ANÁLISIS DE MECANISMOS BIOQUÍMICO-FISIOLÓGICOS INVOLUCRADOS EN LAS RESPUESTAS A SEQUÍA EN SOJA

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TABLA DE CONTENIDO

página

RESUMEN

El déficit hídrico limita la producción y reduce la estabilidad del rendimiento en el cultivo de soja. Muchos parámetros se han asociado a tolerancia al déficit hídrico, siendo el control de la pérdida de agua foliar el más determinante. Dado que la mayor pérdida de agua se da a través de los estomas, el objetivo de la tesis fue profundizar en las respuestas al déficit hídrico en soja a través del control del cierre estomático y determinar cómo este puede afectar el consumo de agua y la tolerancia al déficit hídrico. En este sentido, nos centramos en el óxido nítrico ($^{\bullet}$NO) como molécula clave en el control del movimiento estomático. Se analizó su cinética de acumulación a partir del establecimiento del déficit hídrico, y su asociación con el cierre estomático. Nuestro trabajo mostró una acumulación de $^{\bullet}$NO endógeno en las células guarda previo al cierre estomático, lo cual permite postular que ante la percepción inicial del estrés existe una acumulación de $^{\bullet}$NO que inhibe el cierre estomático potencialmente a través del bloqueo de las respuestas a ABA. Este control fino inicial del cierre estomático por el $^{\bullet}$NO sería determinante para definir las respuestas finales de pérdida/consumo de agua durante el establecimiento del déficit. En esta misma línea de interés, desarrollamos en soja un modelo matemático empírico que describe la cinética del consumo de agua bajo condiciones de déficit hídrico de un sistema controlado de crecimiento planta-maceta-sustrato. El modelo definió dos parámetros que determinan la curva de consumo: el $t_{0.5}$ (tiempo en el cual el sistema perdió la mitad del agua potencialmente evapotranspirable) y el $Gw(t_{0,5})$ (conductancia estomática a $t_{0,5}$). Un análisis de correspondencia entre $t_{0.5}$ y $Gw(t_{0,5})$, y la estructura genética de dos poblaciones de soja, indicó la existencia de asociaciones genéticas con fenotipos de consumo basados en estos parámetros. Por otro lado, utilizando datos del índice de susceptibilidad a sequía (DSI) en campo, generados por un trabajo previo sobre una de las poblaciones de esta tesis, y a través de un análisis de correspondencia, se observa un agrupamiento entre el parámetro del modelo $t_{0,5}$ y DSI. Esto indicaría que la estrategia de fenotipado desarrollada también podría ser de utilidad en la caracterización de genotipos a nivel de campo.

Palabras clave: soja, déficit hídrico, óxido nítrico, fenotipado, estomas

ANALYSIS OF BIOCHEMICAL-PHYSIOLOGICAL MECHANISMS INVOLVED IN RESPONSES TO DROUGHT IN SOYBEAN

SUMMARY

Water déficit (WD) limits soybean crop productivity and yield stability. Many parameters are associated with tolerance to WD, where the control of water loss is the most important. Since most of the water loss occurs through the stomata, the objective of this thesis was to deepen the understanding of the response to WD in soybeans through the control of stomatal closure and to determine how this can affect the water consumption and its tolerance to WD. To achieve this, we focused on the role of nitric oxide ($^{\bullet}$NO) as a key molecule in stomatal opening/closing control. Our work showed an endogenous $^{\bullet}$NO accumulation at the guard cell level before stomatal closure, which allowed us to postulate that in the early perception of stress there is an accumulation of $^{\bullet}$NO that inhibits stomatal closure, potentially through blocking responses to ABA. This initial fine control of the stomatal closure by $^{\bullet}$NO would be decisive to define the final responses of water loss/consumption during the establishment of the WD. Furthermore, we developed an empirical mathematical model in soybean that describes the kinetics of water consumption under WD conditions of a controlled growth system. The model defined two parameters that determine the water consumption curve, $t_{0.5}$ (time at which the system lost half of the maximum amount of potentially evapotranspirable water) and $Gw(t_{0.5})$ (stomatal conductance at $t_{0.5}$). A correspondence analysis (CA) between $t_{0.5}$ and $Gw(t_{0.5})$, and the genetic structure of two soybean populations, indicated the existence of genetic associations with consumption phenotypes based on these parameters. On the other hand, using data from the drought susceptibility index (DSI) in the field, generated by previous work on one of the populations of this thesis, and through a CA, a grouping between the model parameter $t_{0.5}$ and DSI is observed. This would indicate that the developed phenotyping strategy could also be useful in the characterization of genotypes at the field level

Keywords: soybean, water deficit, nitric oxide, phenotyping, stomata

1. INTRODUCCIÓN

La necesidad de expansión de la frontera agrícola y el cambio climático global imponen nuevas restricciones a la producción de los cultivos, que incluyen tanto estrés de origen biótico (diversas enfermedades y plagas) como abiótico (déficit hídrico, temperaturas extremas…) (Pandey et al., 2017).

En Uruguay el cultivo de soja pasó de ser un cultivo marginal para convertirse en el principal cultivo de secano del país desde el año 2010 con más de 1 millón de hectáreas cultivadas; sin embargo, el aumento del área de cultivo no ha sido acompañada por un aumento de la productividad, la cual se encuentra estancada en el entorno de los 2000 a 2500 kg/h (DIEA). Estos rendimientos son sensiblemente menores a los de Argentina, Brasil, Paraguay y EE. UU.; además, la variabilidad anual de los rendimientos obtenidos en Uruguay es notoriamente mayor que la de los mencionados países.

Entre los factores ambientales que más inciden en el desarrollo y rendimiento del cultivo de soja está el déficit hídrico (Fuganti-Pagliarini et al., 2017, Hufstetler et al., 2007), reduciendo tanto los rendimientos como la intención de siembra. La severidad del estrés por déficit hídrico depende tanto del momento, duración e intensidad de este, como de la respuesta de las plantas a dicha situación (Serraj et al., 2005).

En Uruguay los suelos tienen la particularidad de presentar una baja capacidad de almacenamiento de agua, además de elevadas demandas atmosféricas en los meses de verano. Si a esto le sumamos la alta variabilidad en volumen, intensidad y distribución de las precipitaciones, las cuales son la principal fuente de recarga hídrica de los suelos, obtenemos como resultado bajos rendimientos y una alta variabilidad.

1.1. TOLERANCIA AL DÉFICIT HÍDRICO EN PLANTAS

La tolerancia al déficit hídrico puede definirse como la capacidad que manifiesta una especie o variedad de sobrevivir, mantener o disminuir en baja proporción su rendimiento en condiciones de déficit hídrico. Muchos parámetros se han asociado a tolerancia al déficit hídrico en plantas, entre los que se encuentran:

a) marchitamiento lento de la canopia, primer síntoma visible de déficit hídrico (Carter et al., 1999),

b) sistemas radiculares con raíces más fibrosas, los cuales permiten una mayor exploración del suelo en búsqueda de agua (Pantalone et al., 1996),

c) mayor eficiencia del uso del agua (EUA), asociada a una mayor cantidad de biomasa producida por unidad de agua consumida (Mian et al., 1996),

d) acumulación de osmolitos compatibles, los cuales impiden la disminución del potencial hídrico sin interferir con el funcionamiento celular, incluso a altas concentraciones (Turner et al., 2001),

e) mantenimiento del movimiento del agua en presencia de bajas tasas transpiratorias, asociado al movimiento simplástico facilitado por acuaporinas (Steudle y Frensch, 1996, Steudle y Peterson, 1998, Steudle, 2000),

f) control de la pérdida de agua foliar (Xiong et al., 2002).

1.2. CONTROL DE LA PÉRDIDA DE AGUA FOLIAR Y SU IMPACTO EN LA TOLERANCIA AL DÉFICIT HÍDRICO

De los parámetros asociados con tolerancia al déficit hídrico, el control de la pérdida de agua foliar es el que más contribuye en mejorar la tolerancia a este. Dado que la mayor parte de la pérdida de agua foliar se da por transpiración a través de los estomas, el ajuste de la apertura/cierre, el tamaño y la densidad estomática es fundamental para controlar la pérdida de agua (Sirichandra et al., 2009, Xiong et al., 2002). Un dato a tener en cuenta sobre la importancia de la pérdida de agua foliar es que del total del agua absorbida a través de las raíces por las plantas, más del 95 % es perdida por transpiración y solo menos del 5 % es utilizada para el crecimiento y funciones metabólicas.

Resultados obtenidos en mi tesis de maestría, donde se caracterizaron las respuestas tempranas y tardías al déficit hídrico en soja, mostraron que esta especie tiene altos niveles de pérdida de agua por transpiración y se identificó el cierre estomático como una respuesta temprana inducida por el déficit hídrico potencialmente responsable de esta pérdida. Con base en estos antecedentes, centramos nuestro proyecto de tesis en

la generación de herramientas metodológicas útiles en la selección de genotipos con respuestas adecuadas a las características del déficit hídrico y, por lo tanto, contribuir a los programas de mejoramiento de soja.

La tesis presenta una primera parte enfocada en el óxido nítrico ($^{\bullet}$NO) y su rol en el control del cierre estomático bajo condiciones de déficit hídrico. La segunda parte presenta el desarrollo de un modelo matemático basado en aproximaciones biológicas que describe la cinética de consumo de agua en soja a partir de la suspensión del riego. Evaluaciones realizadas al modelo indican que podría utilizarse para el fenotipado y la selección de genotipos de soja en relación con su rendimiento bajo condiciones de déficit hídrico.

1.3. HIPÓTESIS DE TRABAJO

La cuantificación de parámetros bioquímicos y fisiológicos de respuesta a sequía permite explicar la diferencia en la tolerancia a sequía de distintos genotipos de soja y, por lo tanto, dichos parámetros pueden ser usados como una herramienta determinante en el fenotipado por sequía en soja.

Se establecieron las siguientes hipótesis de trabajo que son los fundamentos de los trabajos realizados en los capítulos de esta tesis:

H1: el control de la apertura y del cierre estomático durante el establecimiento del estrés es determinante en la respuesta final y el óxido nítrico ($^{\bullet}$NO) cumple un rol importante en este proceso.

H2: la modelización de la cinética de consumo de agua a partir de la suspensión del riego, bajo condiciones controladas de crecimiento, puede utilizarse como una metodología de fenotipado en soja para la identificación de genotipos con respuestas contrastantes al déficit hídrico.

1.4. OBJETIVOS

1.4.1. Objetivo general

El objetivo general de este trabajo es profundizar en la comprensión de la expresión del fenotipo de soja en condición de déficit hídrico, con el propósito de aumentar la exactitud y precisión del fenotipado y, de esta forma, facilitar la identificación de marcadores fenotípicos que sean de utilidad en el mejoramiento de este cultivo frente a déficit hídrico.

1.4.2. Objetivos específicos

a. Identificar y caracterizar respuestas al déficit hídrico en soja.
b. Identificar factores endógenos de la planta asociados al control del cierre estomático bajo condiciones de déficit hídrico, partiendo de la base de que este proceso es clave en la regulación de la pérdida de agua foliar en soja.
c. Desarrollar una estrategia de fenotipado a partir del consumo de agua en soja, para la identificación de genotipos con respuestas contrastantes al déficit hídrico.

2. CAPÍTULO I:

ROL DEL ÓXIDO NÍTRICO (•NO) EN LA RESPUESTA ESTOMÁTICA INICIAL AL DÉFICIT HÍDRICO EN SOJA

La reducción de las pérdidas de agua durante un período de déficit hídrico es quizás el rasgo más importante asociado a tolerancia en las plantas, siendo el cierre estomático una respuesta fisiológica clave para lograrlo (Xiong et al., 2002), con el ácido abscísico (ABA) como molécula central de dicha respuesta (Davies y Zhang, 1991, Leung y Giraudat, 1998).

En soja, como en muchas otras plantas, el déficit hídrico produce una disminución de la conductancia estomática mientras que aumenta el contenido de ABA en hojas y xilema. Inicialmente estudios utilizando •NO exógeno en plantas modelo sugirieron que el •NO también estaba involucrado en el cierre estomático (Desikan et al., 2002, Neill et al., 2002, Lamattina et al., 2003); sin embargo, de manera controversial, un ensayo utilizando mutantes de *Arabidopsis thaliana* con niveles extremadamente bajos de •NO demostró que los estomas de los mutantes deficientes en la producción de •NO estaban significativamente más cerrados que los estomas de plantas WT en respuesta a ABA. Curiosamente, los estomas de los mutantes también estaban significativamente más cerrados que los WT en respuesta a la deshidratación (Lozano-Juste y León, 2010b).

Más recientemente el efecto del •NO sobre la atenuación de las respuestas a ABA ha sido bien documentado (Castillo et al., 2015, Albertos et al., 2015, Wang et al., 2015, Signorelli y Considine, 2018). De todas formas, y más allá de las controversias respecto al rol del •NO en el cierre estomático, el conocimiento sobre la producción endógena de •NO y su relación con el cierre estomático en plantas de cultivo es escaso, por no decir nulo.

Este trabajo tuvo como objetivo determinar el patrón de acumulación endógena de •NO desde el establecimiento del estrés hídrico y cómo este se relaciona con el cierre estomático y otras respuestas bioquímicas y fisiológicas. Para establecer si el •NO

endógeno está involucrado en el cierre estomático, se analizó la relación entre la acumulación de •NO endógeno, la cinética de cierre estomático y el nivel de estrés nitro-oxidativo en plantas de soja sometidas a diferentes niveles de déficit hídrico.

Los resultados obtenidos sugieren que el cierre estomático es una respuesta muy temprana al déficit hídrico en soja, en contraste con la acumulación de prolina y otros cambios en parámetros fisiológicos como contenido relativo de agua (RWC) y potencial hídrico de hoja (Ψ_L), que aparecen en situaciones más moderadas de estrés.

