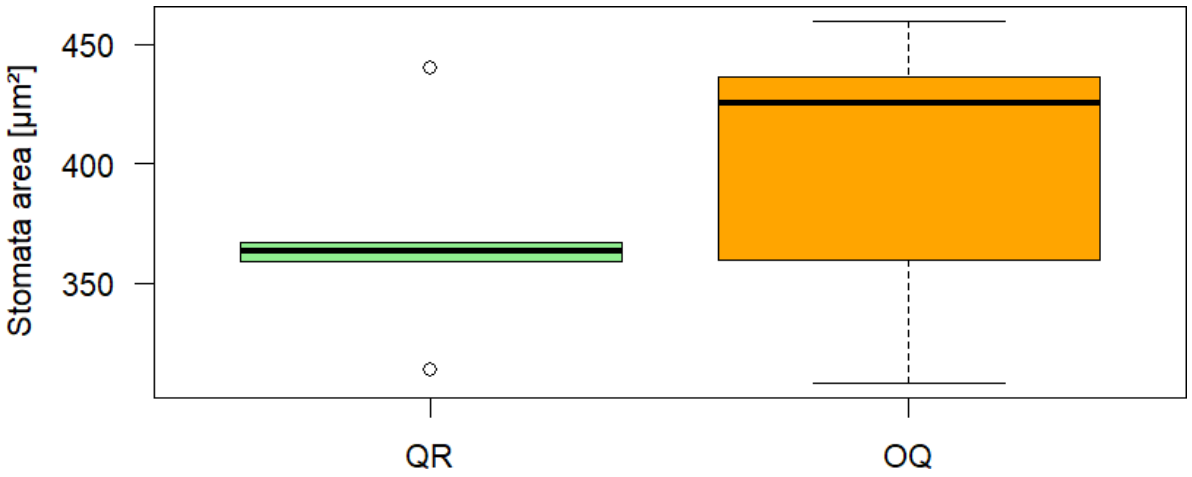

Figure 16 – *Stomata area of samples from the* Quercus robur *species, shown in two boxplots based on the collection period (prior to recent climate change (QR) or after recent climate change (OQ)).*

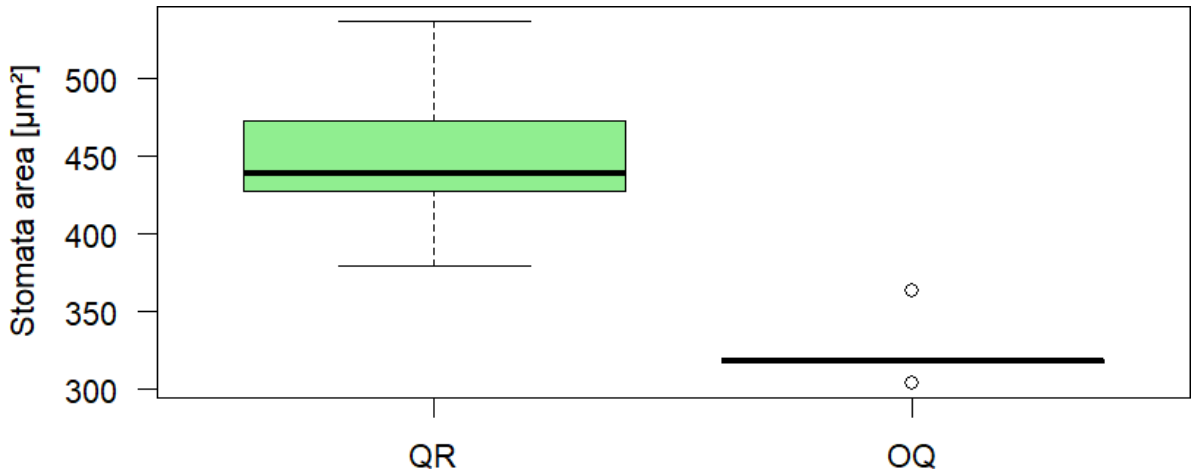

Figure 17 – *Stomata area of samples from the* Fagus sylvatica *species, shown in two boxplots based on the collection period (prior to recent climate change (FS) or after recent climate change (OF)).*

Also in this case (Fig. 16 and Fig.17), the *Fagus sylvatica* samples exhibit a clear difference in their data between the samples prior to climate change effects and those afterward. This is confirmed also by the Wilcoxon-Mann-Whitney test, with a p-value of 0.007937. Conversely, once again, *Quercus robur* samples do not show significant differences, as indicated by the very high p-value of 0.6905, which points to the statistical insignificance of the comparison. Actually, this time the graphs regarding *Quercus robur* species show results, even if not statistically significant, that are opposite to those seen in the case of stomata number analysis (4.2). In that case, stomata number seems to decrease over time, whereas now it appears that the stomata area increases.

