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**RESEARCH NOTE**

# Quantitative trait loci mapping for stomatal traits in interspecific hybrids of *Eucalyptus*

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**Abstract.** *Eucalyptus* is an important industrial species with tolerance to drought and salt stress. Genetic improvement activities including quantitative trait loci (QTL) mapping for pulping and adventitious rooting traits are in progress, but no information is available on the genomic regions on adaptive traits such as stomatal characteristics. In this study, an interspecific cross between *Eucalyptus tereticornis* and *E. grandis* was generated for the development of genetic map and QTL identification for stomatal traits. Simple sequence repeats (SSRs), inter-simple sequence repeats (ISSRs) and sequence related amplified polymorphism (SRAP) markers were used for genotyping the $F_1$ individuals. Parent-specific genetic maps (female, 1023.56 cM; male, 1049.64cM) and consensus map (1049.4 cM) were developed. QTL analysis was carried out to identify the chromosomal regions affecting stomatal density, area and pore length in adaxial and abaxial leaf surface. Seven QTLs were identified with phenotypic variation of 11.36 to 27.30% for stomatal density, area and pore length. Correlation of stomatal traits when combined with growth and wood properties would have greater implications for generation of stress tolerant eucalypt hybrids with higher productivity and adaptability.

**Keywords.** linkage map; quantitative trait loci; stomata; stress tolerance; eucalyptus.

## Introduction

*Eucalyptus* is grown in the tropics mostly for the pulpwood production. Globally, more than 21 Mha of *Eucalyptus* plantations are available (Midgley 2013) and produce 17.5% of world's paper pulp (Hart and Santos 2015). Next to Brazil, India occupies the second position in growing *Eucalyptus* with more than 4.4 Mha plantations (Xie 2015). The major species grown in India are *Eucalyptus camaldulensis* and *E. tereticornis*, due to their adaptability to drought and suitability for paper pulp production. *E. grandis* is a subtropical species targeted for the breeding programmes worldwide because of its high pulp productivity. It is closely related to *E. tereticornis* and production of interspecific hybrids displaying hybrid vigour in terms of pulp yield and drought tolerance is possible (Madhibha *et al*. 2013). Despite, India being a major grower of *Eucalyptus*, studies on hybrid breeding and applied molecular genetics are very limited.

Physiological traits, important for plant growth responses and are often quantitative, suggesting that they are influenced by multiple genes. Patterning of stomata and their function in angiosperms are considered key determinants of growth rate and water balance in plants. This requires a better understanding of molecular mechanisms involved in stomatal distribution. Stomatal parameters like density and pore size have been reported to be correlated with the rate of photosynthesis and transpiration, drought, salinity, yield, elevated $CO_2$ in

SM conducted marker polymorphism analysis in the laboratory, trait measurements, data analysis and drafted the manuscript. BVKW and DB participated in the seedling raising, field planting and maintenance of the mapping population. MGD participated in designing the experiments and finalized the manuscript. MA, NB, RD and SV participated in designing the experiments, conducted controlled pollination, seedling raising and field planting. YR conceived, organized and planned the research and finalized the manuscript.

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woody and crop species (Gailing *et al.* 2008; Liu *et al.* 2017). Considerable progress has been made in detection of stomatal traits related quantitative trait loci (QTL) for salt and drought tolerance in crop species (Shahinnia *et al.* 2016; Liu *et al.* 2017). In *Eucalyptus*, genetic mapping and QTL analyses for various traits including growth and form, wood properties, adventitious rooting, flowering time, biotic and abiotic stresses and secondary metabolism have been reported (Henry and Kole 2014). Currently, only limited information is available on QTL governing stomatal characteristics and such QTL can pave way for the production of high yielding trees with abiotic stress tolerance, especially when combined with QTL for growth and wood properties**.** Hence, the objectives of the study were to develop a consensus linkage map for the interspecific cross *E. tereticornis* and *E. grandis* using simple sequence repeats (SSRs), intersimple sequence repeats (ISSRs) and sequence-related amplified polymorphism (SRAP) markers and to identify QTL associated with stomatal traits.