Por otro lado, bajo condiciones severas de estrés, las plantas de soja responden de manera similar a otras especies de plantas con varias respuestas fisiológicas de estrés comunes (Manavalan et al., 2009) como disminución de la conductancia estomática (SC), RWC y Ψ_L, y respuestas bioquímicas como acumulación de prolina, peroxidación de lípidos, acumulación de peróxido de hidrogeno (H_2O_2) y nitración de proteínas.

No se encontró una correlación clara entre el contenido relativo de agua (RWC), la conductancia estomática (SC) de las hojas y los niveles de •NO. Sin embargo, la cuantificación del •NO en toda la hoja podría no detectar diferencias del contenido de •NO célula específica, por ejemplo, en los estomas. Por esto nos enfocamos en determinar la acumulación de •NO a nivel de estomas y su correlación con la apertura de estos.

Respecto a la relación entre los niveles de •NO endógeno y el cierre estomático, los resultados mostraron un aumento de los niveles de •NO endógeno cuando los estomas comienzan a cerrarse, incluso cuando solo se observan ligeras variaciones en los valores de Ψ_L o RWC; cuando los estomas se cierran por completo bajo condiciones de estrés severo, la concentración de •NO en las células guarda disminuye a los niveles del control.

Los resultados obtenidos de este trabajo aportan evidencia sobre el rol del •NO como regulador negativo de la señalización por ABA a nivel de estomas, algo que ha sido bien caracterizado a nivel bioquímico, pero menos conocido *in vivo*, en condiciones

fisiológicas de déficit hídrico. Además, los resultados demostraron que las conclusiones a las que se había llegado en plantas modelo, utilizando mutantes y moléculas exógenas, son extrapolables a lo que ocurre en condiciones fisiológicas en cultivos como soja sometidos a déficit hídrico.

Dada la importancia del cierre estomático en la fijación de carbono y en la prevención de la pérdida de agua, los resultados de este trabajo sugieren que la modificación del metabolismo del •NO, ya sea mediante mejoramiento convencional o biotecnología, podría ser un enfoque interesante para optimizar la producción de biomasa vegetal o aumentar la tolerancia a la sequía. Por ejemplo, los cultivos que crecen en zonas sin restricciones hídricas podrían manipularse o seleccionarse para producir más •NO en las células estomáticas, de forma tal de minimizar el cierre estomático y fijar más dióxido de carbono. Por el contrario, los cultivos que crecen en zonas sujetas a períodos de déficit hídrico podrían manipularse para reducir la acumulación de •NO en las células estomáticas y, así, minimizar la inhibición del cierre estomático por •NO. Esto contribuiría a un cierre estomático más temprano y sincronizado que redundará en una mayor tolerancia a la sequía.

2.1. ENDOGENOUS ˙NO ACCUMULATION IN SOYBEAN IS ASSOCIATED WITH INITIAL STOMATAL RESPONSE TO WATER DEFICIT

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Endogenous •NO accumulation in soybean is associated with initial stomatal response to water deficit

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Abstract

Drought is the main cause of productivity losses in soybean plants, triggering physiological and biochemical responses, stomatal closure being essential to prevent water losses and thus mitigate the negative effects of drought. Abscisic acid (ABA) is the main molecule involved in stomatal closure under drought conditions along with nitric oxide (•NO). However, the role of •NO in this process is not yet fully understood and contrasting findings about its role have been reported. Most of the assays in the literature have been carried out under in vitro conditions using •NO donors or scavengers, but little is known about the effects of endogenously produced •NO under drought conditions. This study is aimed to determine the pattern of endogenous •NO accumulation from the establishment of water stress and how this relates to stomatal closure and other biochemical and physiological responses. The analysis of soybean plant responses to drought revealed no correlation between whole-leaf •NO accumulation and typical water-deficit stress markers. Moreover, •NO accumulation did not explain oxidative damage induced by drought. However, endogenous •NO content correlated with the early stomatal closure. Analysis of stomatal behavior and endogenous •NO content in guard cells through epidermal peel technique showed a stomatal population with high variation in stomatal opening and •NO content under the initial stages of water stress, even when ABA responses are activated. Our data suggest that upon early stress perception, soybean plants respond by accumulating •NO in the guard cells to inhibit stomatal closure, potentially through the inhibition of ABA responses.

1 | INTRODUCTION

Soybean (*Glycine max L.*) is an economically and nutritionally valuable crop widely grown in the world. The grains of this species have the highest protein content and the second highest oil content of all legumes (Liu, 1997). Like many crops, soybean plants are subjected to different abiotic stresses, which result in a loss of crop productivity and drought is considered the most important factor for yield losses (Bray et al., 2000). The mechanisms of resistance to water deficit in plants can be grouped in three categories: scape, tolerance and avoidance. Scape mechanisms maximize the plant's ability to complete its life cycle, before a severe water deficit occurs. These mechanisms allow an early physiological development with the concomitant formation of seeds. The tolerance mechanism is the ability of plants to survive with low water potentials in tissues and, at cellular level, involve the maintenance of turgor through osmotic adjustment, increased

Abbreviations: Ψ_L, leaf water potential; •NO, nitric oxide; DW, dry weight; Fc, Final control; Fs, Final stress; FW, fresh weight; Ic, Initial control; Is, Initial stress; NOS, nitric oxide synthase; RF, relative frequency; RNS, reactive nitrogen species; ROS, reactive oxygen species; RWC, relative water content; SC, stomatal conductance; TBARS, thiobarbituric acid reactive substances; TW, turgid weight.

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plasticity of cells and decreased cell size. The avoidance mechanism consists of keeping the water potential relatively high in the tissues, despite the lack of soil moisture. Plants that use this mechanism maintain turgor reducing water loss through decreasing stomatal conductance, rolling or folding the leaves for reducing the evaporation surface, and they also have a greater water absorption capacity by having deeper root systems and higher hydraulic conductance (Carrow 1996). The reduction of water losses during stress is maybe the most important trait to avoid drought in plants, stomatal closure being a key physiological response to achieve this (Xiong et al., 2002). Although the area covered by the stomata is generally less than 3% of the total leaf surface, approximately 98% of the absorbed CO_2 and loss of water occur through them (Willmer & Fricker, 1996). Abscisic acid (ABA) is a phytohormone acting as a systemic response to trigger stomatal closure (Davies & Zhang, 1991; Leung & Giraudat, 1998). In soybean, as in many other plants, drought produces a decrease in stomatal conductance while ABA content in leaves and xylem increases (Liu et al., 2003). In particular, ABA inhibits guard cell K_{in} channels and induces hydrogen peroxide (H_2O_2) production in guard cells. This activates Ca^{2+} channels, resulting in an increase of Ca^{2+} concentration, which in turn induces stomatal closure (Pei et al., 2000).

In addition, drought is known to induce the accumulation of reactive oxygen species (ROS) and reactive nitrogen species (RNS; Laspina et al., 2005, Signorelli, Casaretto, et al., 2013) and when these species exceed the antioxidant systems, nitro-oxidative stress is produced (Corpas et al., 2011). Among RNS, nitric oxide ($^{\bullet}NO$) is known to act as a signal molecule during the establishment of stress and due to its small size and low polarity, it can diffuse across membranes. However, overproduction of $^{\bullet}NO$ and $^{\bullet}NO$-derived products can have toxic effects, $^{\bullet}NO$ can react with exposed Cys residues in proteins, leading to S-nitrosothiols (SNOs), a mechanism known as protein S-nitrosylation, altering the functions of those proteins (Wang et al., 2006). In particular, $^{\bullet}NO$ can react with superoxide radicals ($O_2^{\bullet-}$) and generate peroxynitrite ($ONOO^-$), which in turn produces nitrogen dioxide ($^{\bullet}NO_2$), a potent oxidant that can nitrate Tyr residues of proteins and is also able to freely diffuse across membranes (Signorelli et al., 2011). Thus, the rise of protein Tyr nitration is considered a good indicator of nitrosative stress (Chaki et al., 2011; Corpas et al., 2008). Given the multiple impacts of $^{\bullet}NO$ on cell physiology, it is not surprising that $^{\bullet}NO$ has been suggested to participate in seed germination, primary and lateral root growth, flowering, senescence, stomatal conductance, nitrogen fixation, and several other processes (Considine et al., 2017; Corpas et al., 2011; Domingos et al., 2015; Signorelli et al., 2020). Today, we know that the involvement of $^{\bullet}NO$ in these physiological processes is due to its ability to modulate phytohormone responses by affecting their signaling pathways at several levels (Signorelli & Considine, 2018). In an opinion article, Laxalt et al. (2016) recall the dual role of $^{\bullet}NO$ in cell physiology, highlighting the ability of $^{\bullet}NO$ to act promoting or attenuating the ABA signaling in the process of stomatal closure, as a way to avoid the exacerbation of ABA. Initially, exogenous application of $^{\bullet}NO$ donors, such as sodium nitroprusside (SNP) and nitrosoglutathione (GSNO), was shown to induce stomatal closure in *Vicia faba* (García-Mata & Lamattina, 2001). In the model plant *Arabidopsis thaliana*, an induction of ABA-dependent $^{\bullet}NO$ accumulation also has been reported (Guo et al., 2003).

In plants, different enzymatic sources for the synthesis of $^{\bullet}NO$ are consensually accepted. Examples of these enzymatic reactions are the NO_2^- reduction to $^{\bullet}NO$ by the Nitrate Reductase (NR), the oxidation of L-arginine by the activity of nitric oxide synthase ($^{\bullet}NOS$), and the mitochondrial-electron transport chain-dependent (mETC-dependent) nitrite-reducing activity (Astier et al., 2018; Corpas et al., 2009; Planchet et al., 2005). The nitrate reductase loss-of-function double mutant *nia1nia2* showed a reduced accumulation of $^{\bullet}NO$ in guard cells, indicating that in Arabidopsis $^{\bullet}NO$ is produced mainly by Nitrate Reductase activity (Desikan et al., 2002). Moreover, the *nia1nia2* double mutant did not close stomata in the presence of ABA, suggesting that $^{\bullet}NO$ is necessary to induce stomatal closing, acting downstream of ABA in the signaling cascade. However, later evidence contradicts this assumption, revealing that the same mutant has greater stomata closure in the presence of ABA, generating an extreme tolerance to drought (Lozano-Juste & León, 2010a). León et al. (2014) proposed that $^{\bullet}NO$ is not required for ABA-mediated stomatal closure during drought response in experiments performed on whole plants. Moreover, Wang et al. (2015) demonstrated that endogenous $^{\bullet}NO$ acts as a negative regulator of ABA signaling in guard cells, inhibiting the kinase activity of the positive regulator of ABA OST1 (open stomata 1) by *S*-nitrosylation in Arabidopsis. Given that activation of OST1 by ABA is a fast process, while its S-nitrosylation in vivo occurs relatively late after ABA treatment, $^{\bullet}NO$ could play a role in the negative feedback regulation of ABA signaling in Arabidopsis (Wang et al., 2015). Inhibition of the OST1 kinase activity by S-nitrosylation in Arabidopsis *gsnor1* (*S-nitrosoglutathione reductase 1*) mutants, with over accumulation of $^{\bullet}NO$, caused an insensitivity of ABA-induced stomatal closure, with a concomitant higher water loss (Wang et al., 2015).

Using different in vitro approaches, the mechanism of action of ABA and $^{\bullet}NO$ on stomatal closure in Arabidopsis plants has been elucidated as little is known about the kinetics of $^{\bullet}NO$ accumulation under drought and its physiological consequences for the plant. Moreover, we can speculate that the kinetic of endogenous $^{\bullet}NO$ accumulation during drought establishment could have an impact on stomatal opening maintenance. In this work, we analyzed the endogenous accumulation of $^{\bullet}NO$ and how it links to the nitro-oxidative stress and stomatal response produced during drought stress in soybean plants. Our results reveal that endogenously produced $^{\bullet}NO$ plays a role in the maintenance of stomatal opening at the initial stage of drought, whereas once the drought condition is well established, the process of stomata regulation is $^{\bullet}NO$-independent.

2 | MATERIALS AND METHODS

2.1 | Plant materials and growth conditions

Soybean (*Glycine. max* L.) plants (genotype TJ2049) were grown in a growth chamber with a 16/8 h (light/night) photoperiod at 30°C/20°C

(day/night) and an irradiancy regime of 800 μmol photon m^{-2} s^{-1} obtained with Philips metal halide lamps of 400 W.

Three seeds were sown per pot. The pots consisted of PVC tubes of 11 cm in diameter, 30 cm in height and the bottom hole was covered with a mesh. Pots were filled with a mix of sand/vermiculite in a ratio of 1:1. After plantlets emerged, only one plant was left in each pot. The plants were watered daily to field capacity with Rigaud and Puppo (1975) medium supplemented with KNO_3 (10 mM final concentration). Plants were grown for 35 days until reaching the V5 phenological stage and then the treatments were applied.

2.2 | Initial and final drought stress treatments

Drought was induced by withdrawing the irrigation in V5 stage plants. Well-irrigated plants were considered as Controls (Ctrl). The Initial stress (Is) response was defined when the stomatal conductance (SC) decreased significantly (approximately 50% with respect to the control plants) but no visible symptoms of stress were observed. Final stress (Fs) was considered when SC was near 0 and the leaves lost their turgor.

2.3 | Measurements of physiological and biochemical parameters during drought stress

All the measurements listed below started on the last day of irrigation for the drought plants, defined as day 0, and they continued daily over the following six days without irrigation. The last day evaluated was day 6 when plants exhibited an evident loss of turgor and wilting phenotype. All the measurements were made in the third trifoliate leaf.

To determine the relative water content (RWC), leaves were weighed (fresh weight, FW), then hydrated with distilled water for 24 h, weighed to estimate the turgid weight (TW) and then dried at 80°C in the oven to measure the dry weight (DW) afterwards. The RWC was calculated as: 100 × (FW − DW)/(TW − DW). Three biological replicates (n = 3) were used for determinations, consisting of three independent plants per treatment.

Stomatal conductance (SC) was measured on the abaxial surface with a Porometer Model SC-1 (Decagon Device) as instructed by the manufacturer. Five biological replicates (n = 5) were used for determinations, consisting of five independent plants per treatment.

