Stomata morphological traits in two different genotypes of *Populus nigra* L.

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Populus nigra L. (black poplar) possesses amphistomatic leaves, with large (giant) and normal sized stomata. The role of giant stomata in leaf development, and the consequences on stomatal density in adult leaves remains elusive. This paper describes the characteristics of ordinary and giant stomata in leaves of two black poplar genotypes (*58-861* with large leaves from northern Italy, and *Poli* with small leaves from southern Italy). Stomatal traits in both genotypes were studied using light microscopy on mature leaf adaxial and abaxial epidermal impressions. Moreover, scanning electron microscopy was applied to study giant and normal stomata in early, young, and mature leaves. Leaf abaxial surfaces in the two genotypes revealed variable sizes and patterns of stomata related to differences in intrinsic water use efficiency (W_i). These observations provided evidence of different stomatal types in mature black poplar leaves, and new information regarding the presence and potential role of giant stomata in black poplar leaves.

Keywords: Giant Stomata, Poplar Clones, Genotypes, *Populus*, SEM

Introduction

Giant stomata (GS), as the name implies, are distinct from normal-sized stomata primarily due to size. In a treatise on the anatomy of dicotyledons, Metcalfe & Chalk (1979) described large stomata of approximately 40 μm in relatively few families. Shiraishi et al. (1975) reported structures in Satsuma Mandarin (*Citrus unshiu* Marc) similar to large-sized stomata, surrounded by a number of normal-sized stomata on juvenile leaves immediately after full leaf expansion. The giant type stomata observed in *C. unshiu* were consistent with GS examined and characterized by Sitholey & Pandey (1971) and Stace (1965) in different taxa.

Electron microscopy studies on subfossil Holocene *Salix herbacea* L. (Salicaceae) samples in Sweden suggested the presence of GS (Rundgren & Beerling 1999). Finally, Pautov (2009) reported an eterostomatal or-

ganization in *Populus tremula*, with three stomatal types, *i.e.*, paracitic, laterocitic, and intermediate anamo-paracytic.

Poplars (*Populus* spp.) are the fastest growing tree in temperate latitudes in Europe and North America (Marron et al. 2006) when raised under short rotation intensive culture conditions. Poplar is considered a model system for plant biology. *P. tricocarpa* is the first tree genome to be fully sequenced, and is now of high quality and relatively contiguous. Therefore, the genus offers many possibilities to study questions not easily addressed by genera considered model plant systems, *e.g.*, *Arabidopsis thaliana* (Jansson & Douglas 2007). Stomatal traits have been widely studied in *Populus*, as these traits are a useful criterion for clone discrimination in the genus. In particular, a series of clones have been explored that confirm general poplar micromorphologic fea-

tures, including amphistomatic leaves, with evidence some stomatal traits change depending on environmental conditions (Al Afas et al. 2006), and in response to growing conditions (Woodward et al. 2002, Hermle et al. 2007, Di Baccio et al. 2010, Jaime et al. 2014). For example, in *P. trichocarpa* stomata were not found on the adaxial leaf surface when grown under field conditions, however low stomatal density was observed on the adaxial surface under glasshouse conditions (Ceulemans et al. 1988, Radoglou & Jarvis 1990).

Despite frequent studies on stomata in poplar (Reich 1984, Ceulemans et al. 1988, Al Afas et al. 2006, Pearce et al. 2006, Cocozza et al. 2010, Woo 2010), reports of giant stomata in *Populus* remain equivocal.

Recently, interest in several poplar morphological and physiological traits has increased, with the aim of evaluating new breeding strategies for industrial applications (Kauter et al. 2003, Sebastiani et al. 2004). Additional knowledge of poplar stomata might support industrial objectives, since stomatal density has been demontrated to affect biomass production in different poplar clones (Al Afas et al. 2006).

Materials and Methods

Plant material and growth conditions

Observations were conducted using homogeneous 25 cm long woody stem cuttings obtained from two different *P. nigra* genotypes, *58-861* and *Poli*. The *58-861* genotype was the maternal parent from Val Cenischia (Torino, northern Italy - 597 m a.s.l., 45° 09′ N, 07° 01′ E), and *Poli* was obtained from Policoro (Matera, southern Italy - 7 m a.s.l., 40° 09′ N, 16° 41′ E).