## Material and methods

### *Plant materials*

The mapping population used in this study consisted of the parents of the cross, *E. tereticornis* (Et86) × *E. grandis* (Eg9) and their $F_1$ individuals. Et86 is a selection from a seed production area (Pudukottai, 10°23′N, 78°51′E), while Eg9 is a selection from the provenance trail (13017, Lorne) located at Osamund (11°26′N, 76°37′E). Controlled pollination experiments were carried out during May 2010 and $F_1$ were field planted in 2011 at Panampally (10°47′N; 76°45′E), Kerala.

### *Marker genotyping*

Two parents (Et86 and Eg9) and 98 $F_1$ individuals were genotyped with 114 SSR loci (table 1 in the electronic supplementary material at http://www.ias.ac.in/jgenet/), 13 ISSR primers (table 2 in the electronic supplementary material) and 13 SRAP primer combinations (table 3 in the electronic supplementary material). PCR amplifications and electrophoresis for SSR, ISSR and SRAP markers were carried out as reported by Arumugasundaram *et al.* (2011), Balasaravanan *et al.* (2005) and Li and Quiros (2001), respectively.

### *Generation of genetic linkage maps*

ISSR and SRAP markers were named after the primer serial number and the approximate fragment size. Chi-square ($\chi^2$) test was performed and markers that deviated from the theoretical expected ratios were considered as distorted with a significance level ($P < 0.01$, $P < 0.001$ and $P < 0.0001$) and used for analysis. Linkage analysis was performed using JoinMap 4.0 software (Van Ooijen 2011) with cross pollinator (CP) option. Linkage phase determination and grouping was done independently for the parental datasets. Linkage group (LG) was estimated by applying independence logarithm of odds (LOD) ranging from 1 to 10. SSR markers were assigned to the LG based on the information from published literature (Grattapaglia *et al.* 2015). Marker ordering was performed with regression mapping using the standard parameters and the markers that were mapped in the first and second round were included, maps were generated using Mapchart 2.1 software (Voorrips 2002). The parent-specific maps were integrated using the 'Combine groups for map integration' function of JoinMap to produce a consensus map using common SSR markers.

### *Stomatal morphology measurements and data analysis*

Leaves of coppice shoots from the parents and hybrid individuals were selected for stomatal morphology studies. Stomatal observations were carried out in the 3rd and 4th fully expanded leaves on the 30th day after coppicing the plants, following the methodology described by James and Bell (2000). From each plant, two leaves were smeared with a thin film of nail varnish at the centre of the leaf blade on the abaxial and adaxial surface to make an imprint. Stomatal density per unit area (number of stomata per $mm^2$) was determined at 400× magnification from 10 field views per slide using photomicroscope (Leitz, Japan). Images of the stomata were taken using an ultrascope fitted with a microscope and stomatal length (SL), width (SW) and pore length (PL) were measured using Scope Image 9.0. SL, SW and PL values were measured in micrometre ($\mu$m) from abaxial and adaxial leaf surfaces.

Stomatal area ($\mu m^2$) was calculated using the following formula (Wang *et al.* 2012),

$$\frac{SL \times SW \times \pi}{4}$$

Descriptive statistics and one-way analysis of variance (ANOVA) were used to analyse the variation in stomatal traits between the parents and $F_1$ individuals using the SPSS ver. 22.0 software (IBM, Armonk, USA).

