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Heterosis and reciprocal effects for physiological and morphological traits of popcorn plants under different water conditions

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ABSTRACT

In spite of the benefits of heterosis in maize breeding, little is known about the physiological mechanisms of this phenomenon and its genetic control under different water regimes. This study aimed to understand the heterosis effects on plant growth, the photosynthetic and transpiration traits, and the root traits of four inbred popcorn lines and their hybrids, including their reciprocal combinations. Plants were grown in lysimeters, inside a rain shelter, under two water conditions (water stress – WS; well-watered – WW) until anthesis. Plant growth traits included shoot biomass, plant height, and leaf area. Photosynthetic traits comprised leaf pigment and total nitrogen content, chlorophyll fluorescence, gas exchange, water use efficiency and stomatal index and density, along with the stable carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) isotope compositions of the last developed leaf. Root weight density and specific root length were also recorded. Greater heterosis effects were observed for traits related to plant growth and root weight density, and specifically under WS. Traits related to root weight density in deeper soil layers benefited markedly from heterosis, but there were no advantages in terms of stomatal conductance and water status in general. Apparently, only $\delta^{13}C$ supported a better water status under WS, and was observed in the hybrids in particular. Non-additive gene effects were predominant in controlling of most of the growth and root traits studied, supporting the conclusion that the heterosis effect is especially favorable under water-limiting conditions. Moreover, the choice of the female parent is essential for traits related to gas exchange when breeding for better resilience to drought.

Abbreviations: A, leaf net CO_2 assimilation rate; E, transpiration rate; ET, cumulative plant evapotranspiration; Fv/Fm, maximum quantum yield of PSII; g_s, stomatal conductance; NPQt, non-photochemical quenching parameter describing regulated dissipation of excess energy; RWD, root weight density; SRL, specific root length; WC, water condition; WS, water stress; WUE_{Agro}, agronomic water use efficiency; $WUE_{Instant}$, instantaneous water use efficiency; $WUEi_{ntrin}$, intrinsic water use efficiency; WW, well-watered; $\delta^{13}C$, stable carbon isotope composition; ΦNO, quantum yield of non-regulated non-photochemical energy loss in PS II; ΦNPQ, quantum yield of regulated nonphotochemical energy loss in PS II; ΦPSII, quantum yield of photochemical energy conversion in PS I.

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1. Introduction

The continuous increase in maize grain yield that started in the late 1930s was essentially due to the expression of heterosis (Tollenaar and Lee, 2006). Currently, maize breeding programs are still largely based on the exploitation of this phenomenon (Zhang et al., 2016) but the increasingly negative effects of climatic change worldwide (IPCC, 2019) makes it necessary to accelerate breeding for resilience to abiotic stress conditions such as drought. Heterosis is the expression of an adaptive advantage of a progeny in relation to its parents, which can be quantified by faster growth, higher final biomass, greater flower fertility, and consequently larger yields (Birchler et al., 2010). The positive effects of heterosis have been especially relevant for biomass and yield (Cairns et al., 2012) and have been evidenced from early growth through to adult plant stages (Chairi et al., 2016; Holá et al., 2017; Rockenbach et al., 2018). Furthermore, heterosis has been suggested to positively affect crop adaptation to abiotic stresses such as drought (Araus et al., 2010), which opens the possibility of identifying phenotypic traits in maize that confer stress resilience.

Water stress-induced physiological and biochemical disturbances, which can reduce leaf expansion and alter the metabolic cell activity that leads to stomatal closure and photosynthetic inhibition, are known to affect carbon partitioning and result in a fall in yield (Dalal and Sharma, 2017). Some studies have reported that hybrids have adaptive advantages compared to parent lines under water limitation, resulting in higher final productivity (Araus et al., 2010; Chairi et al., 2016). In the case of subtropical maize hybrids under drought conditions, Araus et al. (2010) suggested that heterosis was due to constitutive differences in plant water status and an enhanced water use by hybrids. Along the same lines, Chairi et al. (2016) reported heterosis for root traits and total transpiration in a dry soil environment, regardless of the water regime, as well heterosis for photosynthetic traits (leaf net CO_2 assimilation rate, stomatal conductance, and transpiration rate) being more evident under water stress.

A root system that secures higher transpiration and water use may also have a positive role in improving the uptake of nutrients like nitrogen (Lynch, 2019). Therefore, a root system that is highly resilient to soil water stress represents an adaptive advantage of high agronomic relevance (Trachsel et al., 2011). In this sense, Gao and Lynch (2016) described that maize genotypes with a lower number of crown roots have greater rooting depth under water stress, contributing to a better adaptation in terms of water use and improved growth and yield. According to Zhan et al. (2015), the reduction in root branch density increases drought tolerance in maize, preventing excessive metabolic costs of soil exploration while allowing deeper rooting. In this line of work, the occurrence of heterosis in maize hybrids under drought has been reported for root traits, enhancing agronomic performance either due to higher root weight density or to lower specific root length (Ali et al., 2016; Chairi et al., 2016). Additional root traits may also be involved. Thus, in a recent study in popcorn, the higher agronomic water use efficiency and photosynthetic rates of hybrids compared to inbred lines under water stress seems, as least in part, to be the result of a synergistic association of wider root angles in relation to the soil (about 90º) and longer roots (Leite et al., 2021).

Understanding the physiological mechanisms associated with heterosis can reveal ways to increase the grain yield potential and to improve plant adaptation to water stress. This information, associated with a deeper knowledge about the genetic control of these traits, may guide more efficient parent selection and management of superior segregating populations. The performance data of parents and their hybrid combinations, derived from diallel crosses, provide valuable information for breeders. Based on these crosses, the general combining ability can be estimated, which is associated with additive effects, as well as the specific combining ability, which is associated with non-additive effects (Cruz et al., 2014). Diallel crosses that include parents in a reciprocal sense allow conclusions about the influence of extrachromosomal genes (Cruz et al., 2014). Thus, the interactions between nuclear and cytoplasmic genes have been reported as negligible in explaining heterosis (Zhang et al., 2016), despite their effects on the phenotypic expression of some traits in F_1 hybrids (Fan et al., 2014) such as grain yield and its components (Yao et al., 2013) including grain resistance to disease spread (e.g. Zhang et al., 1997 for *Aspergillus flavus*) and the early vigor of maize seeds (Santos et al., 2017).

Despite the importance of heterosis for maize yield, as well as the known action of reciprocal effects on crosses, the genetic effects and physiological mechanisms associated with heterosis performance under soil water stress conditions needs further study. While the effect of heterosis is relatively well known in terms of increasing growth, biomass and yield (Ahmadzadeh et al., 2004; Araus et al., 2010; Chairi et al., 2016), the genetics and especially the physiological mechanisms behind it are still largely unclear. Among the mechanisms contributing to the greater growth of hybrids compared to inbred lines, higher photosynthetic rates (Chairi et al., 2016), greater transpiration and better water status (Araus et al., 2010), or a more efficient root system (Ali et al., 2016; Chairi et al., 2016) have all been proposed, but so far the debate remains mostly open. To date, besides leaf greenness (Kamphorst et al., 2020a, 2020b, 2021b), traits related to leaf photosynthetic status and stomatal conductance have been shown to be good indicators of the agronomic performance of popcorn under water constraints (Kamphorst et al., 2020a). In addition, the current body of research has reinforced the importance of root physiological and morphological traits in explaining agronomic water use efficiency and the possibility of advances through exploitation of heterosis, given the superior performance of roots in the hybrids relative to the parent lines (Leite et al., 2021).

The objective of this study was to evaluate differences in growth, photosynthesis, transpiration, nitrogen uptake, and root architecture that may be involved in the expression of heterosis in popcorn, as well as to investigate the genetic mechanisms that control these traits under different water conditions. The results of our study may support breeding strategies and even agronomic practices aimed at improving popcorn resilience to drought. Since the goal of this study was to provide insights into which phenotypic traits are associated with maize genotypes better adapted to water stress, we therefore analyzed a wide range of traits. To this end, we performed an exhaustive evaluation of different shoot growth and root traits, together with a wide range of physiological traits informing about the photosynthetic and transpirative status of the plants. In terms of shoot growth, total biomass as well as leaf characteristics were assessed to quantify leaf growth (e.g. total area, density of epidermal cells) and leaf thickness/compaction (e.g. the specific leaf area) (Zhang et al., 2015). To enable a detailed study of the plants' root architecture while controlling water status, plants were grown in a 1.5 m deep lysimetric system where root characteristics at different depths were assessed. Concerning leaf photosynthesis and transpiration, instantaneous measurements (gas exchange and chlorophyll fluorescence), together with leaf structural traits (pigment content on a per area basis, nitrogen content, and stomatal density) and time-integrated indicators of the water and nitrogen status (such as the stable carbon and nitrogen isotope signatures when analyzed in leaf dry matter) were assessed. Thus, the carbon isotope signature when analyzed in plant dry matter, expressed either as composition ($\delta^{13}C$) or discrimination ($\Delta^{13}C$), is affected by water status even in C_4 species (Farquhar, 1983; Farquhar et al., 1989). Consequently, the carbon isotope signature has been used to indicate water status in maize (Araus et al., 2010; Chairi et al., 2016; Kamphorst et al., 2020a). With regard to the nitrogen isotope composition ($\delta^{15}N$), even though its signal in the plant seems more complex to model and less resolved (Cui et al., 2020) it gives insights about how water affects nitrogen uptake and downstream metabolism, with $\delta^{15}N$ usually decreasing in response to water stress (Yousfi et al., 2009, 2012).

2. Material and methods

2.1. Genotypes and growth conditions

Four S_7 popcorn (*Zea mays* L. var. Everta) inbred lines were used, namely: P6 (derived from Zaeli, a hybrid adapted to temperate/tropical climates), P7 (derived from the IAC112 hybrid, adapted to temperate/tropical climates), L61 (derived from the open-pollinated variety BRS Angela – EMBRAPA, adapted to tropical climates), and L75 (derived from the open-pollinated variety Viçosa, adapted to temperate/tropical climates) and their possible hybrids (12), including reciprocal combinations. These inbred lines were selected based on a previous field study under soil water stress and classified as agronomically efficient (P6 and P7) and inefficient with regard to water use (L61 and L75) (Kamphorst et al., 2018). Following the order of female (♀) and male (♂) parents, the hybrids P6 x P7, P6 x L61, P6 x L75, P7 x L61, P7 x L75, L75 x L61, P7 x P6, L61 x P6, L75 x P6, L61 x P7, L75 x P7, and L61 x L75 were used.