A precision pump, Scholander style, Model 600 (PMS Instrument Company) was used to determinate the leaf water potential (Ψ_L) in the petiole of the third trifoliate complete leaf following the instructions of the manufacturer. Three biological replicates (n = 3) were used for determinations, consisting of three independent plants per treatment.

For the proline content determination, 100 mg of leaf tissue was ground in 4 ml of methanol-chloroform-water (MCW 12:5:1) solution. After vortexing, the homogenate was centrifuged at 5000g for 2 min at room temperature, and the supernatant was collected in another tube, 1 ml chloroform and 1.5 ml water were added to the collected supernatant and then centrifuged at 5000 g for 1 min. In the upper phase, free proline was estimated with ninhydrin reagent according to a modified method of Troll and Lindsley (1955) in which toluene replaced benzene and the resin treatment was omitted (Borsani et al., 1999). Proline from the upper-phase was quantified by a spectrophotometer at 515 nm. Five biological replicates (n = 5) were used for proline determinations, consisting of five independent plants per treatment.

2.4 | Lipid peroxidation

Lipid peroxides were determined as thiobarbituric acid reactive substances (TBARS). Lipid peroxides were extracted from 80 mg of ground leaves using a 750 μl of 5% (w/v) metaphosphoric acid and 15 μl of 2% (v/v) butyl hydroxytoluene diluted in ethanol. Samples were homogenized using micro pestles in 1.5 ml microtubes and centrifugated at 15 000 g for 20 min at 4°C. 400 μl of the supernatant was transferred into new 2 ml microtubes and 40 μl of 2% (v/v) butyl hydroxytoluene diluted in ethanol, 200 μl of 1% (w/v) thiobarbituric acid dissolved in 50 mM NaOH, and 200 μl of 25% (v/v) HCl was added. The mixture was incubated at 95°C for 30 min for the color development and then placed on ice to stop the reaction. To extract the chromogen, 1.1 ml of 1-butanol was added and tubes were vortexed for 20 s. A 2 min centrifugation at 2000g was performed to separate the phases and the upper phase was collected for spectrophotometrical determination of malonaldehyde at 532 nm. The concentration was determined using an extinction coefficient of 156 mM^{-1} cm^{-1} (Rusterucci et al., 1996).

2.5 | *In vitro* detection of nitric oxide ($^{\bullet}NO$)

Sampled leaves were selected in the V5 phenological stage. Selected leaves were frozen in liquid nitrogen and ground in a mortar. The powder was suspended in a homogenizing medium composed of 50 mM Tris–HCl pH 7.8, 0.1 mM ethylenediaminetetraacetic acid (EDTA), 5 mM dithiothreitol (DTT) and 0.2% (v/v) glycerol (Signorelli, Corpas, et al., 2013). Homogenates were centrifuged at 20 000g for 20 min at 4°C and the supernatant was used immediately for the spectrophotometric assays. Crude extracts were mixed with 4,5-diaminofluorescein (DAF-2) to a final concentration of 10 μM. Reaction mixtures were incubated at 37°C in the dark for 2 h. Fluorescence was measured in a spectrofluorometer (RF-1501, Shimadzu) at excitation and emission wavelengths of 485 and 515 nm, respectively (Nakatsubo et al., 1998). The $^{\bullet}NO$ content was calculated as arbitrary units of fluorescence per mg of protein.

2.6 | Protein nitration

Aliquots containing 30 mg of protein from crude leaf extracts obtained as indicated above were separated by electrophoresis on a 10% (w/v) sodium dodecyl sulfate (SDS) polyacrylamide gel according to the method of Laemmli (1970). The separated proteins were then electroblotted onto polyvinylidene fluoride (PVDF) membranes

(Immobilon P, Millipore), and rinsed with TBS (20 mM Tris–HCl; 0.5 M NaCl, pH 7.5). After transfer, membranes were used in cross-reactivity assays with a rabbit polyclonal antibody against 3-nitrotyrosine (Chaki et al., 2009) diluted to 1:10000. For immuno-detection, an affinity-purified goat anti-(rabbit IgG)-horseradish peroxidase conjugate (Bio-Rad) and an enhanced chemiluminescence kit (ECL PLUS, Amersham) were used. The molecular protein marker is indicated by a drawn line corresponding to a Precision Plus Protein™ Standards All Blue (#161–0373; Bio-Rad Laboratories). Total protein content was determined using the Bradford method (Bradford, 1976).

2.7 | H_2O_2 and lipid peroxidation histochemical assays

Leaf discs of 14 mm diameter from the third trifoliate leaf were taken out with a hole punch. The most representative images of four independent replicates were shown.

In situ detection of H_2O_2 was done following the protocol proposed by Thordal-Christensen et al. (1997). Leaf discs were vacuum infiltrated under dark conditions with 10 mM potassium phosphate buffer, 10 mM NaN3 and 0.1% (w/v) 3,3- diaminobenzidine (DAB), pH 7.8. Leaf discs were incubated overnight under dark conditions and then clarified with 0.15% (w/v) trichloroacetic acid in 4:1 (v/v) ethanol:choloroform for 48 h before being photographed.

Histochemical detection of lipid peroxidation was done with Schiff's reagent, which detects aldehydes that are generated from lipid peroxides (Yamamoto et al., 2001). Leaf discs were incubated in Schiff's reagent for 60 min and then were bleached by immersing in boiling ethanol for 60 min. The visualization of purple color was used as a qualitative indicator of lipid peroxidation.

2.8 | *In vivo* determination of stomatal $^{\bullet}NO$ content and stomatal opening

Soybean leaves are amphistomatic with more stomata on the abaxial side compared to the adaxial side. The determinations of stomatal $^{\bullet}NO$ content and opening were therefore made on the abaxial side. Nitric oxide was detected in leaf sections of plants subjected to Initial and Final stress conditions with 10 μM 4-aminomethyl-2′,7′-difluorofluorescein diacetate (DAF-2 DA) prepared in 10 mM Tris–HCl pH 7.4 (Signorelli, Casaretto, et al., 2013). Epidermal peels were examined with a confocal laser scanning microscope (Leica TCS SL, Leica Microsystems) and an epifluorescence microscope (Zeiss Axio Imager M2). Fluorescence was quantified as the mean of pixel intensity in the whole guard cells for each stoma. These measures were made on over 40–50 stomata per condition using ImageJ software (National Institutes of Health) for confocal images, and ZEN 2 Pro software for epifluorescence images. The means ± SD of each condition were expressed.

The stomatal opening was determined by measuring the width and length of the opening on the stomata with Image J (National Institutes of Health) and ZEN 2 Pro software from the bright clear images of the CLSM and EFM at 40x. The measurements were made from 40–50 stomata per condition and data were expressed as the means of width/length ± SD.

Relative frequency histograms from values of $^{\bullet}NO$ contents and stomatal opening obtained were performed by the InfoStat Software (Di Rienzo et al., 2018).

2.9 | Stimulation and inhibition of endogenous $^{\bullet}NO$ accumulation in leaves

To stimulate the accumulation of endogenous $^{\bullet}NO$, plants were treated with NO_2^-. Initially plants were supplemented with 10 mM KNO_3 as the sole nitrogen source during the whole growth period and at the last watering before the stress induction, the nutrient solution was substituted by a $NaNO_2$ 0.1 mM solution. Likewise, for inhibition of endogenous $^{\bullet}NO$ content a tungstate treatment was applied by using a Na_2WO_4 0.1 mM solution for the last watering before the stress induction.

2.10 | Transcript accumulation of ABA responsive genes

TJ2049 soybean plants at V5 stage were subjected to drought stress after 35 days of growth by terminating water irrigation. Leaf samples were taken at initial (Is) and Final stress (Fs). In order to evaluate the environmental impact on the expression of the selected genes, it was decided to use controls at each sampling moment: control for initial time point (Ic), and control for final time point (Fc).

Total RNA from leaf samples of drought-stressed plants and well-watered plants was extracted by a phenol/chloroform extraction followed by LiCl precipitation. 50 μg of total RNA of each trifoliate leaf was extracted from three plant replicates and pooled into one sample, thereby generating a single total RNA sample corresponding to drought-stressed plants, and a single total RNA sample corresponding to non-stressed control plants.

10 μg of total RNA from the pools of plants under different treatments was resolved in agarose (1.2% w/v) under denaturing conditions and then transferred to a nylon membrane (Hybond N+). Membranes were pre-hybridized and hybridized at 65°C in 5X SSPE, 5X Denhardt's solution, 0.2% SDS and 0.5 mg ml^{-1} denatured salmon sperm DNA. Hybridization was performed overnight at 65°C with [a-32P] dCTP cDNA labeled probes. The sequence of the dehydrin probe consisted of a region from nt 388 to nt 762 from the CDS of the gene, while the probe corresponding to the ABA/WDS induced gene consisted of 273 pb of the 5' UTR of the transcript and the first 311 bp of the CDS. For labeling probes, 50 ng of purified DNA fragments from genes corresponding to the ABA and drought-regulated gene ABA/WDS-induced (Glyma.20G167500) and the drought-induced 27 kDa dehydrin gene (Glyma.09G185500) were labeled using the Amersham Rediprime II DNA Labeling System (GE Healthcare Life Sciences).

2.11 | Statistical analysis

Analyses of variance were performed with data from at least three independent biological replicates, and means were compared using the LSD Fisher test at the $P \leq 0.05$ level. Analysis of means was performed using *t*-test and Welch's *t*-test at the $P \leq 0.05$ level.

3 | RESULTS

3.1 | Physiological and biochemical responses of soybean to drought

Drought imposition was monitored by measuring the relative water content (RWC) of leaves and leaf water potential (Ψ_L) during 6 days after water withholding. The RWC decreased from 88 to 62% and the Ψ_L decreased from −0.65 MPa to −3.6 MPa from day 0 to day 6 (Figure 1A,B). These parameters indicated that after 5 days of water deficit, drought stress was clearly established, and both RWC and Ψ_L showed a significant drop between the fourth and the fifth day of stress (Figure 1A,B).

Proline content was determined in leaves, as it is considered a stress marker of several abiotic and biotic stresses (Díaz et al., 2005; Díaz et al., 2010; Signorelli, 2016). In our conditions, proline was found to increase only on the fifth day of stress, being unchanged for the first four days of stress (Figure 1C). A number of studies report a simultaneous induction of stomatal closure and proline accumulation, and ABA is considered as the key molecule behind these responses (Schroeder et al., 2001; Yoshiba et al., 1995). Our results show that during the first 2 days of drought imposition the stomatal conductance (SC) decreased dramatically and then it remained constant until the fourth day (Figure 1E). A further decrease was observed at the fifth and sixth day of the experiment (Figure 1E). A Pearson correlation analysis showed that the earlier induction of stomatal closure does not coincide in time with proline accumulation; however, a strong negative correlation was found between proline and RWC and Ψ_L (Table S1). Total •NO content of leaves increased 44% during the first day of stress, although two days after drought imposition •NO content returned to control values. Differences in •NO values between stressed or non-stressed plants were not statistically different due to the high standard deviation observed in samples from day 1 (Figure 1D). The simultaneous existence of full open and closed stomata in day 1 might be the cause of such variability. This high variability was also detected when the stomatal conductance began to fall in response to water deficit (Figure 1E). The performed Pearson correlation analysis between •NO content, RWC, SC, and Ψ_L suggested that no clear correlation exists between RWC, SC, and •NO values (Table S1). According to

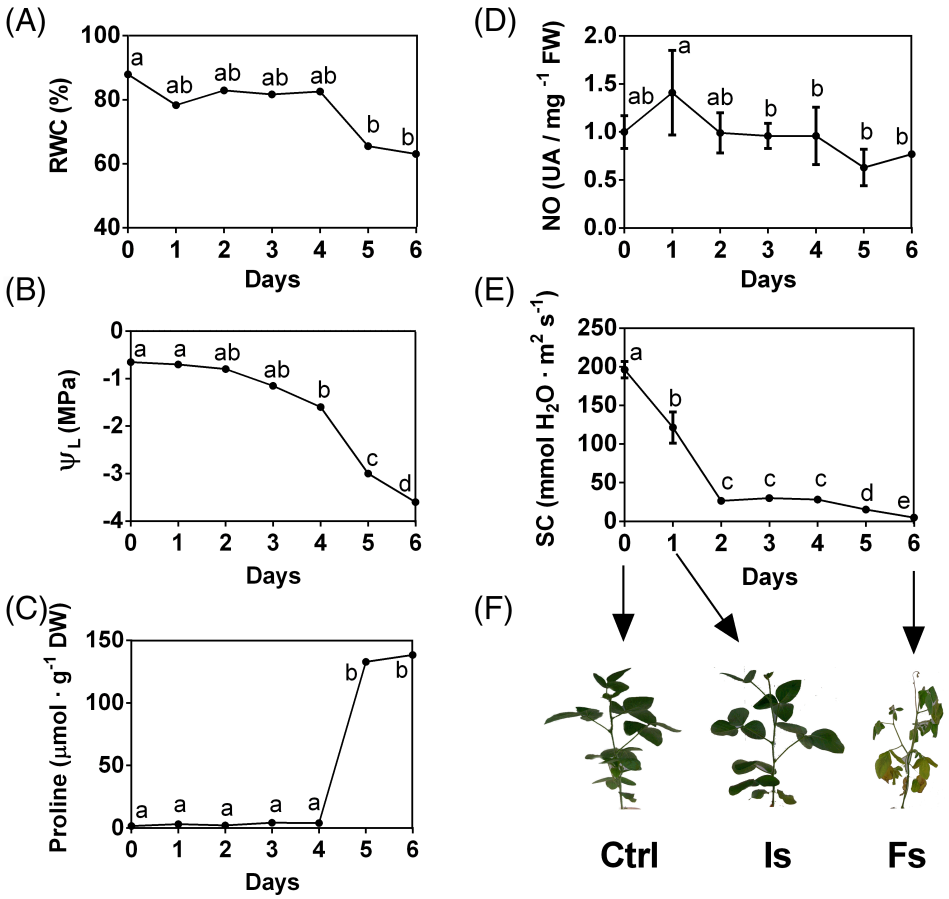

FIGURE 1 Physiological and biochemical responses of soybean to drought after 6 days of suspended irrigation. (A) Relative water content (RWC). (B) Leaf water potential (Ψ_L). (C) Proline accumulation. (D) Nitric oxide contents in leaves. (E) Stomatal conductance. (F) Phenotype of 30-day-old plants during the drought imposition at each defined condition: Ctrl, Control; Fs, Final stress; Is, Initial stress. Different letters indicate significant differences among treatments based on Fisher's LSD test at $P \leq 0.05$