Full-sib families of both genotypes were planted in April 2008 in an open field at *Azienda Didattico Sperimentale* "*Nello Lupori*" in Viterbo (Italy, 42º 25′ N, 12º 05′ E - 309 m a.s.l.). The 0.1230 ha experimental site, considered a short rotation coppice, had alternating inter-row distances of 2 m and 0.75 m between plants in a single row. Soil was composed of sand (57.5%), lime (34.6%), and clay (7.9%), with a pH of 7.1. A randomized complete block design (five blocks) was arranged; each block included a group of 160 F1 clones, and two pairs of parental clones (two *Poli* and two *58-861* clones). A total number of 820 plants were cultivated in the entire plantation (frequency of 6666 plants ha^{-1}). Due to edge effects, the two poplar rows on the outermost border were not included in the experimental design. The plantation was irrigated shortly after planting, and a third class herbicide with weed control measures was applied to obtain optimal field establishment. Average daily temperature and rainfall in the 2008 growing

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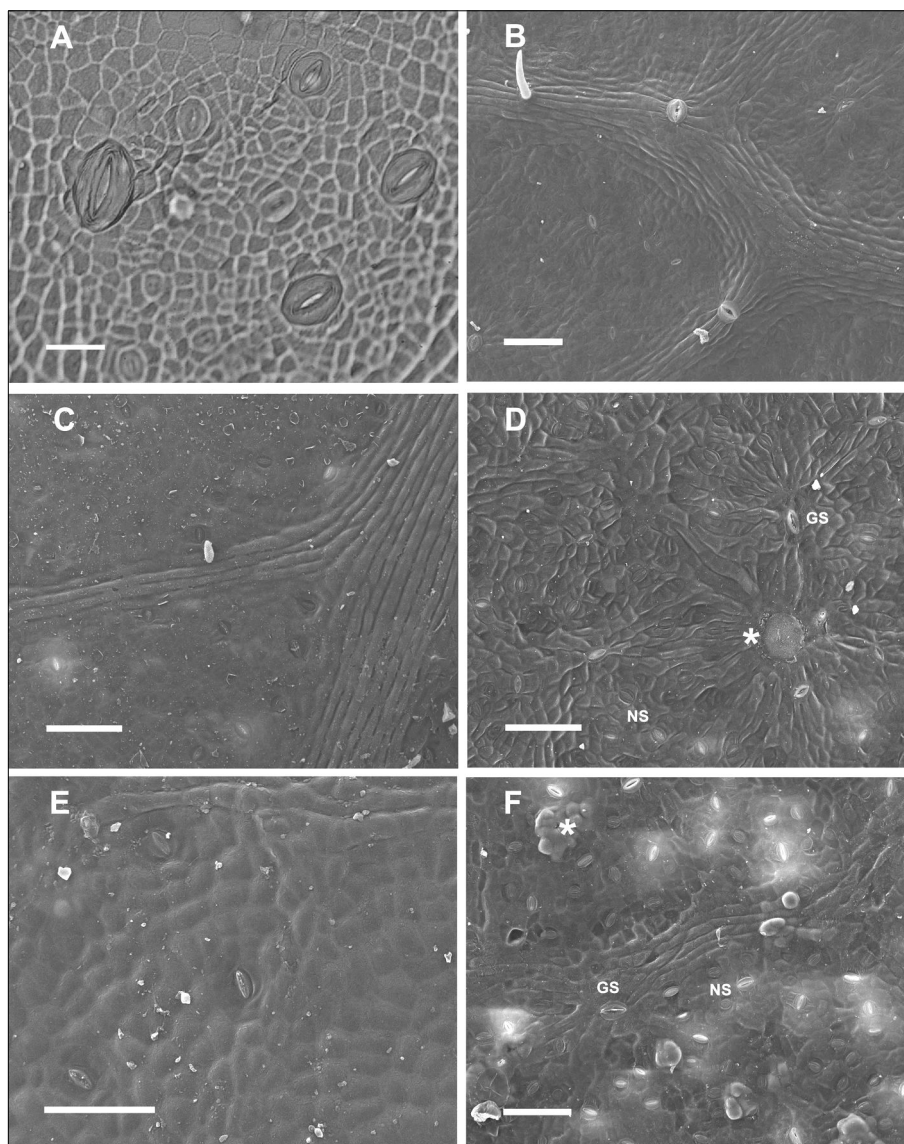
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Fig. 1 - *Populus nigra* L. (A-D): genotype *Poli*; (E-F); genotype *58-861*. (A): Light image from nail polish impression of the adaxial surface of early leaf showing developing stomata, bar = 20 μm. (B): SEM image of the adaxial surface of a young leaf showing trichomes and water stomata, bar = 100 μm. (C): SEM image of the adaxial surface of a mature leaf, bar = 100 μm. (D): SEM image of the abaxial surface of a mature leaf showing anomocytic giant stomata (GS), GS covered by wax (asterisk), paracytic normal stomata (NS), bar = 100 μm. (E): SEM image of the adaxial surface of a mature leaf, bar = 100 μm. (F): SEM image of the abaxial surface of a mature leaf showing GS frequently covered by wax plugs, bar = 100 μm.