Just out of curiosity, it is also possible to analyze whether the area changes proportionally in both axes or if one dimension experiences a greater difference than the other (especially in the case of Fagus sylvatica, where there is actual statistical significance).

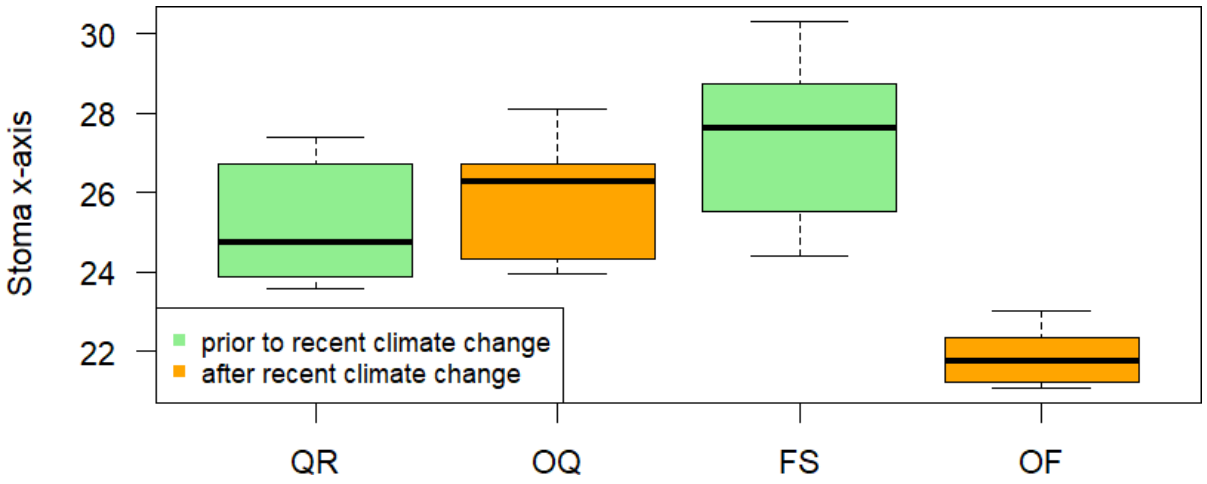

Figure 18 – *Lengths of the first of the two axes of the ideal elliptical shape of stomata, shown in four boxplots based on samples species (QR and OQ are referred to* Quercus robur *and FS and OF are referred to* Fagus sylvatica*) and on the collection period (as explained in the legend, QR and FS concern samples prior to recent climate change, while OQ and OF concern samples after recent climate change).*