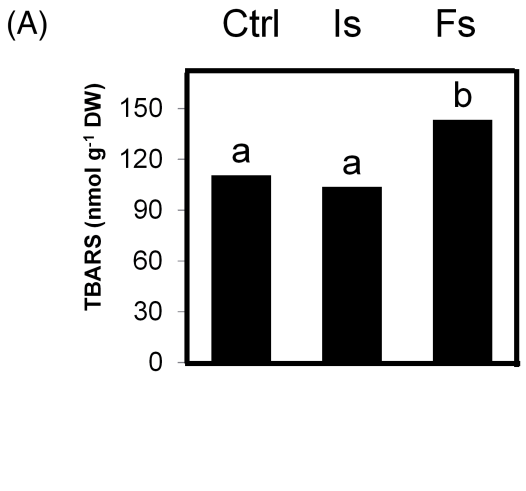

FIGURE 2 Drought-induced nitro-oxidative stress in soybean leaves. (A) Lipid peroxidation measured by TBARS. (B) Image of leaf discs stained with DAB (H_2O_2 indicator) and Schiff's reagent (lipid peroxidation indicator). (C) Protein nitration by immunodetection of 3-nitrotyrosine. Ctrl, Control; Fs, Final stress; Is, Initial stress. Different letters indicate significant differences among treatments based on Fisher's LSD test at $P \leq 0.05$

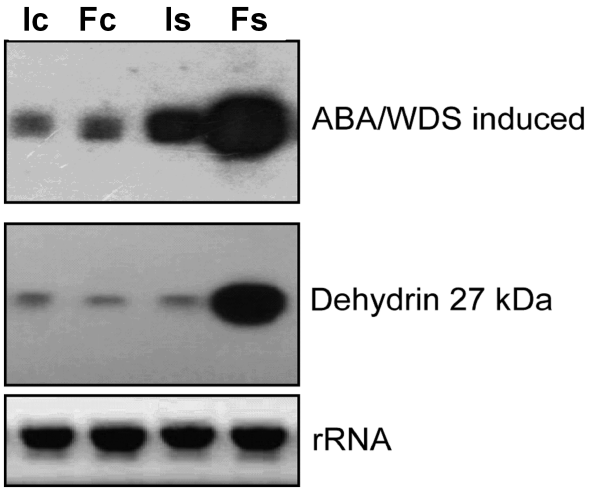

FIGURE 3 Transcript accumulation of ABA and drought-regulated genes in the soybean TJ2049 cultivar. Expression of ABA/WDS (Glyma.20G167500) and dehydrin (Glyma.09G185500) genes in response to moderate or severe drought stress in TJS2049. 10 mg of total RNA from TJS2049 cultivar was analyzed by northern blot. Controls correspond to samples from well-irrigated plants (Ic: Initial control, Fc: Final control). Dehydration-stressed samples correspond to drought stressed plants, at a 50% of its water retention capacity: Initial stress (Is) or at 25% of field capacity: Final stress (Fs). Ethidium bromide staining of rRNA was used to ensure equal loading of RNA samples. The genes and their accession number used as probes were ABA/WDS induced (Glyma.20G167500) and Dehydrin-27 kDa (Glyma.09G185500)

this results, we defined as Initial stress (Is) the condition in which the SC decreased significantly (approx. 50% respect to control) but no visible symptoms of stress were observed (i.e. day 1) and Final stress (Fs) was defined when the SC was near to 0 and the leaves lost turgor (i.e. day 6), whereas well-irrigated plants are considered Controls (C). Figure 1F shows the phenotype of plants in the defined conditions.

3.2 | Drought-induced nitro-oxidative stress in soybean leaves

Oxidative stress is a condition associated with drought imposition, so in order to analyze the oxidative stress, lipid peroxidation was measured by assessing thiobarbituric acid reactive substances (TBARS). Figure 2A shows that lipid peroxidation was only induced at Fs. Furthermore, oxidative damage correlated with an increase of H_2O_2 and aldehydes accumulation at Fs (Figure 2B). To evaluate nitrosative damage, protein nitration was determined by immunoblotting using anti-3-nitrotyrosine antibodies. At Fs, protein nitration was induced (Figure 2C) in agreement with the literature; however, protein nitration did not correlate with the levels of $^{\bullet}NO$ in leaves (Figure 1D and 2C). This might be explained by the fact that $^{\bullet}NO$ reacts with superoxide and generates peroxynitrite ($ONOO^{-}$).

3.3 | Estimation of ABA levels by expression of ABA responsive genes

According to the model in Laxalt et al. (2016), which includes the pathway proposed by Heidari et al. (2011), ABA in guard cells can induce the generation of $^{\bullet}NO$ via the dephosphorylation and activation of nitrate reductase (NR). Subsequently, $^{\bullet}NO$ could attenuate the

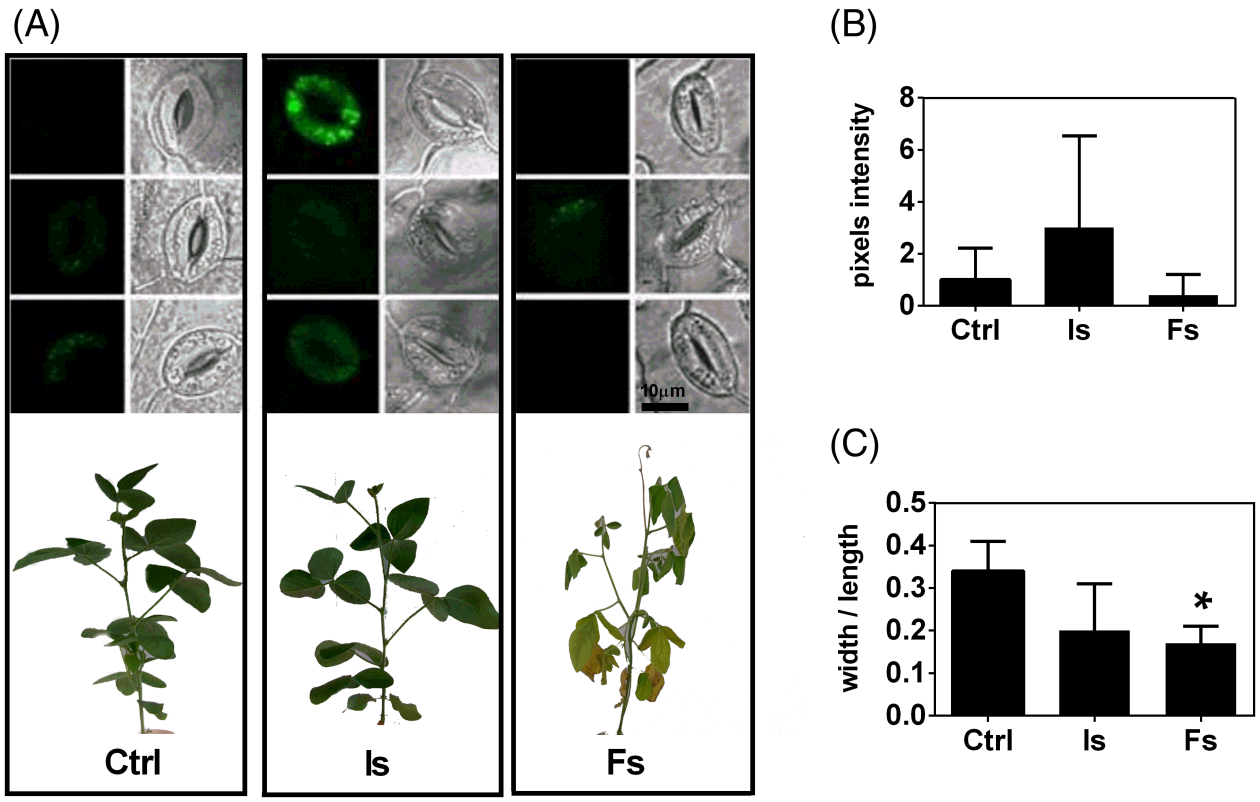

FIGURE 4 Endogenous •NO content and stomatal opening. (A) Representative CLSM images showing bright green fluorescence corresponded to the detection of •NO accumulated in guard cells. Images are accompanied by bright field microscopy of the same stomata. (B) Relative stomatal content respect to control of •NO determined by pixel intensity of CLSM images. Bars indicate SD of 15–20 stomata. (C) Stomatal opening measured as width/length from bright clear images from CLSM and is expressed as means ± SD. Ctrl, Control; Fs, Final stress; Is, Initial stress. Asterisk indicates that differences from Ctrl values were statistically significant at $P \leq 0.05$ (LSD fisher test). Scale bar is applicable for all the microscopic pictures

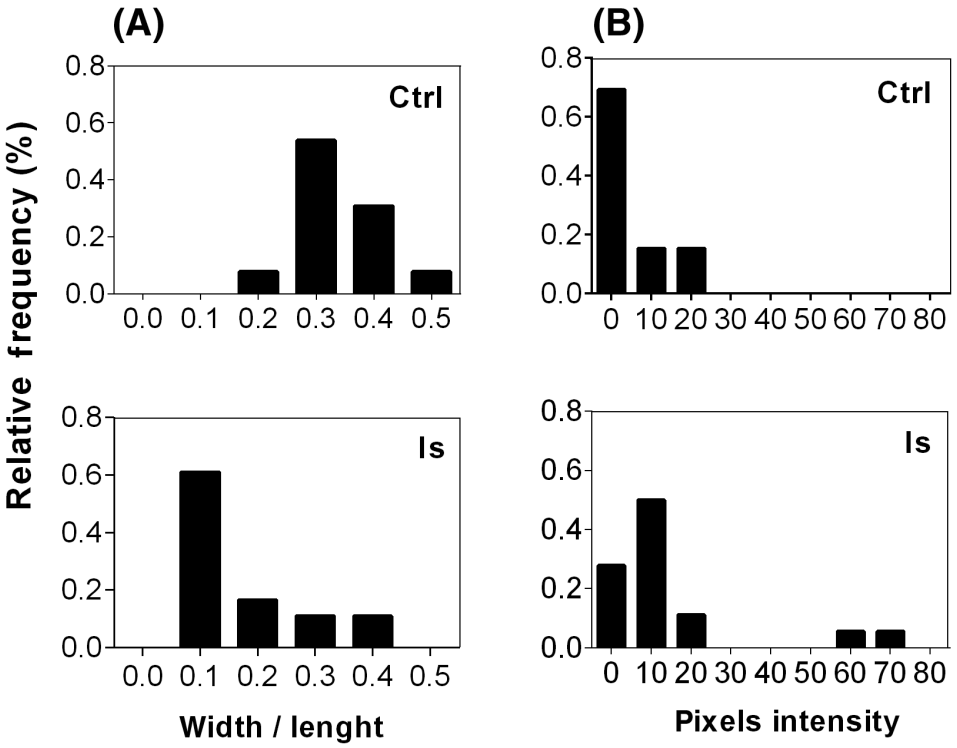

FIGURE 5 Relative frequency (RF) of (A) stomatal opening and (B) stomatal •NO content. Ctrl, Control; Is, Initial stress. Means of Ctrl and Is were statistically significant at $P \leq 0.05$ (*t*-test)

ABA role in the stomatal closure. To evaluate if ABA levels were already enhanced during the Initial stress (Is) state, we analyzed the expression profile of an ABA-responsive gene (ABA/WDS Induced, Glyma.20G167500) and an ABA-independent drought-responsive gene (Dehydrin-27 kDa, Glyma.09G185500). As shown in Figure 3, the ABA-inducible gene (ABA/WDS induced) was more expressed under Is conditions than at control conditions. Both genes were induced during the Final stress conditions (Figure 3), supporting the fact that under these conditions, the water deficit is well established (Figure 2). However, during the Initial stress, only the ABA-inducible gene (ABA/WDS induced) was induced with respect to the controls. In contrast, the dehydrin gene was not induced under the Initial stress. Together, these results indirectly suggest that ABA levels were likely to be increased during the Initial stress condition.

3.4 | Endogenous •NO content and stomatal opening

Since we were unable to detect significant changes in •NO levels using the whole leaf, we determined •NO content by focusing on the guard cells. We analyzed leaf epidermal tissues using CLSM, and quantified •NO in the guard cells as well as the stomatal opening. In control conditions, •NO was detected at low levels in guard cells. The highest levels of •NO accumulation were found at Initial stress conditions, while at Final stress, •NO decreased, reaching even lower levels than the control (Figure 4A,B). The stomatal opening gave concordant results with the SC indicated in Figure 1E, and revealed that at Initial stress the stomatal opening was reduced to 50% with respect to control conditions, and at Final stress the stomata were completely closed (Figure 4C). Analysis of numbers of stomata with certain values of opening and •NO content with respect to the total number of stomata analyzed give us the relative frequency histogram of each variable. Relative frequency histograms of opening and •NO content in a stomata population showed an evident change in the distribution of this frequency in response to stress (Figure 5). The greater variations in •NO contents and stomatal opening, as reflected by standard deviations, were found coincidentally in first stages of stress establishment at Initial stress conditions (Figure 4B,C and 6).

In order to clarify how the levels of endogenous •NO accumulated can affect the relative frequency of stomatal opening in response to water deficit, endogenous •NO levels in response to water deficit were manipulated by hampering or stimulating its synthesis. In plants, •NO is synthesized mainly from NR (Corpas et al., 2009; Neill et al., 2008), meaning the amount of endogenous •NO present can be decreased by inhibiting NR activity with tungstate (Bright et al., 2006). Conversely, •NO synthesis can be stimulated using nitrite at a concentration of 0.1 mM (Bright et al., 2006).