season were respectively 22.89 °C and 14.4 mm. Only parentals (10 *Poli* and 10 *58-861* clones) were examined for observations.

WUE measurements

Complete leaf development was examined by observing leaves throughout the entire growing season (June - September 2008) in 30-day intervals from different leaf generations. Selected leaves marked by small ribbons around the petiole were observed daily, beginning with a young (~4.8 cm in *Poli* and ~5.6 in *58-861*) to mature totally expanded leaf condition, following the Plastochron Index method (Erickson & Michelini 1957). The same leaves were analyzed to obtain physiological measurements (assimilation and stomatal conductance) in the hours before dawn. Excised leaves were placed in water filled test tubes to avoid cavitation, protected in a portable freezer, and transported to the laboratory. A LI-6400 photosynthesis system (LICOR, Nebraska, USA)

was programmed to register gas exchange at 380 ppm relative to a CO_2 reference value. Intrinsic water use efficiency (W_i) was determined as the ratio between assimilation (A), expressed in μmol CO_2 m^{-2} s^{-1} and stomatal conductance (g_s), expressed in mol H_2O m^{-2} s^{-1}.

Statistical analyses

Data management and statistical analyses were performed with SYSTAT® 12 (Systat software, Chicago, IL) using 20 replicates of poplar genotypes (10 *Poli* and 10 *58-861* clones). A preliminary GS frequency analysis revealed a Poisson-shaped, zero-bounded distribution of counting data (x), which were therefore transformed as √x.

General Linear Model (GLM) was applied to compare different generations of leaves because of their small sample size and non-normal distribution. Differences among mean values were tested by *post-hoc* analyses using the Bonferroni's test (α = 0.05),

which was considered the most appropriate due to the limited number of samples analyzed. Bonferroni methods for multiple comparisons are extended to sequential setting and have shown to attain an approximately 50% reduction in the expected sample size compared with earlier approaches (De & Baron 2012).

Linear regression was applied to evaluate relationships between intrinsic W_i and GS pore length traits. Statistical analyses were not applied to young leaf attributes, because the sample number was considered insufficient to establish a clear relationship between intrinsic W_i and GS pore length traits.

Light and scanning electron microscopy (SEM)

For light microscopy, mature leaves were used to produce impressions, prepared with clear nail polish parallel to the midrib, from the basal to apical position, and on both adaxial and abaxial surfaces. All impressions

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Tab. 1 - Number and frequency (= nGS / total number of stomata) of GS in different leaf sections and in both genotypes on the abaxial surfaces of mature leaves. The number of leaves/replicates used for each poplar clone was 20. Frequencies were transformed as √x. (n.s.): not significant.