### *QTL analysis*

QTL analysis was carried out using composite interval mapping (CIM) procedure in WinQTL Cartographer v2.5

**Table 1.** Mapping statistics for consensus map of *E. tereticornis* (Et86) × *E. grandis* (Eg9).

| Linkage group | Total number of loci mapped | | Common SSR markers | Total number of loci mapped in Et86 × Eg9 | Size in cM | Mean distance between markers |
|---|---|---|---|---|---|---|
| | Et86 | Eg9 | | | | |
| 1 | 25 | 16 | 7 | 33 | 91.7 | 2.8 |
| 2 | 21 | 12 | 6 | 27 | 99.8 | 4 |
| 3 | 8 | 18 | 3 | 22 | 93.9 | 6.8 |
| 4 | 13 | 28 | 7 | 33 | 83.1 | 2.5 |
| 5 | 22 | 21 | 6 | 37 | 86.3 | 2.3 |
| 6 | 15 | 9 | 2 | 21 | 97.5 | 4.6 |
| 7 | 20 | 9 | 6 | 23 | 92.9 | 4 |
| 8 | 31 | 9 | 7 | 33 | 92.3 | 2.8 |
| 9 | 15 | 8 | 6 | 16 | 107.9 | 6.7 |
| 10 | 22 | 28 | 3 | 47 | 114.7 | 2.4 |
| 11 | 12 | 14 | 4 | 22 | 89.3 | 4.1 |
| Total | 204 | 172 | 57 | 314 | 1049.4 | – |
| AVG | 18.55 | 15.64 | 5.18 | 28.55 | 95.40 | 3.9 |

(Wang *et al.* 2007) to identify QTLs for stomatal density, stomatal area and pore length in adaxial and abaxial leaf surfaces adopting backcross model. The LOD threshold was determined by permutation analysis with 1000 repetitions. The size of the analysis window was maintained at 10 cM with a walk speed (mapping resolution) of 1 cM and control marker number five. Stepwise regression (forward method) under model 6 and the LOD score corresponding to $P = 0.05$ was used to identify significant QTL.

## Results

### *Marker production and linkage map generation*

A total of 114, 115 and 129 SSR, ISSR and SRAP markers were generated, respectively and the details of SSR segregation pattern, ISSR and SRAP polymorphism status are given in tables 1, 2 and 3 in electronic supplementary material. These markers were used for framework map construction in female and male parents separately at LOD 3.0, where 11 groups, equivalent to the haploid chromosome number were formed. The details on marker types, segregation pattern, parent informativeness and chi-square significance are presented in table 4 in the electronic supplementary material. Details on number of markers mapped, segregation deviation (SD) markers for SSR, ISSR and SRAP of parental maps are given in tables 5 and 6 in the electronic supplementary material. The female map had 204 markers covering 1023.56 cM (table 5 and figure 1 in the electronic supplementary material). The male map had 172 markers with 1046.64 cM length (table 6 and figure 2 in the electronic supplementary material). The consensus map with a total length of 1049.4 cM had an average of 28.55 markers per LG, with an average spacing of 3.9 cM (table 1; figure 1).

### *Phenotypic variations in stomatal traits*

The univariate statistics for stomatal density, stomatal area and pore length of the Et86×Eg9 mapping population is provided in table 2. Analysis of variance ($F$-test) indicated significant differences among the parents and $F_1$ hybrids for the traits such as stomatal density and area on the adaxial and abaxial surface of the leaf. However, pore length did not show significant difference for the abaxial surface. The absolute values of skewness and kurtosis were less than 1 for all traits, indicating that the phenotypic values of these traits were normally distributed and suitable for QTL analysis.

### *QTL analysis*

Phenotypic variance (PV; $R^2$) controlled by these QTL ranged from 11.36 to 27.3% (table 3). No QTL were identified for the stomatal density on the abaxial leaf surface. One QTL of large effect (explaining 27% of phenotypic variance) in LG8 associated with marker locus M14E12-350 for stomatal pore length at the abaxial surface was identified. Two SSR loci (Embra204 in LG9 and Embra2 in LG11) were associated with the stomatal area at the abaxial surface and the PV explained were 12 and 14%, respectively. Two SRAP markers and three ISSR markers were associated with adaxial stomatal area, abaxial and adaxial stomatal pore length and stomatal density in adaxial surface of the leaves.