In Figure 7, we present the results of the effect of repressing and stimulating stomatal •NO accumulation on stomatal opening. In

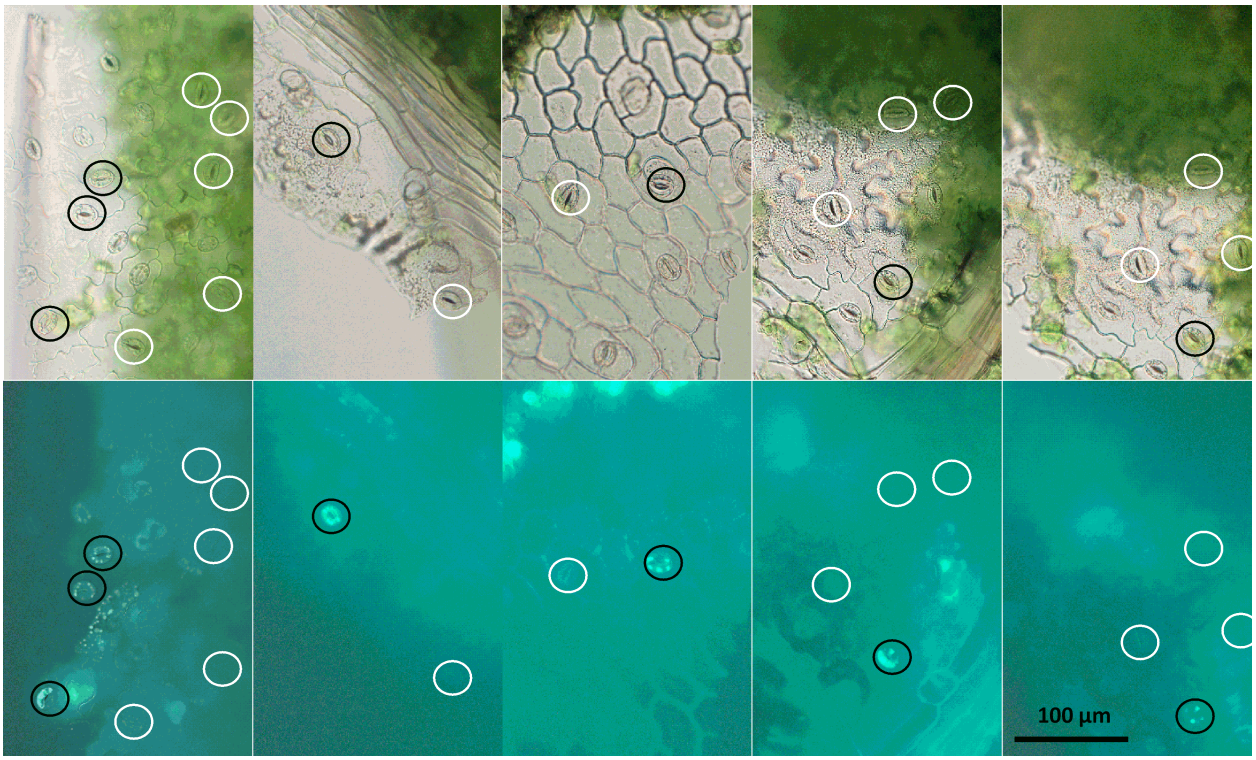

FIGURE 6 Representative bright field microscopy and EFM images showing differential opening and endogenous •NO accumulation in stomata under initial water deficit condition (Initial stress). Bright green fluorescence in EFM images correspond to the detection of •NO accumulated in guard cells. Black circles indicate fully opened stomata and white circles show fully closed stomata. Scale bar is applicable for all the microscopic pictures

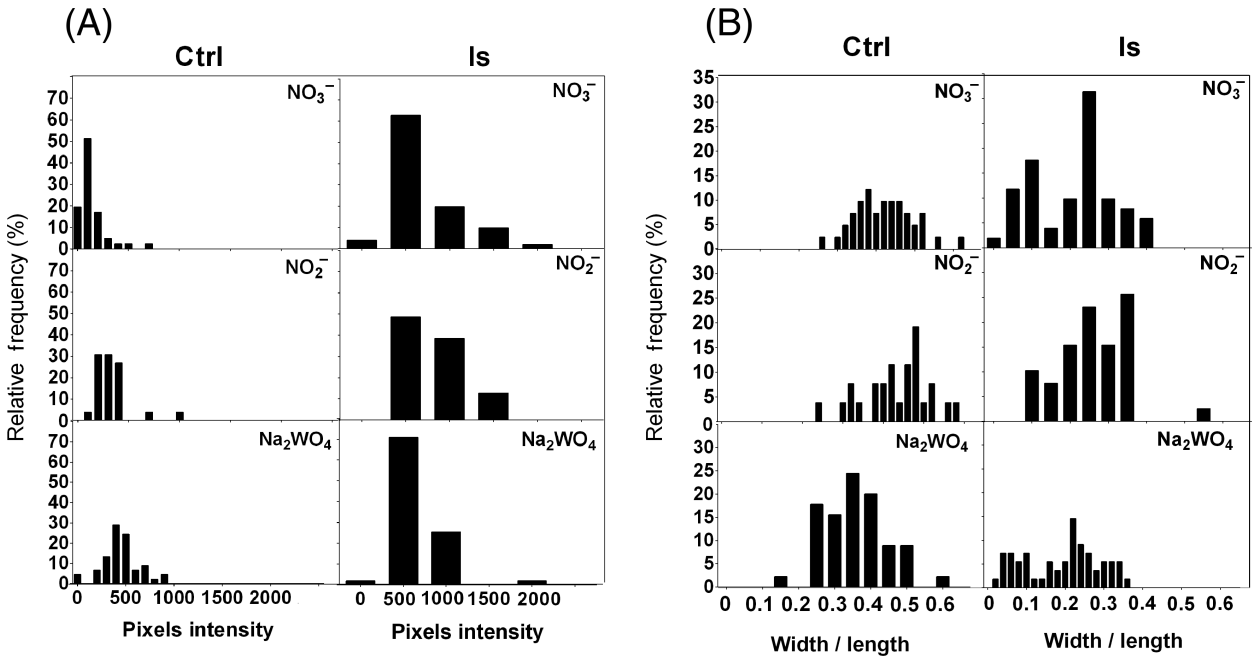

FIGURE 7 Endogenous •NO levels and relative frequency of stomatal opening in response to drought. (A) Relative frequency of •NO accumulation in stomata under Control (Ctrl) and Initial stress (Is) conditions, in plants grown with a •NO synthesis inhibitor (tungstate: Na_2WO_4) and nitrite as a •NO generator. (B) Relative frequency of stomatal opening under ctrl and is conditions, in plants grown with a •NO synthesis inhibitor (tungstate: Na_2WO_4) and nitrite as a •NO generator. Means of Ctrl and Initial stress were statistically significant at $P \leq 0.05$ (Welch's *t*-test)

particular, we analyzed the relative frequency of opening/closing stomata and •NO concentration under Initial stress conditions. In control plants grown with nitrate, approximately 90% of stomata had a signal intensity between 0 and 300 pixels, whereas in the nitrite treatment 90% of the relative frequency corresponds to stomata with a signal intensity between 300 and 500 pixels (Figure 7A), indicating that the •NO synthesis in the presence of nitrite was stimulated. In the case of the tungstate application, the relative frequency distribution is more heterogeneous with intensities ranging from 300 to 900 pixels (Figure 7A) showing that under non-stressed conditions tungstate treatment has a not clear effect.

When plants grown with nitrate underwent water deficit (Is), a greater •NO accumulation was found compared to the stomata of control plants. 60% of the stomata of stressed plants show a signal intensity of 500 pixels, while with nitrite application 90% of the relative frequency of •NO content is found in stomata with a signal intensity ranging from 500 to 1000 pixels, demonstrating that in stressed plants the •NO synthesis was stimulated in the presence of nitrite. Although the effect of tungstate as inhibitor of •NO accumulation was not significant, the frequency of stomata with lower signal intensity in pixels increased during the stress conditions. The effect of nitrite and tungstate application on the stomata opening under Initial stress conditions (Figure 7B) shows that the higher frequency of more opened stomata is observed in plants treated with nitrite, coinciding with a greater •NO accumulation (Figure 7A). It should be noted that in this treatment the frequency of relatively closed stomata (width/length: between 0 and 0.1) is null. In the case of the tungstate treatment, a greater variability in stomata opening was observed, but a higher relative frequency of closed stomata (width/length: between 0 and 0.1) was observed, confirming that reduction in •NO accumulation during hydric deficit contribute to the stomata closing.

4 | DISCUSSION

4.1 | Whole leaf-level analysis of correlation between •NO content, the kinetics of stomata closure, and the level of nitro-oxidative stress

Initially, •NO was suggested to be involved in stomatal closure (Desikan et al., 2002; Lamattina et al., 2003; Neill et al., 2002). However, these studies were performed using exogenous •NO. Less is known about the endogenous •NO production and its relation to stomatal closure in crop plants. The importance of measuring the •NO accumulation in the whole plant—not only in epidermal peels—and to analyze its role in stomatal control has been previously highlighted (León et al., 2014). To see if endogenous •NO is involved in stomatal closure, we determined its levels in leaves. The relationship between the accumulation of endogenous •NO, the kinetics of stomata closure, and the level of nitro-oxidative stress was analyzed in soybean plants subjected to different levels of water deficit. No clear correlation was found between RWC, SC, and •NO values (Table S1). However, measuring •NO in the whole leaf might attenuate differences of •NO content into the stomata, and it could explain the absence of studies reporting endogenous •NO involvement in stomatal movement when quantified at the whole plant level (León et al., 2014). To fill this gap in the current knowledge, we work with three water conditions to

further explore the relationship between •NO and stomata opening in soybean plants. Together, the results obtained in our study suggest that stomatal closure is a very early drought response in soybean plants (Flexas et al., 2004). This is in contrast to what was found in *A. thaliana* (Schroeder et al., 2001; Yoshiba et al., 1995) because in soybean under moderate stress conditions leaves stomatal closure occurs independently of proline accumulation and other physiological parameter changes such as RWC and Ψ_L. However, under severe stress conditions, soybean plants respond similar to other plant species with several common physiological stress responses (Manavalan et al., 2009) like decrease in stomatal conductance, RWC and Ψ_L, and biochemical responses such as proline accumulation, lipid peroxidation, H_2O_2 accumulation, and protein nitration.

4.2 | The link between •NO contents and stomatal opening

Our results showed that an increase of endogenous •NO levels occurs when the stomata begin to close, even when only slight variations are observed in the Ψ_L or RWC values. This result also highlights the tight regulation of both, endogenously produced •NO and stomatal closure, evidencing an important role of these factors in the response of plant acclimation to drought. When stomata were completely closed at Final stress conditions, the concentration of •NO in guard cells decreased to control levels. These results show that greater variations in •NO contents and stomatal opening were found under Initial stress conditions (Figure 1D,E). This may be due to the heterogeneity in stomatal opening that is found during the Initial stress establishment as shown in Figure 6. It is important to remark that this heterogeneity occurs upon initial drought treatment only, no other physiological changes in leaf water content and water potential were applied, which could have lead to alterations in the stomata behavior. This approach is quite different from those wherein exogenous applications of known amounts of sodium nitroprusside (SNP), 2-4-carboxyphenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO) or ABA are used to induce or inhibit the stomata closure specifically (García-Mata & Lamattina, 2001; Guo et al., 2003; Lozano-Juste & León, 2010a).

Initially some authors showed that exogenous applications of SNP produced stomatal closure (García-Mata & Lamattina, 2001) and then they showed that c-PTIO (a •NO scavenger) could reverse the stomatal closure mediated by ABA (García-Mata & Lamattina, 2002). Likewise, Desikan et al. (2002) showed that the *nia1nia2* mutant did not close the stomata in presence of ABA, suggesting that •NO is necessary for the stomatal closure. The reports about the effect of •NO on drought responses sometimes present contradictions, making it questionable whether the application of pharmacological compounds reflects the true physiological effect of •NO (Santisree et al., 2015). In most studies, •NO accumulation in model plants was artificially generated by SNP, ABA, or nitrate, but no data were available regarding stress-induced endogenous •NO accumulation and its role in stomatal closure, and less is known in agronomically important crops. The artificial generation (in vitro) of •NO from donors depends on many factors, such as the reactivity and concentration of the donor, the environment, the active duration of the exposure, the type of tissue, the light, etc. In the case of SNP, it leaves cyanide and iron ions as by-products (Wodala et al., 2010), cyanide is involved in the inhibition of nitrate reductase (NR) and CYTOCHROME C OXIDASE, which also regulates the production of •NO. Furthermore, stability and interaction with other molecules in vitro and in vivo under the given experimental conditions should be considered before drawing conclusions (Santisree et al., 2015).

Controversially, a report using the *A. thaliana nia1nia2noa1–2*, mutant with extremely low levels of •NO showed that stomata from mutants deficient in •NO production were significantly more closed than stomata of WT in response to ABA. Interestingly, stomata of mutants were also significantly more closed than WT in response to dehydration (Lozano-Juste & León, 2010b). Together, this evidence suggested that •NO was attenuating ABA-mediated stomatal closure. Indeed, the effect of •NO on attenuating ABA responses has recently been well documented (Albertos et al., 2015; Castillo et al., 2015; Signorelli & Considine, 2018; Wang et al., 2015). In particular, in guard cells of *A. thaliana* plants grown under in vitro conditions, •NO acts as a negative regulator of ABA signaling by inhibiting OST1 kinase activity through S-nitrosylation (Wang et al., 2015). In concordance with Wang et al. (2015), we showed that accumulation of endogenous •NO may avoid stomatal closure in soybean, but when leaves are dehydrated (wilting phenotype) the absence of •NO cannot prevent the stomatal closure (Figure 4A,B). Our results showed that the conclusions that have been drawn in model plants, using mutants and exogenous hormones, are translatable to what happen at physiological conditions in crops subjected to drought.