The experiment was carried out in a lysimetric system placed under a glass rain shelter at the greenhouse at the Experimental Field Facilities of the University of Barcelona, from early May to mid-July 2018. The lysimetric system is described by Elazab et al. (2016). In short, the system consists of PVC tubes (diameter 14 cm, length 150 cm) cut in half longitudinally, and the two halves fixed together with adhesive tape. The lower parts of the tubes were tied with wire (which was threaded through holes) and the bottom of each tube closed with a pot of the same diameter as the tubes, allowing adequate drainage. The substrate consisted of 80% perlite and 20% peat moss, to which the fertilizer complex NPK (MgO, SO_3) 20 – 5 – 8 (5 – 17) was added, together with micronutrients and a slow-release (2 – 3 months) nitrogen source (isobutylidenediurea / 17 g tube^{-1}). In absolute values, the fertilizer corresponded to 144.7 kg N ha^{-1}, 36.2 kg P ha^{-1}, and 57.8 kg K ha^{-1}.

The experiment was arranged in complete randomized blocks, evaluated under two water conditions (WC) with three replicates per genotype and water condition, each replicate consisting of one plant within an individual lysimeter. Three seeds were germinated directly on substrate in each PVC tube for each genotype and replicate. Fifteen days after germination, seedlings were thinned to one per tube. The plants were spaced 25 cm apart and 94 cm between rows, corresponding to a density of 42,553 plants ha^{-1} (in the range of densities under field conditions). Temperature, humidity, and solar radiation followed the seasonal pattern (Fig. 1).

The tubes were irrigated abundantly with water before sowing, left to stand for 72 h to drain excess water, and then weighed. The water-holding capacity of each tube (at 100% field capacity) was calculated as the difference between the wet substrate (after draining excess) and dry substrate weight (before saturation, oven-dried). The mean water content per tube at relative field capacity was 6.772 L (100%). The plant weight was not included when calculating the relative field capacity of the tubes.

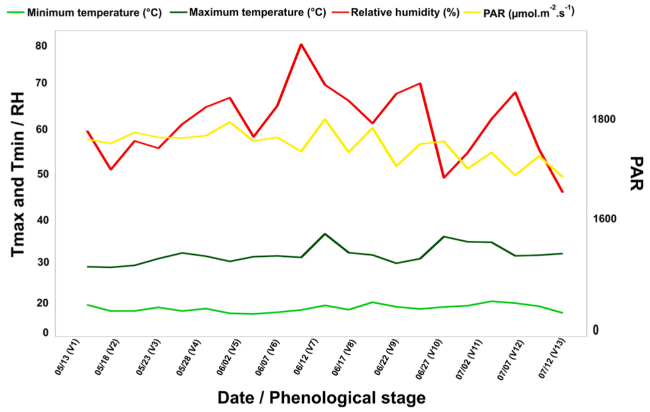

Fig. 1. Mean minimum (T_{min}) and maximum (T_{max}) air temperatures (°C), relative humidity (RH, %) and photosynthetically active radiation (PAR, µmol m^{-2} s^{-1}), across dates and phenological stages (V) of popcorn growth (May–July 2018).

To establish well-irrigated (WW) conditions, the tubes were maintained at relative field capacity until the final evaluations (pre-flowering period). To this end, every 2–3 days the tubes were weighed and then replenished with the corresponding amount of water to restore relative field capacity. In water-stress (WS) conditions, limited irrigation was imposed 15 days after the emergence of seedlings. The amount of water available in the tubes gradually reduced according to plant consumption, until it reached 35% of relative field capacity; thereafter, water content was maintained for 15 days until harvest. The calculations of relative field capacity did not consider the weight of the (growing) plants, causing a certain underestimation of the amount of water needed to reach 35% of the relative field capacity in the tubes. The decrease in moisture in the tubes was homogeneous because the plants that consumed the most water received the precise amount of irrigation to return to the substrate water conditions of the plants that consumed the least. This regime was established to simulate field growth conditions, with a slow decrease in water content, thus avoiding the typical situation of potted plants, which can be affected by severe water stress within a few days. To that end, the tubes were weighed and irrigated at intervals of 2–3 days.

2.2. Morphological traits

At harvest, plant height (cm) was measured with a ruler, from the tube surface to the last developed leaf (visible ligule). Then the plant stalks were separated from the leaves and inserted into paper envelopes for oven drying at 70 °C for 72 h to determine leaf and stalk biomass (g).

The leaf area (m^2) was calculated, based on pictures of each plant (Supplementary Material – Fig. 1). The images were acquired with a Sony α6000 digital SLR (Sony Corporation, Japan), 24.5-megapixel resolution camera with a 23.5 × 15.6 mm sensor size, native resolution of 6000 × 4000 pixels and equipped with a 35 mm focal length lens. The pixel size was calculated using the Ground Sample Distance (GSD) calculator tool developed by Pix4D (https://support.pix4d.com/hc/en-us/articles/202559809-Ground-sampling-distance-GSD). The following equation was used: $\frac{(Sw.H.100)}{(Fr.imW)}$, where GSD represents the distance between two consecutive pixel centers, *Sw* indicates the sensor width of the camera (mm), *H* is the height (distance between camera and object) (m), *Fr* is the real focal length of the camera (mm) and *imW* is the image width (pixels). In our case, *Sw* was 23.2 mm, $H = 1.95$ m, $Fr = 18$ mm and $imW = 4608$ pixels. Therefore, images were analyzed with ImageJ image processing software using a GSD of 0.054 pixels cm^{-1} (Schneider et al., 2012).

2.3. Leaf pigments

Chlorophyll, flavonoids, anthocyanins, and the nitrogen balance index were determined for the middle third of the last developed leaf, one day before harvest, with a portable Dualex® meter (FORCE-A, Orsay, France).

2.4. Fluorescence measurements

To measure chlorophyll fluorescence, we used a MultispeQ device (Michigan State University, Michigan, USA), controlled by the PhotosynQ software platform (Kuhlgert et al., 2016). The last developed leaf at the time of harvest (the same used for all other evaluations) was used for measurements. Prior to fluorescence emission evaluations, part of the sampled leaf was dark-adapted for 15 min, in a box with no light entry. To estimate fluorescence-based parameters, we measured the quantum yield of photosystem II (PSII) photochemistry (ΦPSII), a non-photochemical quenching parameter describing regulated dissipation of excess energy (NPQt), non-photochemical quenching (ΦNPQ), other unregulated (non-photochemical) losses (ΦNO), and the maximum quantum yield of PSII (Fv/Fm).

2.5. *Leaf gas exchange measurements*

Gas exchange was evaluated in the last two days of the experiment, first in WW conditions and the second day in WS conditions, between 11:00 am and 2:00 pm, using a portable infrared gas analyzer, model LI-6400 (LI-COR, Lincoln, NE, USA), equipped with a light source (6400–40 LCF, LI-COR). During the evaluations, the PAR was set to 1500 μmol m^{-2} s^{-1}, the CO_2 concentration to 400 μmol mol^{-1}, relative humidity to between 55% and 60%, and temperature to 25 °C. The net photosynthetic rate (A), stomatal conductance (g_s), and transpiration rate (E) were measured in the last developed leaf of each plant. The instantaneous ($WUE_{instant} = A/E$) and intrinsic ($WUE_{intrinsic} = A/g_s$) water use efficiencies were also calculated.

2.6. *Stomatal and epidermal cell density*

A section about 1 cm in length and 0.5 cm wide from the adaxial and abaxial epidermal surface of the middle third of the last developed leaf, specifically between the central rib and the leaf edge, was varnished with nail polish. After drying for 10 min, the dried varnish layer was lifted off with adhesive tape and transferred to a glass slide. The numbers of stomata (s) and epidermal cells (e) were counted under a microscope, with a 10 X ocular and 40 X objective lens. Three microscope fields were counted in each (adaxial and abaxial) leaf replicate, according to Radoglou and Jarvis (1990).

Stomatal density (SD, stomata mm^{-2}) and epidermal cell density (ECD, cells mm^{-2}) were calculated with the following equations: $SD = \frac{s}{0.152}$; $ECD = \frac{e}{0.152}$; where 0.152 mm^{-2} is the surface area of each microscope field (radius 0.22 mm).

The stomatal index (SI, %) of each leaf surface was then calculated with the following equation $SI = 100x \left(\frac{SD}{ECD}\right)$.

2.7. *Relative leaf water content and specific leaf area*

Before harvest, fresh leaf discs (diameter = 1.65 cm) of the last developed leaf of each plant were collected and immediately weighed to determine the fresh weight (FW). Then the leaf discs were immersed in distilled water in darkness in a refrigerator (4 ºC) for 12 h. After imbibition, the discs were gently dried with paper towels and weighed again to determine the turgid weight (TW). Finally, the discs were oven-dried at 70 ºC for 72 h and the dry weight (DW) was determined.

The relative leaf water content (RWC; %) was calculated with the equation: $RWC = 100x\frac{(FW-DW)}{(TW-DW)}$.

For the same leaf discs, with a leaf area (LA) of 2.1282 cm^2, the specific leaf area (SLA; cm^2 g^{-1}) was calculated with the equation: $SLA = \frac{LA}{DW}$.

2.8. *Cumulative plant evapotranspiration and agronomic water use efficiency*

The total amount of water transpired from each plant during all the growth periods (the cumulative plant evapotranspiration) (ET, dm^3 plant $^{-1}$) was recorded throughout the growth cycle. To this end, before irrigation, each tube was weighed and the substrate surface of each was covered with plastic to avoid direct evaporation from the substrate surface.

In addition, based on the shoot (leaf + stalk) biomass weight (AB) and ET, the agronomic water use efficiency (WUE_{Agro}; g kg^{-1}) at harvest was calculated as: $WUE_{Agro} = \frac{AB}{ET}$.

2.9. *Stable carbon and nitrogen isotope signatures*

The isotope ratios of stable carbon ($^{13}C/^{12}C$) and nitrogen ($^{15}N/^{14}N$), together with total nitrogen content, were determined in samples of the last developed leaf in all plants, under both WCs.

Measurements of carbon and nitrogen isotope composition were carried out at the Scientific Facilities of the University of Barcelona, using an elemental analyzer (Flash 1112 EA; Thermo Finnigan, Berman Germany) coupled to an isotope ratio mass spectrometer (Delta C IRMS, Thermo Finnigan), operating in continuous flow. Samples of 0.7–0.8 mg of leaf dry matter from each plant, together with reference materials, were weighed and sealed into tin capsules. Isotopic results were expressed in standard δ-notation (Coplen and Zhu, 2008): $X = \left[\left(\frac{R_{sample}}{R_{standard}} - 1\right) * 100\right]$, where X is the $\delta^{13}C$ or $\delta^{15}N$ value, and R is the $^{13}C/^{12}C$ or $^{15}N/^{14}N$ ratio, respectively. The $\delta^{13}C$ values (‰) were based on the Vienna Pee Dee Belemnite isotope standard, and $\delta^{15}N$ values on N_2 in air (Farquhar et al., 1989).