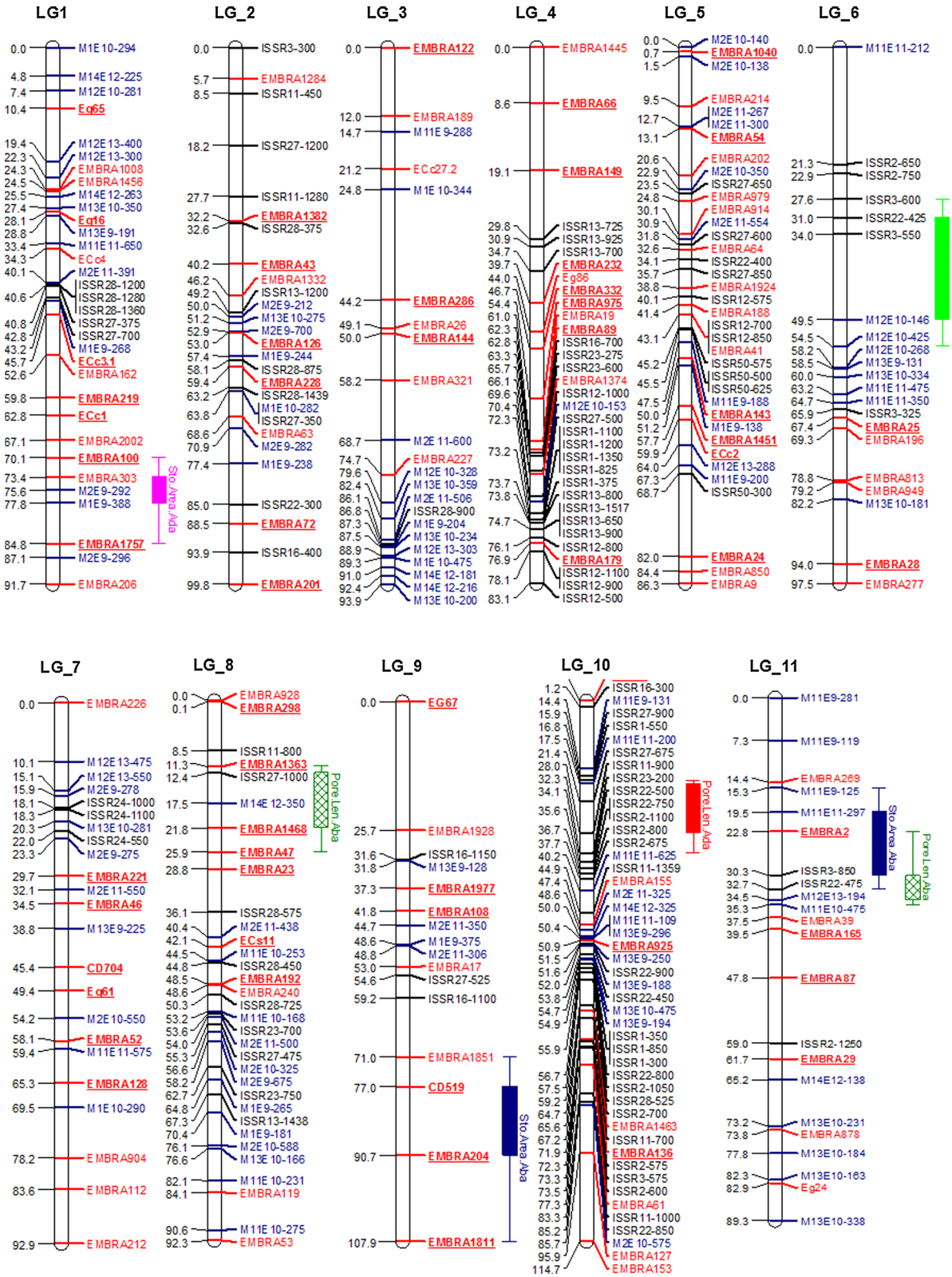


**Figure 1.** Consensus linkage map of the cross *Eucalyptus tereticornis* (Et86) and *E. grandis* (Eg9) showing QTL regions for stomatal traits. Distance among markers are indicated in cM to the left of the linkage groups; markers in red, blue and black colours are SSR, ISSR and SRAP, respectively and underlined markers are common between maternal and paternal maps. QTL for the traits analysed for depicted as coloured vertical bars to the right of the linkage groups 1 (stomatal area adaxial), 6 (stomatal density adaxial), 8 (pore length abaxial), 9 (stomatal area abaxial), 10 (pore length adaxial) and 11 (pore length abaxial).

**Table 2.** Descriptive statistics and ANOVA for stomatal density, stomatal area and pore length of *E. tereticornis* (Et86), *E. grandis* (Eg9) and $F_1$ individuals.

| Genotype | Density (no. mm$^{-2}$) | | Stomatal area ($\mu$m$^2$) | | Pore length ($\mu$m) | |
|---|---|---|---|---|---|---|
| | Adaxial | Abaxial | Adaxial | Abaxial | Adaxial | Abaxial |
| Et86 | 132.00 ± 22.9 | 213.00 ± 50.8 | 135.19 ± 26.6 | 92.26 ± 33.3 | 7.53 ± 1.2 | 5.22 ± 1.0 |
| Eg9 | 92.00 ± 14.7 | 168.00 ± 20.4 | 204.22 ± 34.0 | 133.60 ± 20.2 | 7.69 ± 1.6 | 6.17 ± 1.0 |
| $F_1$ individuals ($n = 98$) | | | | | | |