The use of an *A. thaliana gsnor1-3* mutant deficient in GSNO reductase allowed the over accumulation of •NO in the guard cells causing the S-nitrosylation of (OST1)/(SNF1), (SnRK2.6) and the inability of the ABA-induced stomatal closure, highlighting the role of •NO in desensitizing ABA signaling after a period of drought (Wang et al., 2015). In this sense, Santisree et al. (2015) rose the importance of determining if the increase in •NO levels in stomata cells during dehydration stress is a direct consequence of stress or the indirect effect of ABA. In this work, the kinetics of •NO accumulation in a crop plant species were analyzed, through epidermis samples taken from whole plants subjected to drought, revealing higher levels of •NO at very early stage of the stress treatment which could be involved in the stomatal movement. Results obtained from soybean plants subjected to different levels of water deficit and grown with nitrate as nitrogen source allowed us to conclude that the accumulation of endogenous •NO in response to water deficit at the stoma level could act as a negative regulator of the mechanism involved in stomata closure, allowing stomata to remain open for longer time during water deficit installation. If the water deficit is not prolonged and the hydric conditions improve, cultivars that are able to keep the stomata open will benefit of keep growing during a longer period than compared to those closing the stomata immediately after sensing a drop in water potentials. This study provides evidence about the role of •NO as a negative regulator of ABA signaling in soybean, something that has

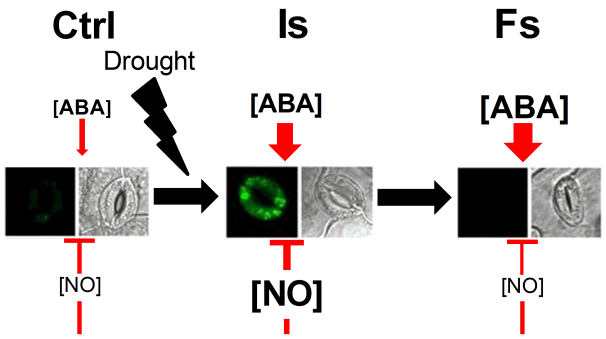

FIGURE 8 Simplified model showing the putative interactions between •NO and ABA during the stomatal closure in soybean plants in response to drought conditions. Arrows and bars represent positive and negative effects on stomatal closure, respectively. Thickness of lines is proportional to the magnitude of the regulatory effect. •NO and ABA concentrations are proportional to the size of the letters. Ctrl, Control; IS, Initial stress; Fs, Final stress

been well characterized at biochemical level but less known under drought conditions. Figure 8 represents the antagonistic action of ABA and •NO in the control of initial stomatal closure in response to drought in soybean. Once the drought is established, •NO accumulation decreases, allowing ABA-induced stomatal closure. Given the importance of stomatal closure in both carbon fixation and water loss prevention, the results of this work suggest that modifying •NO metabolism, either by conventional breeding or biotechnology, could be an interesting approach to optimize plant biomass production or increase drought tolerance. For instance, crops grown in tropical areas with intensive rainfalls could be manipulated or selected to produce more •NO in the stomatal cells so they minimize stomatal closure and fixate more carbon dioxide. Conversely, crops grown in areas often subjected to droughts can be manipulated to reduce •NO accumulation in the guard cell and thus minimize the inhibition of stomatal closure by •NO. This would contribute to an earlier and more synchronized stomatal closure which will result in more tolerance to drought.

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AUTHOR CONTRIBUTIONS

Esteban Casaretto performed the experiments related to plant drought responses characterizations and Nitric Oxide analyses. Santiago Signorelli contributed to the experiments related to oxidative stress markers and nitric oxide detection. Juan P. Gallino and Sabina Vidal were involved in the gene expression analyses, provided suggestions and revised the manuscript. Omar Borsani supervised the study, provided suggestions and revised the manuscript. Esteban Casaretto and Santiago Signorelli conceived and directed this study, analyzed the data, and wrote the manuscript.

DATA AVAILABILITY STATEMENT

"n/a"

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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2.1.1. MATERIAL SUPLEMENTARIO

El material suplementario del artículo precedente se encuentra en el siguiente enlace: https://onlinelibrary.wiley.com/doi/10.1111/ppl.13259

3. CAPÍTULO II.

DESARROLLO DE UN MÉTODO BASADO EN UN MODELO DE CONSUMO DE AGUA PARA EL FENOTIPADO DE GENOTIPOS DE SOJA BAJO CONDICIONES DE DÉFICIT HÍDRICO

Los programas de mejoramiento de soja y, en particular, los programas públicos, no utilizan la respuesta al déficit hídrico como un criterio de selección, debido a los altos costos de los equipos de fenotipado masivo actualmente disponibles. Por lo tanto, la selección de genotipos tolerantes al déficit hídrico en los programas de mejoramiento requiere del desarrollo de estrategias de fenotipado que sean de alto rendimiento, precisas y de bajo costo.

Actualmente está bien documentado que la extracción de agua durante las etapas criticas del cultivo informa en gran medida del rendimiento en escenarios de déficit hídrico (Vadez et al., 2013, Ratnakumar et al., 2009, Zaman-Allah et al., 2011). Varios trabajos relacionan las respuestas en la etapa vegetativa con la tolerancia a la sequía durante las etapas reproductivas (Kron et al., 2008, He et al., 2016, Sinclair et al., 2010). Según Kron et al. (2008), existe una "ventana de desarrollo" en la etapa V4, en la que las plantas sometidas a déficit hídrico mejoran su tolerancia posterior al estrés por déficit hídrico en la etapa reproductiva.

El balance hídrico de un cultivo depende en gran medida de la evaporación del contenido de agua en la superficie del suelo y de la pérdida de agua contenida en los tejidos vegetales por transpiración (Consoli y Vanella, 2014). La transpiración está determinada por la demanda y controlada por los estomas; durante la desecación del suelo, el agua extraíble por las raíces se reduce continuamente, lo que determina que no se pueda mantener la demanda total de transpiración. En esta situación, las plantas responden cerrando los estomas para evitar la desecación.

En este trabajo, desarrollamos un modelo simple e informativo basado en enfoques biológicos y matemáticos para evaluar, bajo condiciones controladas de crecimiento, la respuesta de las plantas de soja al déficit hídrico a través de la cuantificación del consumo de agua en una etapa vegetativa. El modelo caracteriza y predice la curva de consumo de agua de un sistema planta-maceta-sustrato (PPS) con bajos requisitos de muestreo de una variable simple y fácilmente cuantificable como es el peso del PPS. Se confirmó una

fuerte relación entre la cinética de consumo de agua y la conductancia estomática, lo que significa que el agua transpirada por el PPS está regulada por la conductancia estomática y esta última, por la disponibilidad de agua en el sustrato. Además, el modelo puede predecir el valor del agua cuando la conductancia es 0 y, de esta forma, definir el límite de extracción de agua. Este es un rasgo importante porque genotipos con elevados umbrales hídricos comienzan a cerrar sus estomas aun con contenidos de agua relativamente altos, conservando agua del suelo.

A partir de la curva de consumo de agua generada por el modelo se obtienen dos parámetros, $t_{0,5}$ (tiempo necesario para que el PPS alcance la mitad de la cantidad máxima de agua evapotranspirable) y Gw($t_{0,5}$) (conductancia estomática [Gw] en $t_{0,5}$), los cuales se identificaron como biológicamente relevantes en una población segregante biparental y en una población de mejoramiento, por lo que, al menos en soja, pueden ayudar al análisis de las respuestas al déficit hídrico. Además, un análisis de correspondencia entre los parámetros $t_{0,5}$ y Gw($t_{0,5}$) con la estructura genética de ambas poblaciones muestra una asociación genética. Un trabajo reciente (Quero et al., 2021) clasificó la misma población de mejoramiento utilizando índices, como el índice de susceptibilidad a la sequía (DSI) y el índice de estabilidad del rendimiento (YSI), en relación con el grupo de madurez de los genotipos. En este trabajo, los autores pudieron identificar QTLs asociados con esos rasgos. Un análisis de correspondencia preliminar entre el parámetro del modelo $t_{0,5}$ y DSI mostró la existencia de agrupamientos definidos, lo que indica que la estrategia de fenotipado discutida en este trabajo podría ser útil para mejorar la selección de genotipos en relación con el rendimiento en condiciones de campo.

Los resultados muestran que el modelo propuesto es una opción válida para ser incluido en protocolos de fenotipado de respuesta a sequía en programas de mejoramiento, en los que la mano de obra calificada y la infraestructura pueden ser limitantes. Además, el modelo contempla la variable tiempo, por lo tanto, considera los cambios en los parámetros a partir del comienzo del déficit hídrico. Este tipo de modelos, en donde se incluyen variables dinámicas, son escasos y permiten una caracterización más precisa de fenómenos biológicos complejos como la adaptación de las plantas a cambios en los niveles de agua disponible.

3.1. A SIMPLE AND ACCURATE METHOD BASED ON A WATER-CONSUMPTION MODEL FOR PHENOTYPING SOYBEAN GENOTYPES UNDER HYDRIC DEFICIT CONDITIONS

agronomy

MDPI

Article

A Simple and Accurate Method Based on a Water-Consumption Model for Phenotyping Soybean Genotypes under Hydric Deficit Conditions

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Abstract: Drought limits crop productivity and reduces yield stability. Drought tolerance as a selection criterion in breeding programs requires the development of high-throughput, precise, and low-cost phenotyping strategies. We developed a mathematical model, based on biological approaches, for evaluating soybean plants' response to drought under controlled growth conditions. The model describes the kinetics of water consumption of a plant pot substrate system (PPS) with low sampling requirements. The model generated two parameters, $t_{0.5}$ (time necessary for the PPS to reach half of the maximum amount of evapotranspirable water) and $G_w(t_{0.5})$ (stomatal conductance [G_w] at $t_{0.5}$), which determined the water- consumption curve of each genotype. An analysis of the kinetics of water consumption in response to a progressive water deficit in a biparental and breeding population was performed as a preliminary test of the model. A correspondence analysis between the $t_{0.5}$ and $G_w(t_{0.5})$ parameters with the genetic structure of the populations shows a genetic association. The phenotyping methodology presented in this work and drought susceptibility in field conditions are discussed based on previous results. This work could be useful for improving the selection of soybean genotypes in relation to their performance under drought conditions.

Keywords: drought; stomatal conductance; mathematical modeling; crop breeding

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1. Introduction

Despite increases in soybean (*Glycine max* L.) crop yields achieved by breeding and better agricultural practices, its productivity and yield stability are especially susceptible to drought events [1,2]. Drought stress affects both the vegetative and reproductive stages in soybean, reducing leaf area, increasing flower and pod abortion, and diminishing pod and seed size [3]. Several works relate the response in the vegetative stage to drought tolerance during reproductive stages [4–6]. According to Kron et al. (2008) [4], there is a "developmental window" in the V4 stage, in which plants subjected to water stress improve the subsequent drought-stress tolerance in the reproductive stage. Furthermore, Sinclair et al. (2010) [6], using a simulation model, determined that water conservation through an early decrease in stomatal conductance and reduced transpiration rate explains the increase in soybean yield throughout 70 years with drought events.

Soybean breeding programs are mostly focused on increasing total yield and yield stability, which are mainly affected by water shortage during the crop cycle. These programs, particularly public breeding programs, do not use drought response as selection criteria due to the high costs of the massive phenotyping equipment currently available. Most of the high-throughput phenotyping platforms are based on the diagnosis of changes in the plant's physiology, such as leaf conductance, leaf area, and root system development, using a complex and expensive system of analysis [7]. Hence, including drought tolerance as a selection criterion in breeding programs requires the development of high-throughput, precise, and low-cost phenotyping strategies [8]. However, as Valdez et al. (2013) [9] indicate, strategies could include the quantification of two crucial aspects of the plant's water budget: (i) the ability to capture more water, and (ii) the ability to conserve and use captured water more efficiently. Nowadays, it is well documented that water extraction during the key crop stages greatly informs crop performance in water restricted scenarios [9–11].

Water balance in a land crop is greatly dependent on evapotranspiration phenomena. This is the combination of two independent processes involved in water losses from the soil. One is the evaporation of the water content in the soil surface, and the second is the loss of water contained in the plant tissues by transpiration [12]. In crops, the transpiration increases as the plants grow due to the increase of leaf area. At the same time, this causes a contrary effect on the evaporation, which decreases progressively. Evapotranspiration can be determined by measuring several components of water balance in the soil. Specifically, in a controlled close system with no run-off or percolation, the total water content of the system within a certain period can be quantified as weight.

At the whole plant level and under constant water demand, water uptake or water use depends mainly on root-system development, leaf area, transpiration rate, and leaf conductance [9]. Under conditions with increasing water restriction, each of the abovementioned parameters has a different role on water use. However, there is a consensus within the scientific community that transpiration changes are a critical component in contexts where the available water content in the soil is changing [13–16]. Transpiration is determined by the demand and controlled by stomata; during soil desiccation, the water extractable by the roots is continuously reduced, which determines that full transpiration demand cannot be supported. In this situation, the plants respond with stomatal closure to avoid shoot desiccation. In addition, stomatal opening is quite sensitive to the evaporative demand, and a high vapor-pressure deficit (VPD) reduces stomatal opening to restrict water losses. Soil water content and atmospheric demand ultimately determine the dynamic of water consumption [17]. Therefore, leaf conductance measurements using gravimetric methods have been explored as robust parameters for breeding programs [18–21]. Moreover, the variation of stomatal conductance in soybeans in response to VPD and soil water content has been shown to be dependent on the genotype [22,23].

The increase in the number of new genotypes evaluated by breeding programs calls for a high capacity for data acquisition and processing, yield prediction, and responses under different environmental conditions. To this end, the use of tools such as sensors, data analysis programs, algorithms, and models is essential [24]. However, the evaluation of the models with experimental data is necessary for their improvement and adjustment [25,26]. Under optimal development conditions, mathematical models can predict plants' responses more accurately. Under stress conditions, however, a greater range of responses are generated and the prediction accuracy of the models becomes weaker [24]. The results of models focused on the water absorption of crops [27–29] show a great variation among themselves as a consequence of the many factors involved. For example, the substrate information must be improved, as well as the plant-soil interaction [24]. It is necessary to have a better theoretical and empirical basis and an appropriate knowledge of the environmental conditions where the model will be applied. Another aspect that stands out that is not always taken into account when determining the performance of the model is the competence of its users. Therefore, it is necessary to simplify and facilitate the input data models. Most of the relevant mathematical models that we know of are designed to explain crop behavior

in response to field environmental changes. Moreover, the time variable is not included in these models, which would not account for the physiological changes at the plant level.