Genotype	Parameter	GS counts			GS frequency		
		Apical	Medium	Basal	Apical	Medium	Basal
Poli	Mean	1.29	1.00	1.05	0.08	0.06	0.07
	Std. Err.	0.18	0.14	0.19	0.01	0.01	0.01
58-861	Mean	1.45	1.20	1.10	0.08	0.06	0.06
	Std. Err.	0.15	0.19	0.16	0.01	0.01	0.01
-	p-value	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

were fixed on glass slides, and examined under a Leica DM4000B light microscope connected to a Leica DFC420 camera at 200x magnification. Leica Application Suite (LAS) software was used to analyze images. Three sampling areas (apical, medium, and basal; 126 588 μm^2 each) were randomly chosen from every slide sample for microscopic observations. Pore length and giant and normal stomata were studied on the abaxial surface of mature leaves. The absence of a cuticular ridge on the adaxial surface impressions resulted in unreliable data for GS statistical analyses on this surface.

To identify qualitative differences in stomata morphology during leaf development, further observations were performed on three categories of leaves: (i) early (~2.3 cm long in *Poli* and ~2.5 cm long in *58-861*); (ii) young (~4.8 cm long in *Poli* and ~5.6 cm long in *58-861*); and (iii) mature (~6.00 cm long in *Poli* and ~10.00 cm long in *58-861*). Each leaf developmental stage from each genotype was used to make impressions prepared with clear nail polish parallel to the midrib for light microscopy.

For SEM, samples from early, young, and mature *Poli* and *58-861* leaves were preserved in 1:1 H_2O:ethyl alcohol at 4 °C. Specimens were dehydrated in an ethanol series, critical-point dried in liquid CO_2, and coated with 30 nm of gold. Specimens were subsequently observed under a FEI-Quantas 200 ESEM, at the CISME center of the *Università degli Studi di Napoli "Federico II"*. Stomatal types were classified according to Carpenter (2005) and Metcalfe & Chalk (1979).

Results and discussion

Observations from light and SEM microscopy

Large stomata, approximately 40 μm in length, with anomocytic type structure were surrounded by normal stomata with paracytic structure and meristemoid cells on the developing lamina in early *Poli* (Fig. 1A) and *58-861* leaves (data not shown). Trichomes and presumed water stomata (~50 μm long) were observed on adaxial surface venation in both genotypes; however only genotype *Poli* is reported here (Fig. 1B). In mature *P. nigra* leaves, GS with anomocytic structure were

surrounded by normal stomata 20 to 30 μm long with paracytic structure (Fig. 1C, F). Initiation of normal stomata might continue on adaxial and abaxial surfaces, until the leaf has reached approximately 60% of its final size.

A progressive decrease in stomata length (from ~40 μm in GS to ~20 μm in normal stomata) was observed in subsequent divisions, with a concomitant transition from anomocytic to paracytic structure. The sequence of events in stomatal development