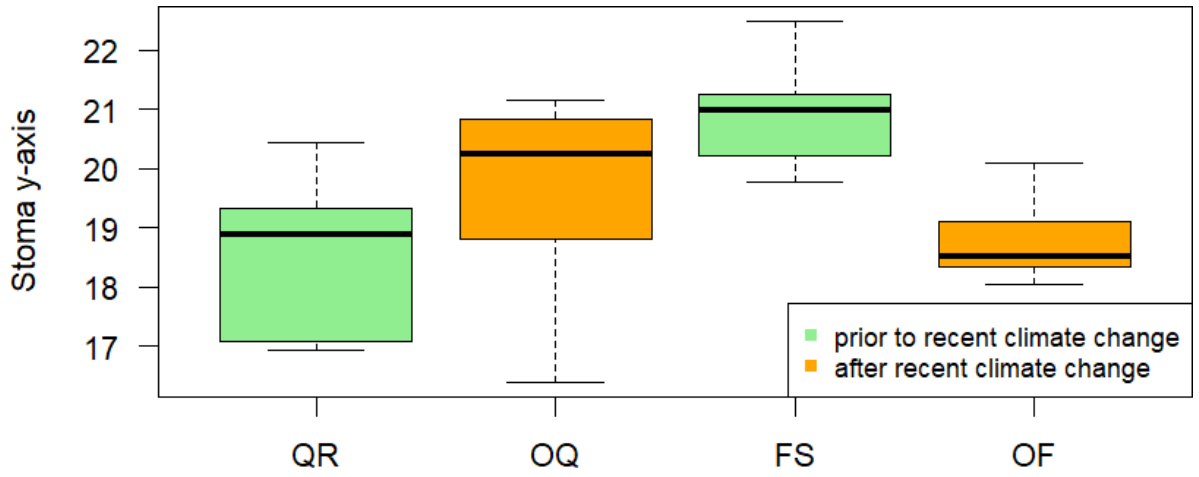

Figure 19 – *Lengths of the second of the two axes of the ideal elliptical shape of stomata, shown in four boxplots based on samples species (QR and OQ are referred to* Quercus robur *and FS and OF are referred to* Fagus sylvatica*) and on the collection period (as explained in the legend, QR and FS concern samples prior to recent climate change, while OQ and OF concern samples after recent climate change).*

In both graphs (Fig. 18 and Fig.19), the data from *Fagus sylvatica* are consistent with the analysis of the area obtained above (4.3), showing a reduction in the length of both axes of the ideal ellipse. In contrast, the data for *Quercus robur* are more ambiguous, as the x-axis dimension does not seem to change, while the y-axis appears to vary but not in a statistically significant way, according to the Wilcoxon-Mann-Whitney test (the p-value is 0.5476).

DISCUSSION

The primary aim of this study was to investigate how climate change has influenced, and continues to influence, plant physiology, with a particular focus on stomatal traits. Stomatal density and size were selected as key indicators, given that stomata regulate water–gas exchange and are directly affected by three of the most critical environmental variables associated with climate change: elevated atmospheric CO_2, rising temperatures, and drought stress (Hetherington & Woodward, 2003; Franks et al., 2017).

Historical and contemporary studies provide a foundation for interpreting stomatal responses under changing climatic conditions. Woodward (1987) was among the first to report a ~40% decline in stomatal density over the last two centuries, a trend interpreted as an adaptive response to rising atmospheric CO_2 concentrations. More recent reviews have reinforced this inverse relationship, although they caution against reductionist interpretations that overlook the interplay of multiple stressors (Yan et al., 2017). In particular, drought and elevated temperatures often drive reductions in stomatal number and size, creating potentially confounding effects when isolating the influence of CO_2 alone (Lawson & Blatt, 2014).

The findings from *Fagus sylvatica* in the present study are broadly consistent with these established patterns. Both the data presented here and results from Condorelli (2024), based on Bertoloni's herbarium collections, indicate a clear reduction in stomatal density, accompanied by a decrease in stomatal size. Such changes may represent a physiological strategy to prevent excessive CO_2 uptake while limiting transpirational water loss, consistent with predictions of stomatal optimization theory (Franks & Beerling, 2009).

Conversely, *Quercus robur* did not exhibit the expected trend, with no statistically significant differences in stomatal density between pre- and post-industrial samples. Several factors may explain this discrepancy. First, the limited sample size reduces the statistical robustness of the findings, as manual verification was required to supplement digital image analysis. Second, differences in sampling methodology may have introduced bias: while *F. sylvatica* samples could be obtained from young individuals within beech forests, *Q. robur* samples were restricted to sites with fewer individuals, where trunk diameter criteria were not always met. If stomatal plasticity is indeed more pronounced in younger trees, as suggested by our working hypothesis, the inability to standardize age classes in *Q. robur* could partly account for the absence of a detectable trend. This interpretation

aligns with the view that stomatal traits may be subject not only to short-term plastic responses but also to long-term evolutionary adjustments affecting plant fitness (Hetherington & Woodward, 2003; Assmann & Zeiger, 2020).

Overall, the evidence highlights both the potential and the limitations of using stomatal traits as proxies for climate change impacts. Although numerous studies support a consistent inverse relationship between atmospheric CO_2 and stomatal density (Woodward, 1987; Yan et al., 2017b), the interaction of additional stressors—such as drought, temperature extremes, and species-specific genetic factors—complicates interpretation. Indeed, there is still no consensus as to whether climate change universally induces a decline in stomatal density, or whether responses are species- and context-dependent (Xu et al., 2016). The lack of reproducible experimental criteria further underscores the need for caution in drawing definitive conclusions.

The use of historical herbarium collections, such as Bertoloni's "Erbario," represents a powerful approach to studying long-term stomatal responses under natural environmental conditions. These collections provide invaluable time-series data that may circumvent some of the limitations of short-term experiments conducted under artificial growth conditions. At the same time, advances in automated digital image analysis, including the application of artificial intelligence and machine learning tools, could significantly improve the accuracy and scalability of stomatal trait quantification, thereby enhancing the statistical reliability of future studies (Fetter et al., 2019).