In this study, we developed a simple and informative model based on biological and mathematical approaches for evaluating, under controlled growth conditions, the response of soybean plants to water restriction through the quantification of water consumption in the vegetative stage. To find a physiological explanation of the variables generated by the model, they were correlated with the stomatal conductance dynamic. Also, a preliminary test of the model was performed separately in a biparental segregating population and in a breeding population. Results show that the approach proposed is a valid option to be included in plant phenotyping protocols with limited manpower and infrastructure.

2. Materials and Methods

2.1. Phenotyping Method Based on Water Consumption under Controlled Environmental Conditions

For the development of the mathematical model, we performed an experiment with a soybean genotype (GENESIS 5601). Plants were grown in a 0.5 L plastic bottle (pot) filled with a mix of sand:vermiculite (1:1). This combination of plant, pot, and substrate was defined as a Plant Pot Substrate system (PPS) (Figure S1). Plants were grown in an environment defined by day/night cycle temperatures of 30/20 °C, respectively, and a light/darkness photoperiod of 16/8 h, respectively. Relative humidity (RH) was controlled at 35/40% during the entire growth period. Three seeds per pot were sown, and only one seedling remained after the cotyledon expanded. The homogeneity of the plants was carefully analyzed to avoid any interference related to developmental phenotype. During the first 16 days after sowing (developmental stage V2–3), soybean seedlings were grown without water restriction, and substrate was kept at field capacity with Rigaud and Puppo (1975) [30] medium supplemented with KNO_3 (10 mM final concentration). Since day 17, watering was suspended, and water substrate content was measured daily by gravimetry (water gravimetric content) during the next 10 days of water deficit (dwd) (Figure 1). Stomatal conductance was measured simultaneously with PPS on the abaxial leaf surface with a Porometer Model SC-1 (Decagon Device), as instructed by the manufacturer, since the suspension of watering. Five biological replicates (n = 5) were used for determinations, consisting of five independent PPSs.

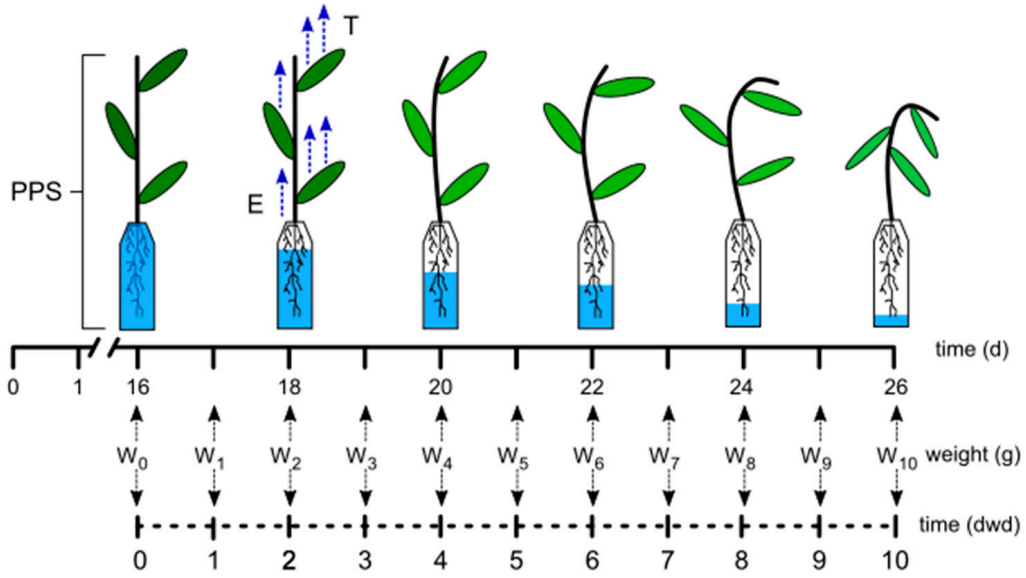

Figure 1. Schematic illustration of the experiment for determining water consumption. Plants were maintained at field-capacity condition until day 16, after which the water supply was suspended. The Plant Pot Substrate system (PPS) was weighted daily for 10 days ($t = 0$ until $t = 10$). Evaporation: E; transpiration: T; days of water deficit: dwd.

2.2. Phenotyping Strategy Applied to an F3 Segregating Population

An F3 segregating population of 177 genotypes derived from the crossing of parental lines SO7.6557 × DM6.8 were phenotyped using the methodology and the mathematical model developed in this study. The phenotyping experiment was laid out in a randomized incomplete block design, with three replications in each experiment. Growth conditions were the same as described in the Section 2.1. PPS weight and stomatal conductance were measured simultaneously.

2.3. Phenotyping Strategy Applied to a Breeding Population

A local breeding population composed of a set of 89 genotypes [31] was also phenotyped using the methodology described above and the mathematical model developed in this study. Five well-known commercial varieties were included as checks in all phenotyping experiments. In this case, plants were grown in 2.851 L PVC tubes (11 cm in diameter and 30 cm long) with a mix of sand:vermiculite (1:1) under the same environmental conditions as described previously. Plants were grown without any watering restriction for 30 days (developmental stage V4–5), after which watering was suspended and the PPS weight was registered at days 0, 4, and 8 after suspending the water supply. PPS weight and stomatal conductance were measured simultaneously.

2.4. Genotyping by Sequencing and SNP Calling

The F3 segregating population of 177 genotypes was genotyped using a SoySNP6k chip [32] in an Iscan system (Illumina, San Diego, CA, USA). SNP calling was done using the GenomeStudio software (Illumina, San Diego, CA, USA). Genotyping-by-sequencing (GBS) data were obtained for the breeding population according to the methodology proposed by Quero et al. (2021) [31].

2.5. Data Analysis

2.5.1. Nonlinear Models

To model the weight of the plant pot substrate system and its evapotranspiration, a one-phase exponential decay and one-phase association function were used, respectively. In both cases, the initial values and parameters estimation is described by Equations (11) and (12) of Section 3.6. It is worth noting that the nonlinear models were fitted using the *nls* function of the *stats* [33] package of the statistical R software [33].

2.5.2. Multivariate Characterization

The multivariate characterization of the soybean populations was accomplished through some exploratory analyses and visualization tools. First, genotypes were clustered in groups based on the PCA of the genotypic variability using a hierarchical clustering algorithm (HCPC). Genotypes were grouped based on similarity from the Euclidean distance using the Ward method, and the number of groups was determined by the highest relative loss in inertia using the function *HCPC* of the *FactoMineR* [34] package in the statistical software R [33]. Second, a correspondence analysis (CA) was performed based on the contingency tables of the HCPC analysis from the genotypic characterization, and the clustering was performed according to $t_{0.5}$ and $G_w(t_{0.5})$. The correspondence analysis was performed to visualize the relationship between the two grouping strategies (i.e., based on genetic and phenotypic variables). The CA was conducted using the *CA* function of the *FactoMineR* [34] package in the R software [33].

2.5.3. Statistical Model and Adjustment of Phenotypic Means

Best linear unbiased estimators (BLUEs) for each advanced inbred line were obtained with mixed models to include experimental design components using the following model:

$$y_{ij} = \mu + \alpha_i + \beta_j + \varepsilon_{ij}$$

where y_{ij} is the response variable, μ is the overall mean or intercept, α_i is a random variable associated with the ith assay with α_i~N $(0, \sigma^2{}_A)$, β_j is the effect of the jth line, and ε_{ij} is the residual error with ε_{ij}~N $(0, \sigma^2\varepsilon)$. The model was adjusted in the R software [33] using the *lme4* package [35], while the BLUEs were estimated using the *emmeans* package [36], also in R [33]. In all cases, BLUEs were further used as genotypic values for the model, PCA, and CA analyses.

3. Results

In this work, we developed an empirical mathematical model for describing the kinetics of water consumption of a PPS (Figure 2) using an important plant such as soybean. This model was applied for developing a phenotyping methodology for water-deficit response in soybean plants under controlled environmental conditions.

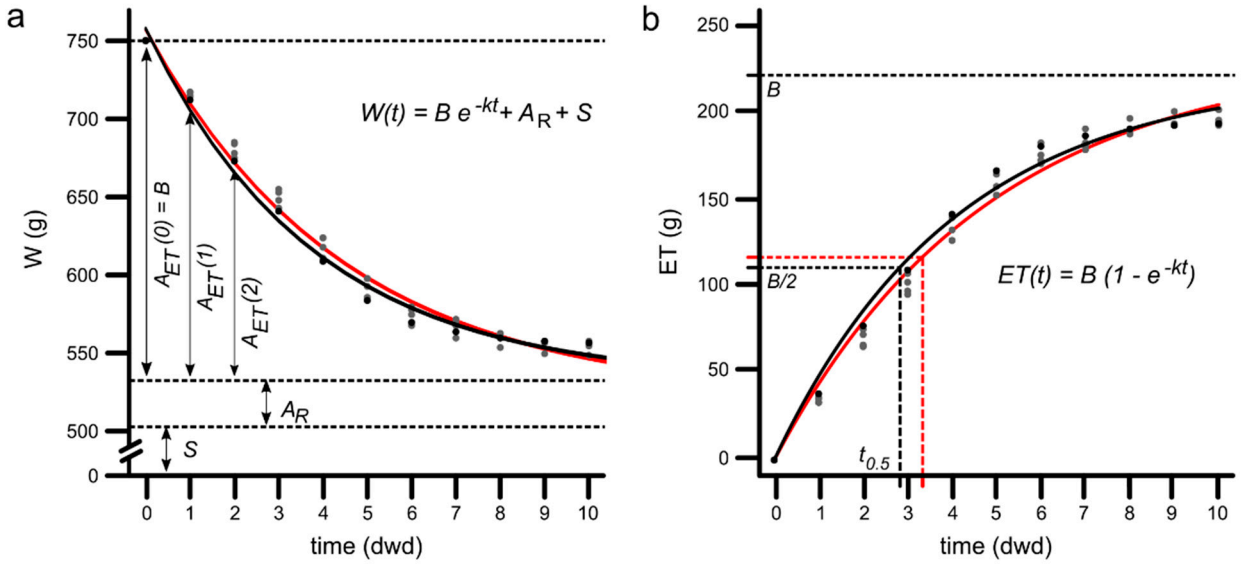

Figure 2. (**a**) Empirical model representing evapotranspiration over time and the empirical model adjustment. Weight (W) of the PPS along the days of water deficit (dwd). Sum of dry weight substrate, pot, and plant weight (S), residual water (A_R), evapotranspirable water (A_{ET}), and potential evapotranspiration (B) of plants at field-capacity conditions. (**b**) Mathematical analysis for modeling $ET(t)$ as a function of time. The parameter $t_{0.5}$ is the time required for the PPS to halve the potentially evapotranspirable water.

3.1. Mathematical Model Development

In the experiment, the PPS system weight (W) is defined as the weight of water (A) plus the rest of the components of the system (S). S is the sum of substrate, pot, and plant weight. The latter was considered constant during the assay because, although it could vary throughout the days, it is insignificant in comparison to the rest of components of S. A is the sum of the transpirable water (A_{ET}) plus a percentage of that which cannot be evapotranspired by the PPS throughout the whole assay. The non-evapotranspirable water is defined as residual water (A_R). The values of A_R depend on the matric potential of the substrate and the transpiratory capacity of the plants (Figure 2a). The amount of potentially evapotranspirable water of the PPS is a function dependent on time (t), and it is named $A_{ET}(t)$. Therefore, $A(t) = A_{ET}(t) + A_R$.

3.2. PPS Weight Modelling over Time

If $W(t)$ is a function of the PPS weight over time (t), then

$$W(t) = A(t) + S = A_{ET}(t) + A_R + S \quad (1)$$

In order to find an algebraic expression for the $W(t)$ function, we assume the following: "The velocity with which the PPS weight varies is directly proportional to the amount of water that can be evapotranspired for the PPS in this fraction of time". This hypothesis can be mathematically expressed through the following differential equation:

$$\frac{dW(t)}{dt} = -kA_{ET}(t) \tag{2}$$

where $k > 0$ is a constant of proportionality. The negative sign of the equation is due to the decrease of the PPS weight over time, as the assay was performed withdrawing watering at $t = 0$.

Since A_R and S are constant magnitudes over time, the derivation of the equality (1) results in $\frac{dW(t)}{dt} = \frac{dA_{ET}(t)}{dt}$, thus, the Equation (2) can be rewritten as follows:

$$\frac{dA_{ET}(t)}{dt} = -kA_{ET}(t) \tag{3}$$

obtaining the first order homogeneous linear differential equation for the function $A_{ET}(t)$. Solving the Equation (3) using separation variables method (Figure S2), the solution can be written as:

$$A_{ET}(t) = Be^{-kt} \tag{4}$$

where B is a positive constant.

By combining the Equations (1) and (4), we obtain the following equation:

$$W(t) = Be^{-kt} + A_R + S \tag{5}$$

The graphic representation and the experimental data adjustment of Equation (5) are shown in Figure 2a.

3.3. Evapotranspiration Modelling as a Function of Time

To quantify the evapotranspiration (Figure 2b) ($ET(t)$) of the PPS from the precise moment when watering was suspended ($t = 0$) to time t, is the difference between the PPS weight in both times, that is

$$ET(t) = W(0) - W(t) \tag{6}$$

Combining the Equations (5) and (6), we have that:

$$\begin{aligned} ET(t) &= W(0) - W(t) = A_{ET}(0) + A_R + S - (A_{ET}(t) + A_R + S) \\ &= A_{ET}(0) + A_R + S - A_{ET}(t) - A_R - S \\ &= A_{ET}(0) - A_{ET}(t) = B - Be^{-kt} \\ &= B\left(1 - e^{-kt}\right) \end{aligned}$$

Therefore,

$$ET(t) = B\left(1 - e^{-kt}\right) \tag{7}$$

The graphic representation and the experimental data adjusting of the Equation (7) are shown in Figure 2b.