2.10. *Root traits*

At harvest, the tubes were opened to separate the substrate from the roots. First, the substrate core was cut into five equal sections, each 0.30 m long, obtained from the upper surface of the tubes to the lower end, in the following layers: 0–30 cm (a), 30–60 cm (b), 60–90 cm (c), 90–120 cm (d), and 120–150 cm (e). Afterwards, samples were gently shaken and then washed with tap water, using mesh to remove the soil. Finally root samples were rinsed with distilled water and lightly dried with paper towels. Specific root length and root weight density were determined separately for each layer.

From each core section, a random sample of 4–5 root segments was collected from the total amount of roots recovered and stored in a fridge (4ºC) to later calculate the specific root length (SRL). To that end, the root samples were spread in a plastic tray (21.0 ×29.7 cm) containing about 1.0 L water (depth 0.5–1.0 cm) and photographed with a Sony α6000 DSLR camera (Sony Corporation, Japan). Thereafter, the images were processed using ImageJ software. Root images (Supplementary Material – Fig. 2) were analyzed with GiA Roots software (Galkovskyi et al., 2012), which includes user-assisted algorithms to distinguish roots from background and a highly automated "pipeline". When calibrating the resolution, the user can set the pixels cm^{-1} scale as desired. In this study, 1.0 cm corresponded to 148 pixels. From a list of root traits provided by GiA Roots, the "root network length (cm)" was selected, which corresponds to the total number of pixels in the network, representing the length of the analyzed root segments. The SRL in each of the five soil sections (SRL_{sample}, m g^{-1}) was determined using the analyzed images as follows: $SRL_{sample} = RL_{sample}/RB_{sample}$, where RL_{sample} represents the total length of the root segments (m) extracted from the root images, and RB_{sample} represents the dry root biomass (g) of the analyzed segments (Elazab et al., 2012).

Root dry weight was determined separately for each section. Each root section was placed in a paper envelope and oven dried at 60 °C for 72 h. The root segments used for the SRL estimation within each root section were dried separately. The root weight density of each soil section (RWD_{sec}, g m^{-3}) was calculated as before (Elazab et al., 2012) using the following expression: $RWD_{sec} = RB_{sec}/\pi * R^2 * L$; where RB_{sec} is the dry root biomass of the soil section (g); R is the tube radius (0.07 m) and L is the length of the tube section (0.30 m).

In addition, the ratio of shoot (aerial biomass - *AB*) to root (RB_{total} – sum of the five RB_{dry}) dry matter was calculated using $\frac{AB}{RB_{total}}$.

2.11. *Heterosis estimation*

For each trait, relative heterosis (*H*) was calculated as the difference between the mean performance of the hybrid (F_1) in relation to the means of its parents (*MP*) in absolute and percentage values, respectively, according to the following expressions: $MP = \frac{(P1+P2)}{2}$ and $H = \left(\frac{F_1-MP}{MP}\right) * 100$. In these cases, *P1* and *P2* indicate the mean of the parents and F_1 the mean hybrid performance (Hallauer et al., 2010).

2.12. Statistical analysis

For each study trait, individual variance analysis was performed within each water condition, whereas combined variance analyses were undertaken including both conditions. Individual variance was analyzed by the following statistical model: $Y_{ij} = \mu + G_i + B_j + \varepsilon_{ij}$, where Y_{ij} is the observation of the i-th genotype in the j-th block; μ is the general constant; G_i is the genotype effect; B_j is the block effect; and ε_{ij} is the experimental error. Combined variance analysis was performed according to the following statistical model: $Y_{ijk} = \mu + G_i + B/WC_{jk} + WC_j + G * WC_{ij} + \varepsilon_{ijk}$, where Y_{ijk} = observation of the ith genotype in the jth environment in the kth block; μ = general constant; G_i = fixed effect of the ith genotype; B/WC_{jk} = effect of the kth block in WC j; WC_j = fixed effect of the jth WC with NID; $G * WC_{ij}$ = fixed effect of the interaction between the ith genotype and the jth WC; and ε_{ijk} = mean experimental random error associated with observation Y_{ijk}, with NID (0, σ^2). Therefore, the parent and hybrid effects were partitioned separately, for each trait. Statistical analyses were performed with SAS software 9.4 (SAS Institute Inc., Cary, NC, USA).

The combining abilities were analyzed by method I diallel analysis, as proposed by Griffing (1956), in which p^2 combinations are included and the genotype effect is considered fixed. The four parents, hybrids, and reciprocal combinations were analyzed by this model.

The statistical model considered for the analysis is: $Y_{ij} = \mu + g_i + g_j + s_{ij} + r_{ij} + \mathcal{E}_{ij}$ where Y_{ij} = mean value of the hybrid combination (i ‡ j) or the parent (i = j); μ = general mean; g_i, g_j = effects of the general combining ability of the ith or jth parent (i, j = 1, 2, 3 and 4); s_{ij} = effect of specific combining ability for crosses between parents of order i and j; r_{ij} = reciprocal effect that measures the differences resulting from parent i, or j, when used as the male or female parent in cross ij; and Ɛ = mean experimental error associated with observation of order ij [*NID* (0, σ^2). The analyses were performed using GENES software (Cruz, 2013).

The quadratic components (ϕ) that express genetic variability in terms of general ($_g$) and specific ($_s$) combining abilities and reciprocal ($_{rc}$) effects were estimated by: $\phi_g = (QMG - QMR)/2p$; $\phi_s = QMS - QMR$; e $\phi_{rc} = (QMRC - QMR)/2$, where QMG is the mean square of the general combining ability, QMS is the mean square of the specific combining ability, QMRC is the mean square of the reciprocal effect, QMR is the mean square of the residue, and p is the number of parents. Lastly, the effects of the quadratic components were expressed as percentages relative to the sum of the total effects.

3. Results

3.1. Growth traits, leaf pigments, chlorophyll fluorescence and N status

The growth, leaf pigments, and N status traits studied differed statistically between inbred lines and hybrids, except for plant area and the specific leaf area under WW conditions (Table 1). Water conditions did not affect the flavonoid, anthocyanin, or the nitrogen contents or stable nitrogen isotope composition ($\delta^{15}N_{dm}$) in dry matter. Moreover, no effect of the genotype by water condition (G*WC) interaction was observed for plant area, plant height, flavonoid or anthocyanin.

All chlorophyll fluorescence traits differed significantly between WCs (Table 1). The maximum quantum yield of PSII (Fv/Fm), the quantum yield of photosystem II (ΦPSII) and the quantum yield of non-regulated non-photochemical energy loss in PS II (ΦNO) were 21.9%, 53.6% and 24.7% lower, respectively, under WS than WW conditions.

Table 1
Summary of combined and individual analysis of variance, means and standard deviations of morphological traits, leaf pigments, chlorophyll fluorescence and N status of popcorn inbred lines and hybrids grown under different water conditions, together with heterosis estimates (H%).

Traits	Water Stress								Well-watered								Comb. Analysis		
	Inbred lines		I	Hybrids		II	III	H (%)	Inbred lines		I	Hybrids		II	III	H (%)	G	WC	G*WC
Leaf biomass (g)	25.26	±2.25	**	34.31	±2.74	*	**	38.98	31.49	±2.49	**	40.27	±3.72	**	**	27.99	**	**	**
Plant area (m^2)	0.40	±0.04	**	0.48	±0.05	*	**	20.63	0.57	±0.03	*	0.64	±0.04	ns	**	12.88	**	**	ns
Stalk biomass (g)	28.28	±3.98	**	47.67	±6.39	**	**	72.97	56.86	±5.54	**	64.74	±12.58	**	*	15.85	**	**	**
Plant height (cm)	55.17	±9.05	*	68.08	±10.18	**	*	25.07	79.71	±15.11	*	86.25	±12.03	**	ns	8.48	**	**	ns
Specific leaf area (cm^2 g^{-1})	209.77	±19.85	ns	198.37	±10.87	**	*	-5.46	187.10	±10.14	*	191.67	±13.24	ns	ns	2.62	**	*	**
Chlorophyll content	28.04	±3.35	ns	25.27	±2.57	ns	*	-9.93	34.93	±3.20	**	27.18	±2.33	**	**	-21.29	**	**	**
Flavonoid content	0.96	±0.10	ns	1.08	±0.07	**	**	11.92	0.99	±0.08	*	1.12	±0.09	**	**	12.83	**	ns	ns
Anthocyanin content	0.19	±0.01	*	0.21	±0.01	ns	**	8.96	0.19	±0.02	ns	0.22	±0.01	**	**	12.16	**	ns	ns
Fv/Fm	0.58	±0.18	ns	0.53	±0.10	*	ns	-8.42	0.70	±0.11	ns	0.69	±0.08	ns	ns	-1.56	ns	**	ns
NPQt	2.41	±2.34	ns	3.62	±1.74	ns	ns	51.60	1.23	±1.56	ns	1.30	±1.11	ns	ns	21.53	ns	**	ns
ΦPSII	0.20	±0.05	ns	0.24	±0.07	ns	ns	22.73	0.57	±0.09	ns	0.46	±0.10	**	ns	-19.05	**	**	**
ΦNO	0.23	±0.07	*	0.17	±0.06	**	ns	-25.66	0.24	±0.04	ns	0.25	±0.05	*	ns	3.26	**	**	**
ΦNPQ	0.43	±0.05	**	0.55	±0.07	**	*	30.94	0.17	±0.04	ns	0.29	±0.07	**	**	69.24	**	**	**
Nitrogen balance index	29.38	±4.91	ns	23.78	±3.21	**	**	-19.22	35.68	±3.76	*	24.86	±3.06	**	**	-29.83	**	*	*
Nitrogen content$_{dm}$ (%)	2.39	±0.16	ns	2.21	±0.26	*	*	-7.33	2.61	±0.40	ns	2.07	±0.37	ns	**	-20.23	**	ns	*
$\delta^{15}N_{dm}$ (‰)	3.59	±1.13	ns	5.20	±0.81	**	**	45.34	4.70	±0.48	**	5.39	±0.86	*	*	17.45	**	ns	**

Fv/Fm: maximum quantum yield of PSII; NPQt: non-photochemical quenching parameter describing regulated dissipation of excess energy; ΦPSII: quantum yield of photochemical energy conversion in PS I; ΦNO: quantum yield of non-regulated non-photochemical energy loss in PS II; ΦNPQ: quantum yield of regulated non-photochemical energy loss in PS II. The values in the Inbred lines and Hybrids columns represent the means ± standard deviations of the respective 4 and 12 evaluated genotypes. Statistical differences (I) between inbred lines and (II) between hybrids under each water condition, according to the partitioning of the parent and hybrid effects. (III) Contrast between inbred line and hybrid means. Combined analysis (Comb. Analysis): genotype (G), water condition (WC) and the genotype*water condition (G*WC) interaction. Levels of significance: * $p < 0.05$; ** $p < 0.01$; ^{ns} = not significant.