In conclusion, while this study corroborates previous evidence of declining stomatal density in *F. sylvatica*, the contrasting results for *Q. robur* illustrate the complexity of plant responses to climate change. This complexity arises from the interplay of environmental variables, sampling limitations, and potential evolutionary differences between species. Continued integration of herbarium-based research with technological innovation in trait measurement will be essential for disentangling these dynamics and for developing a more comprehensive understanding of how climate change shapes plant physiology.

REFERENCES

- Ahuja I., Ric C. H. de Vos, Bones A. M, Hall R. D. (2010). Plant molecular stress responses face climate change. Volume 15, Issue 12, Pages 664-674.
- American Metereological society site
- Anjum S. A., Xie X., Wang L., Saleem M. F., Man C., Lei W., (2011). Morphological, physiological and biochemical responses of plants to drought stress. African Journal of Agricultural Research Vol. 6(9), pp. 2026-2032.
- Assmann, S. M., & Zeiger, E. (2020). Guard cell sensory systems: recent insights on stomatal responses to light, abscisic acid, and CO_2. *Plant Physiology, 182*(4), 1646–1656.
- Chaudhry S., Sidhu G. P. S. (2022). Climate change regulated abiotic stress mechanisms in plants: a comprehensive review. Plant Cell Reports 41:1–31.
- Condorelli, L. (2024). [Study on Fagus sylvatica stomatal responses using Bertoloni's herbarium collections].
- Fetter, K. C., et al. (2019). StomataCounter: a neural network for automatic stomata identification and counting. *New Phytologist, 223*(3), 1671–1681.
- Framework Convention on Climate Change convention (FCCC).
- Franks, P. J., & Beerling, D. J. (2009). Maximum leaf conductance driven by CO_2 effects on stomatal size and density over geologic time. *PNAS, 106*(25), 10343–10347.
- Franks, P. J., et al. (2017). Increasing water-use efficiency directly through genetic manipulation of guard cell signalling. *Nature Communications, 8*, 128.
- Gray J. E., Holroyd G. H., Van der Lee F. M., Bahrami A. R:, Sijmons P. C., Woodward F. I. ed al. (2000). The HIC signalling pathway links CO2 perception to stomatal development. Nature volume 408, pages713–716.
- Hetherington A. M., Woodward F. I. (2003). The role of stomata in sensing and driving environmental change. Nature volume 424, pages901–908.
- Lake J.A., Quick W.P., Beerling D.J., Woodward F.H. (2001). Plant development: signals from mature to new leaves. Nature 411:154.
- Lawson, T., & Blatt, M. R. (2014). Stomatal size, speed, and responsiveness impact on photosynthesis and water use efficiency. *Plant Physiology, 164*(4), 1556–1570.

- Lidner M., Maroschek M., Netherer S., Kremer A., Barbati A., Garcia-Gonzalo J. et al (2010). Climate change impacts, adaptive capacity, and vulnerability of European forest ecosystems. Forest Ecology and Management 259, 698–709.
- Sato H., Mizoi J., Shinozaki K., Yamaguchi-Shinozaki K. (2024). Complex plant responses to drought and heat stress under climate change. The Plant Journal, 117, 1873–1892.
- Urry, Cain, Wasserman, Minorsky (2020). Campbell Biology in Focus. Third edition.
- Woodward, F. I. (1987). Stomatal numbers are sensitive to increases in CO_2 from pre-industrial levels. *Nature, 327*(6123), 617–618.
- Woodward F. I., Lake J. A. Quick W. P. (2002). Stomatal Development and CO2: Ecological Consequences. The New Phytologist, Vol. 153, No. 3, Special Issue: Stomata, pp. 477-484.
- Xu, Z., et al. (2016). Plant responses to drought and rewatering. *Plant Signaling & Behavior, 11*(2), e1189355.
- Yan W., Zhong Y., Shangguan Z. I. (2017a). Contrasting responses of leaf stomatal characteristics to climate change: a considerable challenge to predict carbon and water cycles. Global Change Biology (2017) 23, 3781–3793.
- Yan, W., Zhong, Y., & Shangguan, Z. (2017b). A meta-analysis of the responses of stomatal density to global environmental change. *Frontiers in Plant Science, 8*, 758.