| Mean | 112.85 | 246.91 | 153.97 | 143.25 | 5.12 | 5.13 |
| Max. | 169 | 351 | 244.11 | 238 | 9.17 | 8.85 |
| Min. | 57 | 123 | 54.85 | 73.29 | 2.09 | 2.68 |
| Skewness | 0.19 | − 0.21 | 0.32 | 0.64 | 0.4 | 0.21 |
| Kurtosis | − 0.17 | − 0.45 | 0.74 | 0.33 | 0.6 | − 0.51 |
| SE (±) | 22.43 | 49.37 | 32.81 | 32.27 | 1.32 | 1.28 |
| F-test | * | ** | ** | * | ** | ns |

**$P < 0.01$; *$P < 0.05$; ns, not significant; SE, standard error.

**Table 3.** QTLs for stomatal traits mapped in Et86 × Eg9 using WinQTL Cartographer v2.5.

| Traits | LG | Locus name | LOD | Left marker | Right marker | PV (%) | Additive |
|---|---|---|---|---|---|---|---|
| Stomatal density adaxial | 6 | ISSR3-550 | 4.15 | ISSR22-425 | M12E10-146 | 14.9 | 21.07 |
| Stomatal area abaxial | 9 | EMBRA204 | 4.37 | CD519 | EMBRA1811 | 12.4 | − 23.21 |
| Stomatal area abaxial | 11 | EMBRA2 | 3.93 | M11E11-297 | ISSR3-850 | 14.4 | − 24.81 |
| Stomatal area adaxial | 1 | M2E9-292 | 3.42 | EMBRA303 | M1E9-388 | 11.4 | 22.85 |
| Pore length abaxial | 8 | M14E12-350 | 11.34 | ISSR27-1000 | EMBRA1468 | 27.3 | − 1.69 |
| Pore length abaxial | 11 | ISSR22-475 | 6.8 | ISSR3-850 | M12E13-194 | 14.8 | 1.39 |
| Pore length adaxial | 10 | ISSR27-675 | 7.37 | M11E11-200 | ISSR11-900 | 24 | − 1.51 |

LG, linkage group; PV(%), phenotypic variation ($R^2$).