The non-photochemical quenching parameter describing regulated dissipation of excess energy (NPQt) and quantum yield of regulated non-photochemical energy loss in PS II (ΦNPQ) values were 159.4% and 99.2% higher, respectively, under WS than WW. Differences between inbred lines and hybrids were only significant for ΦNPQ (Table 1). With the exception of Fv/Fm and NPQt, the G*WC interaction affected all other traits related to chlorophyll fluorescence (Table 1).

Compared to WW conditions, soil water stress caused decreases of 15.8%, 25.8%, 31.8%, and 23.4% in leaf biomass, plant area, stalk biomass and plant height, respectively (Table 1). Decreases in leaf biomass, plant area, stalk biomass and plant height were higher for the inbred lines i.e., 19.8%, 28.6%, 50.3%, and 30.8%, respectively, than for the hybrids, which decreased by 14.8%, 24.6%, 26.4%, and 21.1%, respectively. Regardless of the WCs, the values of these traits were higher for hybrids. Heterosis was more marked under WS (Table 1). Thus values for leaf biomass, plant area, stalk biomass and plant height were 38.9%, 20.6%, 72.9%, and 25.0%, respectively. The growth trait least affected in relative terms by water status and heterosis was specific leaf area, which was 5.6% higher under WS than WW ($p < 0.05$), whereas heterosis under WS was just 5.4% (Table 1).

Concerning leaf pigments, the chlorophyll content and the nitrogen balance index decreased more in the inbred lines (19.7% and 17.7%, respectively) than in the hybrids (7.0% and 4.3%, respectively) under WS and WW conditions. In general, under both WCs, the heterosis values of chlorophyll content and nitrogen balance index were negative, but positive for flavonoid and anthocyanin (Table 1). Under both WCs, hybrids had higher flavonoid and anthocyanin content, resulting in positive estimates of heterosis.

The heterosis estimates of ΦPSII, NPQt and ΦNO were more evident under WS, corresponding to 22.7%, 51.6%, and − 25.7%, respectively, than under WW conditions (−19.1%, 21.5%, and 3.3% under WW, respectively). The heterosis estimates of ΦNPQ were 30.9% under WS and 69.2% under WW conditions.

Under both WCs, nitrogen content in dry matter was higher in inbred lines and $\delta^{15}N_{dm}$ was higher in hybrids, and consequently heterosis values were negative for nitrogen content in dry matter but positive for $\delta^{15}N_{dm}$ (Table 1).

For both WCs, we highlight the importance (expressed in %) of the quadratic components related to general (ϕ_g) and specific (ϕ_s) combining abilities, and reciprocal effects (ϕ_{rc}) of the above growth measures, leaf pigments, and N status traits (Fig. 2). General combining ability (related to the quadratic component ϕ_g) and specific combining ability (related to the quadratic component ϕ_s) differed significantly under both WCs for most traits (Supplementary Table 1). Quadratic components (ϕ) with negative general combining ability effects were observed for chlorophyll content (−0.52) and nitrogen balance index (−0.83); these negative values must be interpreted as estimates of the real value equal to zero (Supplementary Table 1). Therefore, the quadratic component was no longer considered (Fig. 2), comprising 0% of the variation. In this way, the component does not explain the genetic variability of the trait. In general, even though mean squares related to general combining ability (related to quadratic component ϕ_g) and reciprocal effect (related to quadratic component ϕ_{rc}) were significant, the most important component to determine the traits (and to explain most of the genetic variability observed) was the quadratic component related to specific combining ability (related to quadratic component ϕ_s), with non-additive effects being the most prevalent (Fig. 2). The values were evident, under WS, for flavonoid (58%), leaf biomass (85%), plant height (50%), and stalk biomass (70%) traits, and under WW, leaf biomass (64%), nitrogen content in dry matter (64%), and PA (53%) (Fig. 2).

Regardless of the WCs, and given the large values of residual percentages, we observed a high environmental influence on chlorophyll fluorescence traits (Fig. 2). However, in ΦNO and ΦNPQ under WS, and ΦNPQ under WW ΦPSII, we found that the importance (expressed in %) of the quadratic components related to specific combining ability (ϕ_s), and with predominant non-additive effects, was the most important to explain the genetic variability for these traits (Fig. 2). The significance of values of the mean squares of general and specific combining ability, and reciprocal effect, and the respective quadratic components related to ϕ_g, ϕ_s, and ϕ_{rc} for chlorophyll fluorescence traits can be found in Supplementary Table 1.

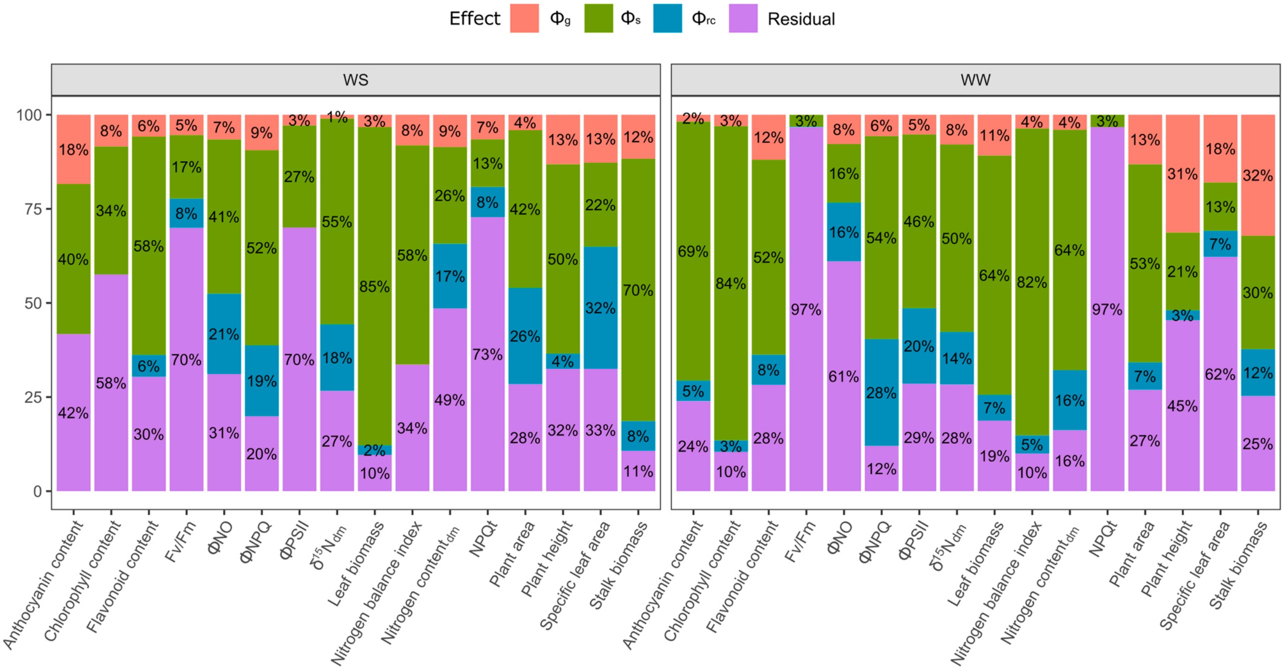

Fig. 2. Importance (expressed in %) of the quadratic components related to general (ϕ_g) and specific (ϕ_s) combining abilities, and reciprocal effects (ϕ_{rc}) for growth traits (leaf biomass, plant area, stalk biomass, plant height, and specific leaf area), leaf pigments (chlorophyll, flavonoid, and anthocyanin content), chlorophyll fluorescence (Fv/Fm, NPQt, ΦPSII, ΦNO, and ΦNPQ) and N status (nitrogen balance index, nitrogen content in dry matter, and $\delta^{15}N_{dm}$).

3.2. *Leaf gas exchange, stomatal characteristics, water status and stable C composition*

The combined analysis revealed a significant effect of water limitation, which decreased the leaf net CO_2 assimilation rate (A) by 17.1%, stomatal conductance (g_s) by 30.0%, the transpiration rate (E) by 28.7%, cumulative plant evapotranspiration (ET) by 29.4, and relative leaf water content by 4.3%, while increasing the intrinsic water use efficiency (WUE_{intrin}) by 18.3% and $\delta^{13}C$ by 7.8% (values averaged through both inbreds and hybrids). The differences between inbred lines and hybrids were significant for the traits ET and $\delta^{13}C$ under WS, and only for ET under WW conditions. Aside from ET, these traits were significantly affected by the G*WC interaction (Table 2).

The estimated heterosis was modest (<10.0%) for A, g_s, E and relative leaf water content under both WCs (Table 2). The heterosis estimates under both WCs for the traits of instantaneous water use efficiency ($WUE_{Instant}$), WUE_{intrin}, agronomic water use efficiency (WUE_{Agro}) and $\delta^{13}C_{dm}$ were low ($< \pm 7.0\%$). The heterosis effects for ET were higher, corresponding to 47.5% under WS and 31.4% under WW conditions (Table 2).

In general, for the stomatal characteristics (adaxial/adaxial index and density), no significant WC and genotype effects were observed in the combined analysis. Regardless of the WCs, these traits did not show contrasts between means in the inbred lines and hybrids. Therefore, the heterosis estimates for the traits were low ($< \pm 13.6\%$).

The ϕ_s effects related to specific combining ability (with non-additive effects being prevalent) were the most important for explaining the genetic variability, for adaxial stomatal density (38%), ET (77%), and WUE_{intrin} (42%) under WS, and for A (58%), adaxial stomatal density (49%), E (49%), and ET (66%) under WW (Fig. 3). Under WS in particular, we observed a strong effect of ϕ_{rc} (related to reciprocal effect) on genetic variability expression in A (30%) and g_s (32%) (Fig. 3). Supplementary Table 1 presents the significance of values of the mean squares of general and specific combining ability, and reciprocal effect, and the respective quadratic components related to ϕ_g, ϕ_s, and ϕ_{rc}, for photosynthetic and transpiration gas exchange, stomatal characteristics, water status and stable carbon isotope composition traits. In general, regardless of the WCs, and given the large residual percentage values, we observed a strong environmental influence on leaf gas exchange, stomatal characteristics, water status, and stable C composition (Fig. 2).