and pattern formation during the early stages of leaf development observed in *P. nigra* genotypes were similar to developmental stages demonstrated in *A. thaliana* (Von Groll & Altmann 2001, Bergmann & Sack 2007, Delgado et al. 2011), and in early divergent angiosperms (Rudall & Knowles 2013). The distinct leaf stomatal pattern likely arose from GS that developed early in leaf ontogenesis, forming anomocytic stomata in primary lineages, and corresponding smaller stomata with paracytic structures in satellite li-
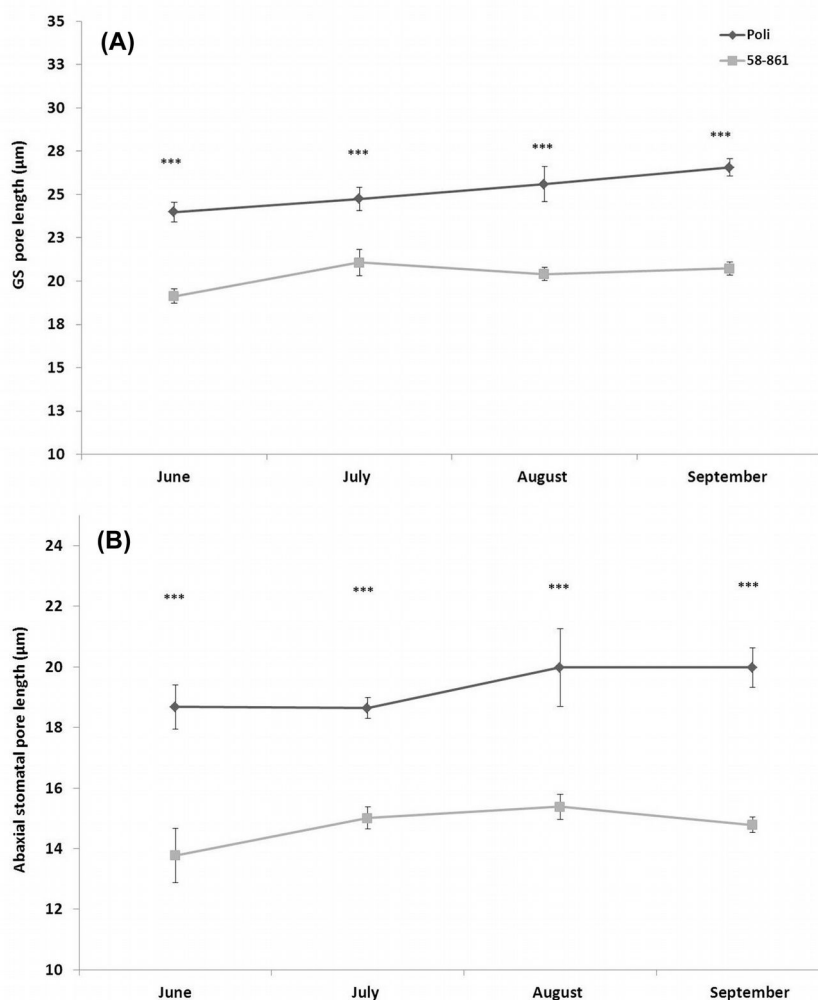

Fig. 2 - Pore length of stomata on the abaxial surface in a mature leaf. (A): Giant stomata; (B): ordinary stomata. The number of leaves/replicates used for each poplar clone was 20. (***): $P \leq 0.001$.
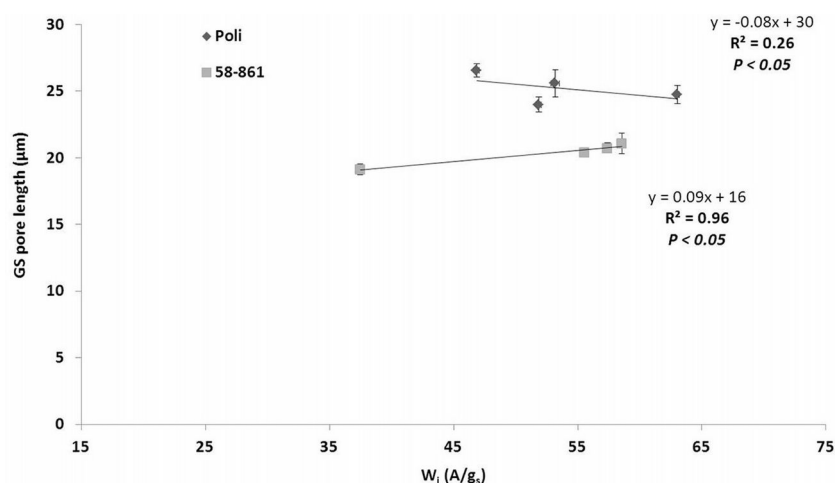
Fig. 3 - Relationships between mean size of giant stomata on abaxial surface and intrinsic WUE ($W_i = A/g_s$) during growing season; mean values are referred to each month of the season. (A): assimilation (μmol CO_2 m^{-2} s^{-1}); (g_s): stomatal conductance (mol H_2O m^{-2} s^{-1}).

neages (Von Groll & Altmann 2001, Casson & Gray 2008).

In mature genotype *Poli* leaves, the wax cover was visible, and GS were occasionally covered by wax (Fig. 1C, D). In contrast, wax plugs frequently occluded GS in mature *58-861* leaves (Fig. 1E, F).