## Discussion

### *Genetic linkage map*

A number of linkage maps are reported in *Eucalyptus* including high-density maps in interspecific crosses of *E. grandis* and *E. urophylla*, the hybrid combinations predominantly grown in Brazil, Australia and South Africa (Freeman 2014). Only very few linkage maps are available for the hybrid combinations with *E. tereticornis* as parent species. The individual parent-specific map generated in this study for *E. tereticornis* (Et86) was 1023.56 cM. Yu *et al.* (2012) reported a map of size 1488 cM with 21 LGs using expressed sequence tag-based cleaved amplified polymorphic sequence markers (EST-CAPS) and random amplified polymorphic DNA (RAPD) markers in *E. tereticornis*. Recently, for the same species, Li *et al.* (2015) generated linkage map of 1241 cM length with 585 loci distributed in 11 LGs. In *E. grandis*, several genetic maps were developed using different types of DNA markers and the linkage map length ranged from 925 cM (Kullan *et al.* 2012) to 1216 cM (Gion *et al.* 2011). The present study also observed the map length of 1046.64 cM with 172 markers belonging to SSR, ISSR and SRAP. Most of the reports on *Eucalyptus* maps constructed till date reported the haploid number of chromosomes, despite of various types of DNA markers, pedigree structure and mapping software packages (Freeman 2014) and the present study also showed marker distribution on 11 LGs.

### *QTL mapping for stomatal traits*

Size, density and pattern of leaf stomata play an important role in plant growth and yield. Genetic relationships between stomatal density and size with yield were documented in cereals (Shahinnia *et al.* 2016). In *Quercus*, based on the QTL for stomatal density, their role in adaption towards climate change was discussed (Gailing *et al.* 2008). The aim of this study was to identify the QTL regions controlling stomatal density, stomatal area and pore length on the adaxial and abaxial surface of the Et86 × Eg9 mapping population. The marker loci M14E12-350 in LG8 showed cosegregation for stomatal pore length at the abaxial surface with PV of 27.3%. The higher PV (> 25%) recorded in this study could be attributed towards the use of smaller populations, lack of multienvironment measurements and wide physical linkage between adjacent causal loci within single crosses (Hall *et al.* 2016).

This result is a step forward in understanding the function of these loci and to correlate their role in yield and biotic and abiotic stress tolerance. The accuracy of QTL mapping depends on several factors such as density of genetic map, genetic architecture of the trait, phenotyping efforts, statistical methods and population size (Semagn *et al.* 2010). In the present study, the size of mapping population is small and established only in a single environment. Nevertheless, practical applications for such QTL-linked markers will require validating them in different genetic backgrounds and environments.

Stomatal parameters like density, area and pore length gain significance in *Eucalyptus* because of the variation expressed towards abiotic stress tolerance, photosynthetic and transpiration efficiency. These characteristics affect the growth and productivity of *Eucalyptus*, where stomata play a major role in the control of water evaporation, gas exchange and pathogen entry (Tong *et al.* 2016). Stomata size and density are under a complex genetic control and thus provide multiple levels of regulation for stomatal functions (Chaves *et al.* 2016). Therefore, a better understanding of the genetic control of stomatal parameters is an important aspect in breeding of *Eucalyptus* for productivity, abiotic stress tolerance and disease resistance (Héroult *et al.* 2013; Tong *et al.* 2016). Wider variations in stomatal density, area and pore length in Et86 × Eg9 provide an opportunity to assess their influence on various physiologically adaptive characteristics of hybrid individuals. The individuals which compromise between high water use efficiency and leaf cooling capacity would be the best-adapted genotypes under semiarid conditions (Chaves *et al.* 2016). Thus, correlation of stomatal traits with yield and adaptability would help to improve the productivity of *Eucalyptus* plantations in arid conditions.

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