3.3. *Root traits*

No effect of water limitation on root weight density (RWD) or specific root length (SRL) was detected in the soil sections b and c, nor in the shoot-to-root dry matter ratio (shoot-root). With the exception of SRLa and SRL under WS, the differences between inbred lines and hybrids of all root traits were statistically significant (Table 3). The G by WC interaction was significant for all root traits. Regardless of the WC, the RWD of the hybrids was higher than that of their parents in the different tube sections (Table 3). A decrease in RWD was observed with increasing root depth under both WCs (Fig. 4A and B). The ratio between WS and WW for the RWD of the parents and the hybrids within each tube section showed a lower reduction in the RWD of the hybrids, mainly in soil sections c, d and e (Fig. 4C). The water stress-induced decrease in RWD was more prominent in inbred lines, with estimates of 47.1%, 55.0% and 52.6% for sections c, d and e, respectively, compared to the hybrid genotypes, with reductions of 42.5%, 50.3% and 39.9%, for the same sections. Regardless of the WC, the values of these sections (c, d and e) were higher for the hybrids than their parents. With the exception of RWD in soil section a, the heterosis of RWD for the other sections (b, c, d and e) was higher under WS. In the deeper soil layers, the heterosis effects increased for RWD-related traits (Table 3).

With the exception of SRL in soil section a, root-related trait estimates were higher under WS in sections b, c, d and e (6.7–18.7%), compared to WW. Specific root length tended to increase with increasing

Table 2

Summary of combined and individual analysis of variance, means and standard deviations of photosynthetic and transpiration gas exchange, stomatal characteristics, water status and stable carbon isotope composition traits of popcorn inbred lines and hybrids grown under different water conditions, together with heterosis estimates (H%).

Traits	Water Stress								Well-watered								Comb. Analysis		
	Inbred lines		I	Hybrids		II	III	H (%)	Inbred lines		I	Hybrids		II	III	H (%)	G	WC	G*WC
A (mol CO_2 m^{-2} s^{-1})	23.23	±4.03	*	24.41	±2.61	**	ns	5.34	27.84	±2.47	**	29.50	±2.75	**	ns	7.50	**	**	*
g_s (mol H_2O m^{-2} s^{-1})	0.10	±0.02	ns	0.10	±0.01	**	ns	0.53	0.15	±0.02	**	0.15	±0.03	*	ns	3.24	**	**	*
E (mmol H_2O m^{-2} s^{-1})	2.99	±0.71	ns	3.05	±0.44	**	ns	2.39	4.20	±0.27	**	4.28	±0.55	*	ns	3.50	**	**	*
Adaxial stomatal density (mm^{-2})	79.22	±6.11	**	72.49	±8.01	ns	ns	-7.58	78.22	±6.90	**	67.51	±6.80	**	ns	-13.55	**	ns	ns
Adaxial stomatal index (%)	19.60	±1.51	*	20.75	±2.92	ns	ns	6.02	21.37	±1.71	ns	20.32	±2.88	ns	ns	-4.90	ns	ns	ns
Abaxial stomatal density (mm^{-2})	103.07	±13.80	ns	98.51	±11.81	ns	ns	-4.37	96.67	±8.97	*	98.16	±10.46	ns	ns	2.07	ns	ns	ns
Abaxial stomatal index (%)	24.05	±1.69	*	25.29	±2.89	ns	ns	5.42	24.54	±1.60	*	26.35	±3.81	ns	ns	7.58	ns	ns	ns
ET (dm^3 $plant^{-1}$)	9.55	±1.66	*	13.80	±1.57	*	**	47.46	14.80	±3.07	**	19.10	±4.26	ns	*	31.37	**	**	ns
Relative leaf water content (%)	88.71	±4.24	ns	89.14	±1.97	**	ns	0.50	93.15	±1.62	ns	92.98	±1.56	ns	ns	-0.17	*	**	*
$WUE_{Instant}$ (mol CO_2 mmol H_2O^{-1})	7.84	±0.77	ns	8.10	±0.60	**	ns	3.37	6.65	±0.49	ns	6.96	±0.68	ns	ns	4.66	*	ns	ns
WUE_{intrin} (mol CO_2 mmol H_2O^{-1})	224.61	±33.24	ns	239.33	±18.20	**	ns	6.79	192.67	±18.81	*	201.40	±22.55	ns	ns	4.61	**	*	*
WUE_{agro} (g kg^{-1})	5.74	±0.38	*	5.85	±0.29	**	ns	2.15	5.84	±1.03	ns	5.68	±0.62	ns	ns	-2.87	*	ns	ns
$\delta^{13}C$ (‰)	-13.27	±0.21	**	-13.05	±0.30	ns	*	-1.65	-12.12	±0.30	ns	-12.17	±0.24	*	ns	0.38	**	**	**

A: leaf net CO_2 assimilation rate; g_s: stomatal conductance; E: transpiration rate; ET: cumulative plant evapotranspiration; $WUE_{Instant}$: instantaneous water use efficiency; WUE_{intrin}: intrinsic water use efficiency and WUE_{Agro}: agronomic water use efficiency. The values in the Inbred lines and Hybrids columns represent the means ± standard deviations of the respective 4 and 12 evaluated genotypes. Statistical differences (I) between inbred lines and (II) between hybrids under each water condition, according to the partitioning of the parent and hybrid effects. (III) Mean contrast between inbred lines and hybrids. Combined analysis (Comb. Analysis): genotypes (G), water condition (WC) and the genotype*water condition (G*WC) interaction. Levels of significance: * $p < 0.05$; ** $p < 0.01$; ^{ns} = not significant.

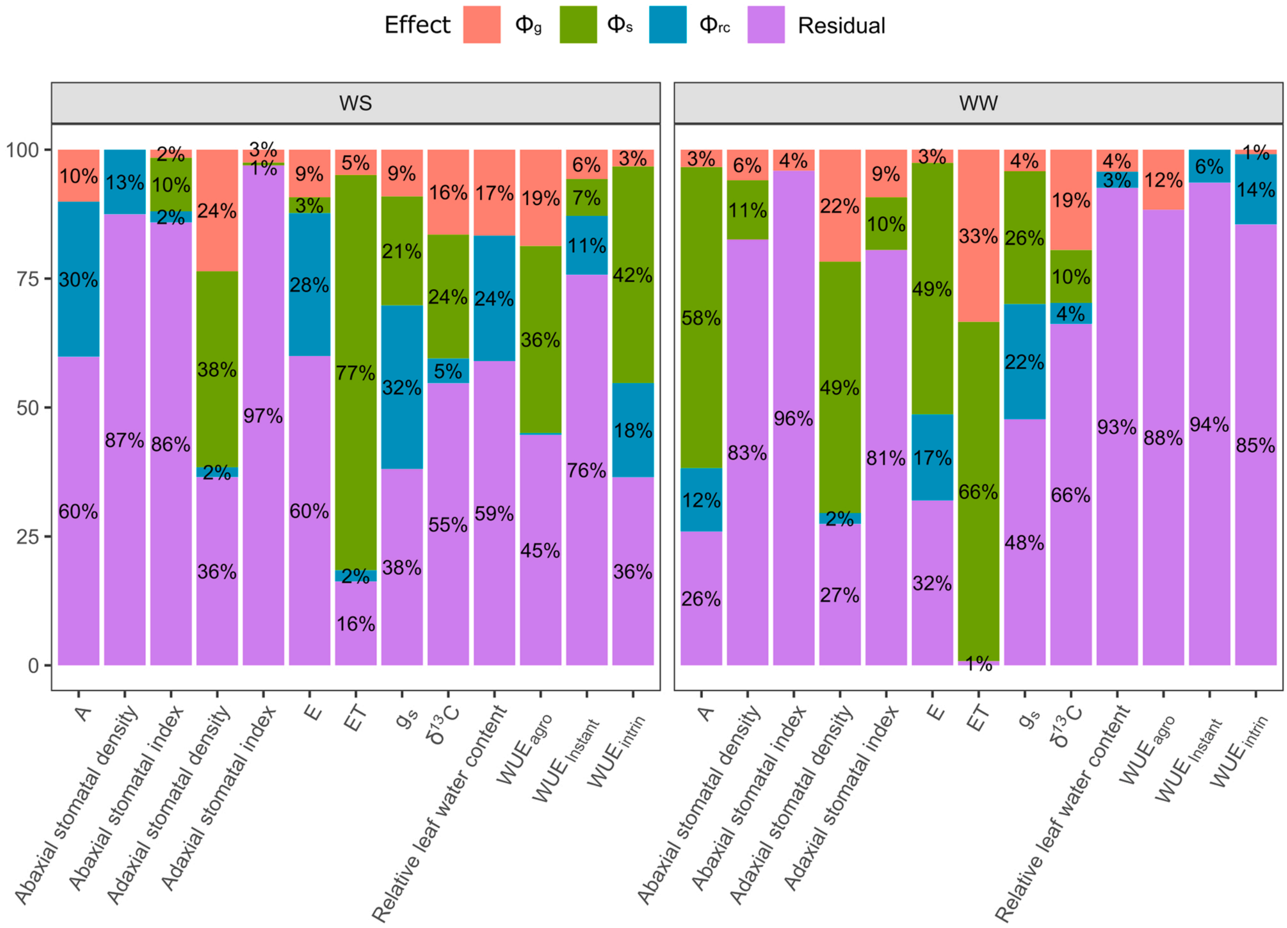

Fig. 3. Importance (expressed in %) of the quadratic components related to general (ϕ_g) and specific (ϕ_s) combining abilities, and reciprocal effects (ϕ_{rc}) for leaf gas exchange (A: leaf net CO_2 assimilation rate; E: transpiration rate; g_s: stomatal conductance), stomatal characteristics (abaxial-adaxial stomatal density; abaxial-adaxial stomatal index), water status (ET: cumulative plant evapotranspiration; relative leaf water content; $WUE_{Instant}$: instantaneous water use efficiency; WUE_{intrin}: intrinsic water use efficiency and WUE_{Agro}: agronomic water use efficiency) and the stable carbon isotope composition ($\delta^{13}C$).

soil depth under both WCs. In general, the heterosis values of SRL in all tube sections, were negative and higher under WW compared to WS conditions (Table 3).

Regardless of the WC, the shoot to root ratio of the hybrids was lower than in the parents. Negative heterosis effects were observed under both WCs (Table 3).

For most root traits in both WCs, the most important quadratic components (importance expressed in %) were ϕ_s (related to specific combining ability), wich non-additive effects being the most prevalent (Fig. 5). The significance of values of the mean squares of general and specific combining ability, and reciprocal effect, and quadratic components related to ϕ_g, ϕ_s, and ϕ_{rc}, respectively, for root traits can be found in Supplementary Table 1.