Mature leaves during growing season (light microscopy)

Results showed *Poli* compared with the genotype *58-861* generally exhibited longer GS (Fig. 2A); these length differences between genotypes were also maintained in ordinary stomata, and observed throughout the growing season (Fig. 2B).

However, significant differences were not detected in GS number and frequency (= number of GS/total number of stomata) in the three leaf sections examined (apical, medium, basal) from both *P. nigra* genotypes (Tab. 1).

Different sized stomata (~40 to ~20 μm) were present on the adaxial and abaxial leaf sides. However, due to the absence of cuticular ridges, stomata were typically associated with GS on impressions obtained from adaxial surfaces. Therefore, statistical analyses would be unreliable if applied to GS on the adaxial surfaces. However, regression analysis performed on abaxial surfaces showed a significant relationship between GS size and W_i (assimilation / stomatal conductance), with high prediction accuracy in *58-861* ($R^2 = 0.96$) but not in *Poli* ($R^2 = 0.26$ - Fig. 3).

Cocozza et al. (2010) characterized the northern Italy genotype *58-861* as more water efficient and drought sensitive, with larger leaves compared with *Poli*, and the current study confirmed these data (Fig. 3). Previous studies on the same genotypes focused on stomatal densities and length, but did not examine the distinction between ordinary and large sized stomata (Regier et al. 2009, Cocozza et al. 2010).

Regier et al. (2009) reported a significant reduction in stomata size in genotype *58-861*, but only on the abaxial leaf surface when water uptake changed, with a subsequent reduction in well-watered status. In addition, the current data suggested GS size in *Poli* was larger than in genotype *58-861*, and might explain the increased W_i verified in the northern Italy poplar clone. Furthermore, GS size could be associated with the stomatal opening and closing mechanism (together with density), which cumulatively provides total leaf stomatal conductance, and more efficient water use in genotype *58-861* despite smaller stomata.

Observed stomatal characteristics did not follow seasonal changes, suggesting the attributes were favored by selection, and represent adaptive-genetic traits. This hypothesis emphasizes the role of GS as an adaptive and functional trait distinguishing different genotypes, and might serve to clarify certain physiological attributes, including WUE in *P. nigra*.

Warren & Adams (2006) indicated leaf anatomy was the primary factor to explain WUE by measuring stable carbon isotopes. Consequently, the role of GS in leaf physiology should take a primary role in developing a greater understanding of processes, including WUE and plasticity, or seasonal acclimation (Nicotra & Davidson 2010). In particular, drought tolerance, as reported in the larger-sized and more water use-efficient genotype *58-861*, can characterize its physiology through several adaptive traits of stomata (density, pore length, structure).

A more developed wax covering on the abaxial leaf surface of genotype *58-861* represents another distinctive trait of the nor-

thern genotype compared to the southern *Poli* genotype. Pearce et al. (2006) described an additional function for wax accumulation on leaf adaxial surfaces in *Populus*, particularly poplars adapted to warmer regions (*i.e.*, *P. angustifolia*). The abaxial epidermal wax covering in mature leaves of genotype *Poli* was reported by Pearce et al. (2006). Surface roughness, caused by ridges, trichomes, or both is the chief factor limiting wetting properties of some species, such as poplar. Superficial wax plays a dominant role in affecting wetting of other taxa (Pinon et al. 2006). The wax covering in the southern genotype *Poli*, and wax plugs in *58-861* might be responsible for additional differences in drought tolerance between these two genotypes, which originated from distinct environments.

Finally, stomatal analysis in the Salicaceae and closely related groups could be useful in clarifying specific features of stomatal patterns, presence of large sized stomata (GS), and the possible phylogenetic origins of normal stomata relative to GS, *i.e.*, are GS the precursors to normal stomata. The prevailing view is that stomata arose once in evolutionary history, and subsequently radiated throughout land plants. Oversized stomata could be a significant factor in land plant evolution, with implications in physiological pathways.

An increased understanding of giant stomata can be useful to industry considering the importance of stomata in a perspective of biomass production. In fact, poplar clones with high abaxial stomatal density (where giant stomata were found) showed an increased biomass production (Al Afas et al. 2006).

Acknowledgements

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