4. Discussion

4.1. Growth measures, leaf pigments, chlorophyll fluorescence and N status

Regardless of the WCs, the hybrid dry biomass was higher than in the inbred lines, both for leaves and stalks, and the plant height and area were higher as well, with more pronounced heterosis values under WS. The greater dry matter accumulation before flowering and larger leaf size (represented by plant area) was associated with a higher whole-plant canopy photosynthesis (Tollenaar et al., 2004) in the hybrids compared to the inbreds (Table 1). The higher photosynthetic area of hybrids compared to inbreds was probably due to greater cell expansion in the former because the density of epidermal cells in the leaves was lower in the hybrids than the inbreds, which indicates that hybrid epidermal cells were larger. The stomatal density of the adaxial side was also lower in the hybrids compared to the inbreds under both water conditions. Moreover the stomatal index, which is a trait that compares the number of stomata to the number of epidermal cells, remained basically constant between hybrids and inbreds, further suggesting that a generally greater expansion of cells was responsible for the larger leaf area of the hybrids In contrast to our results, Blum (2013) and Chairi et al. (2016) concluded that the heterosis observed in plant growth traits was the result of a larger number of cells instead of a larger cell size.

Greater cell expansion in hybrids compared to inbreds would be present not only in leaves but also in the non-laminar parts of the plant. Overall, the greater cell expansion in hybrids occurred under both WW and WS conditions, suggesting constitutive differences (i.e. even under well-watered conditions) in water status between hybrids and lines (Araus et al., 2010) and thus differences in cell turgor also being present. Concerning the non-laminar parts of the plant, the structure most affected by WS was the stalk in both the parent inbred lines and hybrids, and particularly so for the parents. The stalk has both a structural function, due to its high lignification, and plays a role in photoassimilate storage (Forell et al., 2015). When photoassimilation is reduced, the increase in stalk biomass is decelerated, which should also intensify stalk lodging (Robertson et al., 2017). In that sense, the hybrids are also better suited than the inbreds to adapt to water stress conditions.

In our study, the specific leaf area was greater under WS, and under this condition the parents had the highest values, resulting in negative

Table 3

Summary of combined and individual analysis of variance, means and standard deviations of root traits of popcorn inbred lines and hybrids grown under different water conditions, together with estimates of heterosis (H %).

Traits	Water Stress								Well-watered								Comb. Analysis		
	Inbred lines		I	Hybrids		II	III	H (%)	Inbred lines		I	Hybrids		II	III	H (%)	G	WC	G*WC
RWDa (g m^{-3})	1394.31	±183.28	**	2050.81	±236.71	**	**	50.85	2108.55	±234.28	**	3504.94	±583.14	**	**	67.52	**	**	**
RWDb (g m^{-3})	549.32	±55.85	**	1000.46	±194.42	**	**	87.10	711.90	±108.75	**	1290.40	±249.85	**	**	79.60	**	ns	**
RWDc (g m^{-3})	340.38	±37.28	**	655.85	±83.42	**	**	98.15	643.23	±143.28	*	1140.38	±241.30	**	**	75.45	**	ns	**
RWDd (g m^{-3})	224.29	±42.42	**	447.41	±74.28	**	**	106.98	498.47	±123.42	*	900.87	±183.14	**	**	85.57	**	**	**
RWDe (g m^{-3})	258.65	±55.17	**	512.42	±122.57	ns	**	105.32	545.56	±122.00	**	853.07	±197.57	**	**	61.26	**	**	*
SRLa (m g^{-1})	210.74	±54.86	ns	204.75	±26.62	**	ns	-2.49	190.23	±10.64	**	116.28	±29.79	ns	**	-37.33	**	**	**
SRLb (m g^{-1})	183.57	±31.50	ns	143.40	±19.98	**	**	-21.95	189.83	±26.48	*	126.60	±28.13	*	**	-32.96	**	ns	***
SRLc (m g^{-1})	209.73	±28.90	ns	166.70	±20.11	**	**	-20.77	220.06	±23.53	*	148.36	±22.37	**	**	-32.26	**	ns	**
SRLd (m g^{-1})	259.48	±27.76	ns	198.09	±29.49	*	**	-23.30	240.04	±30.54	*	159.79	±29.33	**	**	-33.12	**	**	**
SRLe (m g^{-1})	235.73	±26.70	ns	220.69	±36.65	ns	ns	-6.20	239.63	±28.73	ns	178.76	±31.07	ns	**	-25.59	**	**	*
Shoot root^{-1}	4.32	±0.58	*	3.83	±0.53	**	*	-10.05	4.06	±0.59	ns	3.17	±0.41	**	**	-21.92	**	ns	**

RWD: root weight density; SRL: specific root length; a-b-c-d-e indicate the depth of each soil section, i.e., 0-30 cm (a); 30-60 cm (b); 60-90 cm (c); 90-120 cm (d); and 120-150 cm (e); shoot-root: ratio of shoot-to-root dry matter. The values in the Inbred lines and Hybrids columns represent the means ± standard deviations of 4 and 12 evaluated genotypes, respectively. Statistical differences (I) between inbred lines and (II) between hybrids under each water condition, according to the partitioning of the parent and hybrid effects. (III) Mean contrast between lines and hybrids. Combined analysis (Comb. Analysis): genotypes (G), water condition (WC) and the genotype*water condition (G*WC) interaction. Levels of significance: * $p < 0.05$; ** $p < 0.01$; ^{ns} = not significant.

heterosis values, whereas no clear differences occurred under WW conditions, resulting in negligible heterosis effects. Although water stress is considered to induce plant morphoanatomical changes leading to reduce specific leaf area (Zhang et al., 2015), this trait is also sensitive to other environmental factors (e.g. temperature) and phenology-related metabolic variations (e.g. remobilization of photoassimilates) (Song et al., 2020; Zhang et al., 2015). The slightly higher specific leaf area of inbreds compared to hybrids (under WW conditions), means the former exhibit thinner and/or less dense leaves (Garnier et al., 1997; Vergara-Díaz et al., 2016), probably associated with a reduced cell expansion relative to the hybrids. However, the larger cell expansion of the hybrids may also account for the lower nitrogen content on a dry matter basis, as well as the lower nitrogen balance index and chlorophyll content on a per area basis (the last two measured with a Dualex sensor) of the hybrids compared to the inbreds. In fact, the estimates of heterosis for the chlorophyll and nitrogen contents were negative under both WCs, although the parents showed the greatest reductions in both traits due to WS. Previous studies in maize have found no heterosis effects on leaf N content in field-grown adult plants (Araus et al., 2010) and on N uptake in maize seedlings (Chairi et al., 2016).

The higher vigor of maize hybrids relative to their parents may lead to lower nitrogen content in plant tissues due to the increased demand (Holá et al., 2017). In any case, when net assimilation rates on a per area basis are considered, the higher nitrogen and chlorophyll contents in the inbreds may be counteracted by the better water status of the hybrids (particularly under WS conditions where $\delta^{13}C$ clearly indicated a better water status of the hybrids while specific leaf area was lower). In fact, we found only marginally higher net assimilation rates on a per area basis in the hybrids compared to the inbred lines, which agrees with previous studies where heterosis for net assimilation was marginal, at least under well-watered conditions (Chairi et al., 2016). In the same sense, no differences were reported in the net assimilation of hybrids and their parental inbred lines at silking (Ahmadzadeh et al., 2004).

Therefore, differences between parents and hybrids with regards to assimilation rate on a per area basis were either marginal or simply not significant, particularly under WW conditions. However, it is worth mentioning that hybrids had a considerably greater photosynthetic leaf surface, and therefore the total net assimilation per plant was higher in hybrids than in inbreds. Chairi et al. (2016) described that a marked heterosis effect on net assimilation and transpiration was only evidenced when these traits were expressed per whole leaf or plant rather than on a per unit area basis. However, Araus et al. (2010) under full field conditions also reported higher stomatal conductance and transpiration rates on a per unit area basis in hybrids than in their parents, even under WW conditions. In our study, the higher $\delta^{13}C$ values under WS conditions of hybrids compared to the inbreds supports a better water status in the former (Farquhar et al., 1989; Araus et al., 2010), in spite of no differences in instantaneous gas exchange measures (g_s and T) being recorded. In paralell, the higher $\delta^{15}N$ values in hybrids compared to the inbreds, with heterosis being higher under WS compared to WW conditions, further supports the notion that hybrids experienced less water stress and that even under WW conditions nitrogen uptake and further metabolism were more efficient in the hybrids (Yousfi et al., 2009, 2012). However, Chairi et al. (2016) found no significant differences in $\delta^{15}N$ between lines and hybrids.

In agreement with the literature, water stress induced a generalized decrease in photosynthetic (PSII) efficiency and an increase in heat dissipation (Kuhlgert et al., 2016). Therefore, WS triggered a photoprotective response that contributed to the preservation of the reaction centers through increased heat dissipation at the expense of PSII efficiency, and thus, there was a consequent fall in assimilation rate. However, in spite of the fact that all chlorophyll fluorescence traits were sensitive to WS, only ΦNPQ differed between the hybrids and their parents under any of the WCs. Thus, hybrids showed a significantly higher ΦNPQ, and hence positive heterosis values, regardless of the water conditions. Therefore, our results suggest that maize hybrids

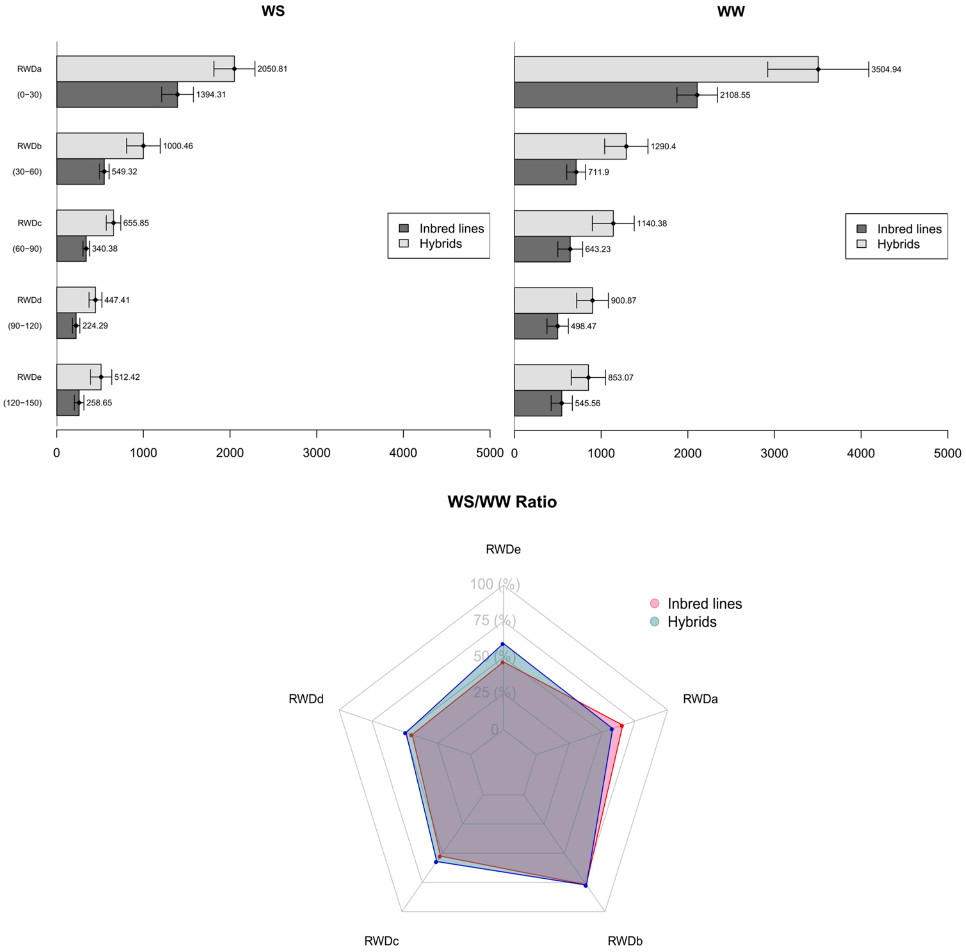

Fig. 4. Root weight density (RWD, g m^{-3}) at different soil depths for popcorn inbred lines and hybrids grown under different water conditions (WS, water stress – A; WW, irrigation at field capacity – B), and WS/WW ratio (C).

possess a greater photoprotective response, which may have conferred an adaptive advantage with regard to the parents, eventually contributing to a better physiological/agronomic performance (Kuhlgert et al., 2016).

In terms of the leaf accessory pigments, hybrids showed higher flavonoid and anthocyanin contents regardless of the WC, resulting in positive estimates of heterosis. Flavonoids and anthocyanins are phenolic compounds of ecophysiological relevance that function as antioxidants, osmoregulators and photoprotective compounds in plant cells (Chalker-Scott, 1999; Gould et al., 2002; Mazza et al., 2000; Steyn et al., 2002). As such, they are implicated in plant responses to environmental stress, including water stress (Nakabayashi et al., 2014). Thus, these compounds prevent the proliferation of reactive oxygen species that may endanger cell metabolism and homeostasis (Kalaji et al., 2017; Wahid et al., 2007). In the present work, the higher content of phenolic compounds in the hybrids than in inbred lines may represent an adaptive advantage that might contribute to a greater drought tolerance through avoidance of photoinhibition.

The other two fluorescence traits, ΦPSII and ΦNO, also differed between inbreds and hybrids, but in the case of ΦPSII, heterosis changed from positive under WS to negative under WW conditions, whereas ΦNO was negative under WS and near zero under WW conditions. Generally, an increase in thermal dissipation by unregulated processes (ΦNO) under severe stress conditions can indicate photoinactivation (Busch et al., 2009). According to these authors, higher A values are positively correlated with ΦPSII (Busch et al., 2009). Our results showed a slight tendency towards higher A and ΦPSII values in hybrids, mainly under WS.

4.2. Water status and cumulative evapotranspiration

Despite the reduction in relative leaf water content under WS compared to WW conditions, this was not an important trait for differentiating the parents from their hybrids, resulting in estimates of null heterosis. It has been reported that genotypes with greater WS tolerance have a higher relative leaf water content, mainly due to the ability to grow a deeper root system and extract water from deeper soil layers, or as a result of greater osmotic adjustment (Gao and Lynch, 2016), or because they control transpiratory losses more efficiently. Higher relative leaf water content may allow greater stomatal opening and thus maintenance of photosynthetic metabolism to some extent (Blum, 2011).

On the other hand, water deficit induced a higher WUE as shown by the significant difference in $\mathrm{WUE}_{\mathrm{intrin}}$ between WS and WW conditions. In the same sense, $\delta^{13}C$ values were more negative under WS than under WW conditions. Opposite to the pattern reported for C3 species, water stress in a C4 species like maize causes more negative $\delta^{13}C$ values, which is associated with a higher time-integrated intrinsic WUE (Farquhar et al., 1989; Cabrera-Bosquet et al., 2009; Araus et al., 2010; Chairi et al., 2016). Concerning the differences between inbred lines and the corresponding hybrids, the later exhibited slightly higher $\mathrm{WUE}_{\mathrm{intrin}}$ and $\mathrm{WUE}_{\mathrm{Instant}}$, regardless of the WCs. However, $\delta^{13}C$ was more negative in the inbreds than hybrids, at least under WS conditions, which suggests that inbred lines suffer more water stress under WS conditions. However, besides that, no significant differences existed between WCs or between hybrids and their parents for $\mathrm{WUE}_{\mathrm{agro}}$, even though heterosis under WS conditions was slightly higher in hybrids than in inbreds. It is

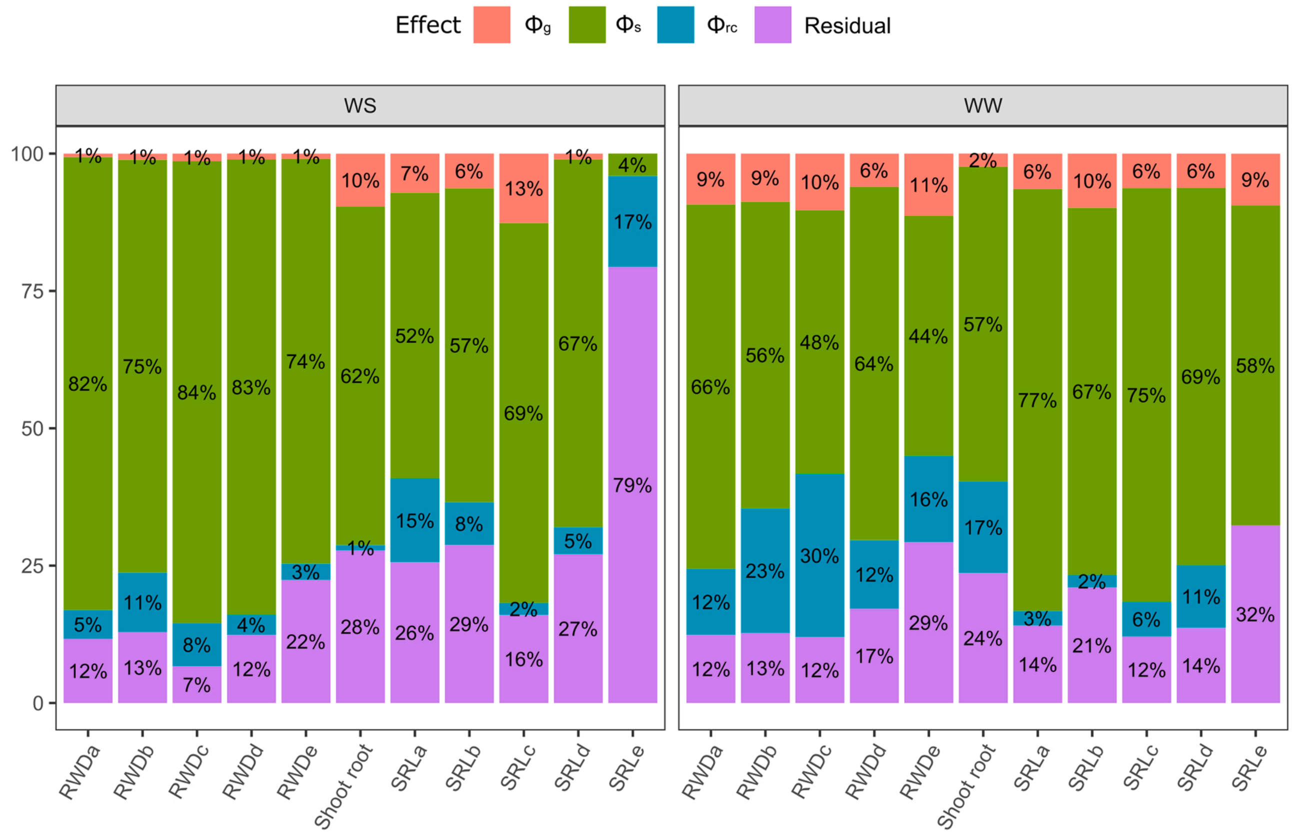

Fig. 5. Importance (expressed in %) of the quadratic components related to general (ϕ_g) and specific (ϕ_s) combining abilities, and reciprocal effects (ϕ_{rc}) for root traits (RWD: root weight density; SRL: specific root length; a-b-c-d-e indicate the depth of each soil section, i.e., 0–30 cm (a); 30–60 cm (b); 60–90 cm (c); 90–120 cm (d); and 120–150 cm (e); shoot-root: ratio of shoot-to-root dry matter).

likely that the level of water stress was moderate, which, while preventing net assimilation rates from differing markedly between inbreds and hybrids, resulted in the plants adjusting the growth of the photosynthetic area to the available water. Also, it should be noted that calculations of cumulative plant evapotranspiration (ET) did not consider the weight of the (growing) plants, thus causing a certain underestimation of the amount of water needed to reach 100% and 35% of the relative field capacity of the tubes, and consequently, the heterosis values of ET tended to be underestimated. As hybrids grew and weight increased, there was increasing underestimation of the ET of these genotypes and consequently its heterosis effect. Under WS in particular, this fact may have influenced the WUE_{Agro} values, which were higher in hybrids, but did not differ statistically between parents and F_1. The above reasons may justify, at least in part, the lack of differences in WUE_{agro} between inbred lines and hybrids.

In any case, the heterosis values for cumulative plant ET were high, especially under WS. In the study of Chairi et al. (2016), the authors described that heterosis in maize seedlings manifests as higher plant water consumption under both WCs, which results in hybrids being more vigorous than the corresponding inbred lines. This agrees with the increased cumulative leaf biomass and plant area. Sustaining a higher cumulative ET in hybrids compared with inbreds requires a suitably adapted root system.

4.3. Root traits

In response to WS, root weight density (RWD) decreased in all studied soil strata (Fig. 4A, B) in both parents and hybrids. Roots play an important adaptive role in drought-prone environments; and in this situation, a better developed system, especially in deeper soil layers, favors higher grain yields (Ali et al., 2016). According to Lambers et al. (2002), under WS, the metabolic cost of the root system to explore the soil is high and can exceed a plant's daily photosynthetic rate by 50%. For this reason, plants under WS tend to have lower RWD, and this was around 39.8% in our observations (Table 3). The hybrids had higher values for RWD than the inbreds, regardless of the soil section (a, b, c, d or e) (Fig. 4A and B), indicating a better developed root system. In addition, the hybrids showed a higher RWD ratio (RWD-WS/RWD-WW) when comparing WCs (Fig. 4C). It is noteworthy that these values were the highest in sections c, d and e, indicating greater investment and deeper root development, in response to drought adaptation (Fig. 4C).

From the five studied sections of RWD, heterosis was more evident in the four deepest soil sections under both WCs, but mainly under WS. The root system developed in deeper soil layers contributes to an efficient water extraction from these more distant profiles (Lynch, 2013), a finding observed in popcorn hybrids that tends to provide adaptive advantages to WS in the field. Ali et al. (2016) reported under WS field conditions a correlation between the biomass of deep maize roots (> 45 cm) and higher grain yield, whereas the development of deeper roots is relevant for drought tolerance. Heterosis effects in root length and root dry matter on popcorn genotypes are already evident during the early stages of plant development, a few days after germination (Rockenbach et al., 2018). Similar results have also been reported in seedlings of other maize categories (Chairi et al., 2016). Under field conditions, the heterosis advantage in terms of deeper roots may be even more relevant than under controlled experimental setups based on lysimeters, even when long lysimeters were deployed, as in our study (1.5 m deep lysimeters were used).

The specific root length (SRL) tended to increase (i.e. roots to become thinner) with increasing substrate depth (strata b, c, d and e), in both genotype groups and WCs. The greater biological activity of thinner roots, which are responsible for nutrient uptake, is well known (Eissenstat, 1992). Also, plants with high SRL tend to have higher water uptake rates (Elazab et al., 2012). However, inbreds exhibited thinner roots than hybrids, which may contradict the positive role of thinner roots. Thus, under both WCs and in the five soil segments, the inbred lines exhibited higher SRL (thinner roots), resulting in negative heterosis values. Contrastingly, Li et al. (2008) reported greater development of fine roots in maize hybrid seedlings as compared to their parents under WS.

It is believed that the lower availability of photoassimilates due to water stress may contribute to higher SRL values under WS compared to WW conditions. The same rationale may be applied for the comparison between inbreds and hybrids, with the former exhibiting thinner roots (i.e. higher SRL) in order to optimize the photoassimilates available for growing roots. A greater SRL may also be a consequence of a less compact structure; for example, with aerenchyma in the cortex (Chairi et al., 2016). In this sense, Elazab et al. (2012) reported a water stress-driven increase in SRL in durum wheat, reinforcing the role of thin roots (i.e., high SRL) for the maintenance of high transpiration rates. In any case, a greater root plasticity of the hybrids compared to their parents was noticed, since the SRL values of the latter were similar under both WCs, whereas they increased in hybrids in response to water stress. In that sense, Chairi et al. (2016) suggested that the acclimation capacity of the root structure to WS is lower in the parents than in the hybrids.

Regarding the shoot to root dry matter ratio, water stress tended to induce higher values, but the differences were not significant. Previous works have reported a decrease in the shoot to root ratio in response to water limitation (Chairi et al., 2016; Kamphorst et al., 2020a). For their part, hybrids showed lower shoot to root ratios than their parents, resulting in negative estimates of heterosis. This was indicative of a greater plasticity in root development and adaptation in the hybrids, particularly in response to WS conditions. Our results are in line with the negative heterosis effect on this ratio reported by Chairi et al. (2016) in maize seedlings.

4.4. Implications for plant breeding

In general, WS discriminated the studied genotypes more clearly as well as the heterosis values, which were also higher under this condition. This can be explained by the fact that the genotypes differed more under WS, which should also result in higher heritability values. In agreement with that, Kamphorst et al. (2019) found higher heritabilities under WS when evaluating popcorn genotypes under different water regimes in the field, indicating greater reliability in the selection under this WC due to the higher genetic variance. Moreover, based on the mean square estimates of the effects, there was no change between the WCs of the quadratic component with highest relevance for the traits evaluated in this study. This implies that the mode of trait expression is the same under both WCs. In an inheritance study, Lima et al. (2019) established that the genetic effects were the same under WS and WW conditions in popcorn, and inferred that the same breeding methods could be applied for both WCs. Nevertheless, the genetic gain will be greater under WS, as stated by Kamphorst et al., (2019, 2021a) and Lima et al. (2019).

There is a greater influence of non-additive effects (related to specific combining ability) on the control of leaf biomass, plant area, the content of chlorophyll, flavonoids, and anthocyanins, and the nitrogen balance index, as well as the leaf nitrogen content and $\delta^{15}N$ in traits under both WCs. Therefore, it can be concluded that the exploration of heterosis is recommended as a strategy to achieve genetic gains (Cruz et al., 2014; Hallauer et al., 2010). Although the mean square of specific combining ability (ϕ_s) is predominant for most traits (as shown by the relative importance in Fig. 2), some of them also have a significant general combining ability (ϕ_g) and reciprocal effect (ϕ_{rc}) component, indicating the influence of both additive and non-additive gene action and the influence of the female parent, even if to a lesser extent. Indeed, ϕ_s was the most important component to determine these traits, and it also explained the genetic variability (Fig. 2). As pointed out by Tollenaar et al. (2004) and observed in the present work, the superiority of the maize hybrids manifested as dry matter accumulation before flowering in the leaf and stalk biomass, and greater plant area in terms of leaf size and leaf area index, and these factors were due to heterosis.

Although the effect of the quadratic component of specific combining ability stood out for chlorophyll and the nitrogen balance index (in other words, ϕ_s was the most important component to determine the traits, and it also explained genetic variability), the heterosis values were negative, indicating that the hybrids had lower values for these traits than their parents. This is due to the fact that a larger plant size, normally observed in hybrids, sees investment of the N in growth; in other words, hybrids exhibit faster leaf growth than the inbred lines, and this is associated with a “dilution” effect with regard to the N content. In this case, N content measured on a dry matter basis in leaves is lower in the hybrids, but positive heterosis in N would be detected by total plant N content instead of N content per unit dry matter. The potential disadvantage of the hybrids in terms of lower leaf N was noticed particularly under WS, as described by Holá et al. (2017). However, we consider that a lower N content on a dry matter basis or on a per unit area basis in the hybrids is a positive trait in the sense that it is an indicator of a faster growth in the hybrids compared to the inbred lines. The same rationale applies to the total chlorophyll content, which was lower in the hybrids than in the inbred lines. Indeed, the leaf chlorophyll content measured under contrasting water environments in a diallel involving WS-tolerant tropical maize parents, was reported to have a predominant general combining ability effect (Erdal et al., 2016). These authors concluded that ϕ_g was the most important component to determine the traits, and it also explained the genetic variability. In fact, additive and non-additive effects have been cited as being involved in the expression of traits related to leaf greenness. To our understanding, increasing these values under field conditions would be advantageous because higher leaf green values are associated with grain production (Cairns et al., 2012).

Given the prevalence of the quadratic effects of specific combining ability, we recommended further exploration of heterosis to increase root weight density (RWD) and specific root length (SRL) in WS and WW environments. Indeed, ϕ_s was the most important component to determine these traits, and it also explained the genetic variability (Fig. 4). In a study carried out under WW and WS conditions, Ali et al. (2016) described highly positive correlations between grain yield and root biomass in maize hybrids. On the other hand, in the evaluation of a maize diallel under abiotic stresses (N and water stress), Chun et al. (2005) observed that for RWD in particular the heterosis values were significant under the different N levels studied, which was attributed to the significant general and specific combining ability effects, and mainly the latter. Based on these reports and our results, we emphasize that the use of hybrids can ensure improved root development under WS.

Unlike the observations under WS, additive effects (related to general combining ability) were predominant for stalk biomass, plant height and specific leaf area under WW conditions This means that $\phi_g$was the most important component to determine these traits, and it also explained the genetic variability (Fig. 2). Evaluating a complete maize diallel in the field under stressful and non-stressful water regimes, Wattoo et al. (2014) described the significance of general and specific combining ability under both WCs, which indicated the influence of both additive and non-additive gene action, with greater influence of the former. Additive effects were also observed for $\delta^{13}C$ under both WCs. In our study, it is believed that the presence of one parent with a high mean (in a negative sense) may cause an interference, resulting in the mean of the hybrids becoming the mean performance of the parents.

It was assumed that the cytoplasmic effect and the nuclear genes of the maternal genotype influenced gas exchange traits (A, g_s and E) under both WCs. However, this effect was even clearer under WS, which means that ϕ_{rc} was the most important component to determine these traits, and it also explained the genetic variability observed. The reciprocal effect in crosses is due to the action of mitochondrial and chloroplast genes (extrachromosomal effect) and genes from the nucleus of the maternal parent (maternal effect) (Cruz et al., 2014). Reciprocal effects on the chlorophyll a, b and ab contents in millet genotypes have also been described, evaluated in a complete diallel of 10 parents (Mehndiratta and Phul, 1983). In this sense, it can be acknowledged that the extrachromosomal genes associated with photosynthesis in the female parent interfere with the agronomic performance of the hybrids. Therefore, when breeding hybrids, it is recommended to prioritize the parent with the highest photosynthetic rate as the female parent, especially under drought conditions.

5. Conclusions

The effects of heterosis in popcorn related to biomass were the result of better growth in the shoots, which increased the photosynthetic area of the plant, as well as the roots, which secured an efficient capture of water. Hybrid vigor was even more evident under water stress conditions, which further highlights the importance of an efficient, albeit plastic, root system in the hybrids. By contrast, the potential role of a higher net assimilation rate per unit area in the hybrid vigor was minor at best, if not completely absent, even when the leaves of the hybrids exhibited somewhat better photoprotective mechanisms than the inbred lines.

Despite a lack of difference in instantaneous measurements of stomatal conductance and transpiration between hybrids and inbreds, the δ^{13}C of dry matter provided a clue about the better water status in hybrids compared to inbreds under WS. In addition, hybrids exhibited higher δ^{15}N in leaves than the inbreds, particularly under WS conditions. This further supported the role of the roots in the hybrids in not only securing more efficient water uptake, but also more efficient nitrogen uptake. Concerning leaf growth, this study also evidenced that the lower nitrogen and chlorophyll content of leaves in hybrids compared with inbred lines is likely the result of faster leaf growth in the former.

Since the non-additive gene effects (due to quadratic components related to specific combining ability comprising most of the genetic variability) are responsible for the control of most shoot growth and root traits, and especially so under water stress, the success of breeding programs for drought adaptation requires appropriate exploitation of heterosis, magnifying wherever possible the traits that confer hybrid vigor. For traits related to gas exchange (leaf net CO_2 assimilation rate, and stomatal conductance), it was assumed that the cytoplasmic effect and the nuclear genes of the maternal genotype were an influence under WS. Therefore, the choice of the female parent is essential to breed for improved performance under drought stress.

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Declaration of Competing Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Data availability

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.agwat.2021.107371.

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