3.4. Potential Evapotranspiration Estimated by the Model

At the moment watering was suspended ($t = 0$), the PPS had the maximum quantity of evapotranspirable water. By definition, this quantity is $A_{ET}(0) = B$, as observed in Figure 2a.

On the other hand, since the constant of proportionality (k) of Equation (7) is positive, we have that

$$\lim_{t\to\infty} ET(t) = \lim_{t\to\infty} B\left(1 - e^{-kt}\right) = B \tag{8}$$

That is, the parameter B is the horizontal asymptote of the function $ET(t)$, and represents the potential evapotranspiration of PPS, as observed in Figure 2b.

3.5. Half-Time of ET

Half-time is, by definition, the time required for the PPS to reduce the potential evapotranspiration in half. As the potential evapotranspiration is B, the half-time, $t_{0.5}$, is expressed as follows:

$$ET(t_{0.5}) = \frac{B}{2} \tag{9}$$

Equation (7) is used to calculate $t_{0.5}$, therefore,

$$t_{0.5} = \frac{1}{k}\ln(2) \tag{10}$$

Thus, the half-time is inversely proportional to the constant k, and independent of B. The graphic representation of $t_{0.5}$ is shown in Figure 2b.

3.6. Parameters of the ET Model Calculated from the Experiment Data

If W_j is the PPS weight registered on the jth day since suspending watering, $\{(j, W_j)\}_{j=0}^{N}$ is the set of data obtained from the assay (Figure 2a).

To determine the ET parameters as a function of time (Figure 2b), with this data set, we can use the least-squares method that requires computational resources or an analytical method from Equation (7). For this last option, we took three specific determinations, W_0, W_n, and W_{2n}, and performed them on days 0, n, and $2n$, respectively. Replacing this data on the Equation (7), we can directly find the parameters, obtaining the following:

$$B = \frac{(W_n - W_0)^2}{W_{2n} - W_n + W_0} \tag{11}$$

$$k = -\frac{1}{n}\ln\left(\frac{W_{2n} - W_0}{W_n - W_0}\right) \tag{12}$$

To develop an empirical model, the PPS weight measurements ($W(t)$) during the whole water-deficit period were used to determine the curve fitting according to the experimental methods described in Figure 2. As shown in Figure 1, 10 days of water restriction determined a curve of PPS weight loss, determined by the water transpired by the plants. As indicated in Figure 2a, W at time t is defined by the parameter B determined by the weight when t is 0 and it represents the potentially evapotranspirable water, A_R indicates the water not extractable, and S indicates the weight of the support and dry substrate.

The evapotranspiration of the PPS over time from the day when watering was withdrawn was determined by Equation (7) and defined by the parameters B and k. It is important to note that B represents the potential evapotranspiration of the PPS. By definition, half-time ($t_{0.5}$) is the time required for the PPS to reach half of the potential evapotranspiration (Figure 2b). These parameters show the kinetics of water consumption of the genotypes and could help in the characterization of soybean genotypes.

3.7. The Model Minimizes Sampling Requirements in Phenotyping Protocols

To simplify the data collection procedure and increase the high throughput capacity, the model parameters were estimated using the minimum sampling. Figure 3a shows the values of K in Equation (7) estimated by the Gauss Newton numeric methods versus the values of K estimated by analytic methods using a sampling of the PPS weight every two days. As observed in Figure 3a, an optimum adjustment was obtained when the values of the PPS weight were sampled on days 4 and 8. The parameters estimated with the weight of these two days are closer to the best model fit. The same results were obtained with

parameter *B* of Equation (7) (Figure 3a), but with an adjustment of 0.96. Both parameters of the model are critical to evaluate the water-consumption curve of a specific genotype. Hence, the PPS weight on days 4 and 8 appears to be enough to describe the kinetics of water consumption throughout the water restriction period. Figure 3b shows that the adjustment curve of Equation (7) with parameters *B* and *K* is the same as the one obtained using all the PPS weight data in the Gauss Newton estimation method.

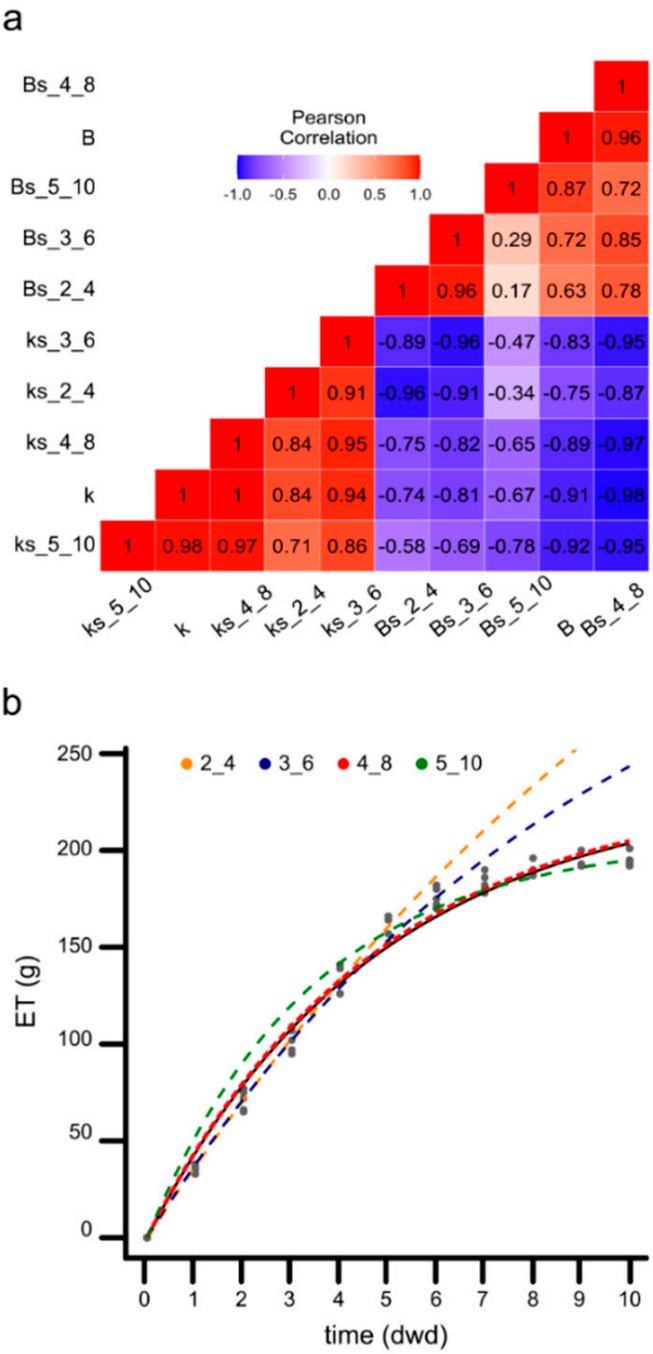

Figure 3. Minimum sampling requirements analysis and adjustment of model. (**a**) Relation between *K* or *B* estimated by the Gauss Newton method and the predicted *K* or *B* generated by analytic methods with two-time sampling, respectively. Axis indicates the parameters and the days of the sampling. (**b**) *ET* over time. Graphic representation of Equation (7) adjusted according to the data of the numeric (solid curve) or analytic methods (dashed colored curve). Colors indicate the two specific sampling days used for modeling the curve. Days of water deficit: dwd.

3.8. Stomatal Conductance as a Function of Time

By definition, stomatal conductance (G_w) is the rate of CO_2 entering the substomatal chamber, or the water vapor exiting through the stomata pore of the leaf. Since most of the water lost by the PPS is from stomatal transpiration, it is possible to suppose that the evaporation is negligible. Thus, the variation in weight can be attributed exclusively to transpiration, that is:

$$\frac{dW(t)}{dt} = -\tau G_w(t) \tag{13}$$

where in τ represents a constant of proportion.

By deriving the Equation (5) and replacing in (13), we obtain the following:

$$G_w(t) = \frac{k}{\tau} B e^{-kt} \tag{14}$$

The graphic representation and the experimental data adjustment of the Equation (14) are shown in Figure 4a.

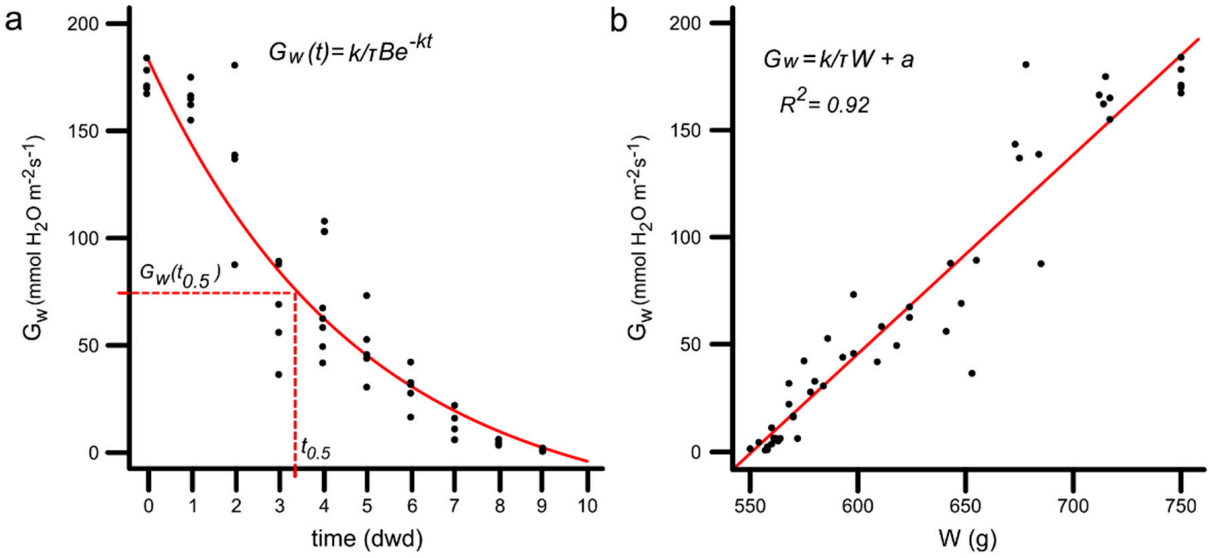

Figure 4. Modeling of stomatal conductance in response to water availability. (**a**) Empirical model representing the conductance over time and the empirical model adjustment. (**b**) Lineal regression between conductance and substrate weight. G_w: stomatal conductance; W: PPS weight; dwd: days of water deficit.

3.9. Conductance as a Function of PPS Weight

By combining the Equation (5) with (14), we obtain the following:

$$G_w(t) = \frac{k}{\tau} B e^{-kt} = \frac{k}{\tau}(W(t) - A_R - S) = \frac{k}{\tau} W(t) + \frac{k}{\tau}(-A_R - S)$$

Thus,

$$G_w = \frac{k}{\tau} W + a \tag{15}$$

where in $a = \frac{k}{\tau}(-A_R - S)$.

Thus, stomata conductance is a linear function of the PPS weight, as shown in Figure 4b.

To understand the physiological component of the kinetics of water consumption dissected by the proposed model, G_w over time was included in the mathematical modeling.

A modeled curve of G_w over the time was performed from Equations (5) and (13) (described in Sections 3.2 and 3.9). Figure 4a shows how the model generated by theoretical tools is adjusted with the experimental data. Using function (4), it is possible to estimate

the half-time for $G_w(t_{0.5})$ from the moment watering is suspended. This parameter is relevant as a useful indicator to identify contrasting responses of different genotypes to the hydric deficit. Figure 4b shows the direct theoretical relation between the G_w obtained from Equation (15) and the PPS weight (red curve), and the high correlation with the experimental data (R^2 = 0.92).

3.10. Application of the Phenotyping Methodology in Two Breeding Populations at Different Plant Developmental Stages

The methodology was tested in a phenotyping approach in two breeding populations. The parameters of the water-consumption curve defined by the model ($t_{0.5}$ and $G_w(t_{0.5})$) were analyzed and related to the genetic variability in order to improve the understanding of the plant's water-consumption behavior in soybean.

3.10.1. Half Time and Stomatal Conductance

An analysis of the kinetics of water consumption in response to a progressive water deficit was performed in a biparental population. Two parameters, $t_{0.5}$ and $G_w(t_{0.5})$, were selected to characterize the variability of the recombinant genotypes (Figure 5a,b). Values of $t_{0.5}$ between 2 and 6 days showed high variability in the kinetics of water consumption. However, 25% of the genotypes had a $t_{0.5}$ lower than 3 days, and the remaining 75% had a $t_{0.5}$ higher than 3 days. The normal distribution observed confirms that the values of the parameters had a biological behavior inside the population. On the other hand, the distribution of $G_w(t_{0.5})$ ranked between 101.8 and 202.8 mmol H_2O m^2 s^{-1} shows normal distribution that accomplishes the behavior of a water-consumption response (Figure 5b).

When the phenotyping protocol was evaluated in V5 plants of a breeding population, a similar range of $t_{0.5}$ values were obtained in comparison to those obtained in the biparental population when evaluating in V3 plants (Figure 5a,c). However, when $G_w(t_{0.5})$ was analyzed, a wider range of values was obtained (6–300), showing that this parameter is more affected by the developmental stage and genotypic variability (Figure 5b,d).

It is important to point out that $G_w(t_{0.5})$ is the conductance reached at the time when the plant has consumed half of the potentially evapotranspirable water, and not a simple calculation of half G_w at the initial time (t_0). This confirms the idea that parameters identified by the model are biologically relevant and, at least in soybean, that could help in the analysis of plant response to water deficit.

3.10.2. Genetic Structure and Correspondence Analysis

Based on the genetic structure of both breeding populations, we identified three main groups in the biparental population and six groups in the breeding population (Figure 6a,b). The latter shows a higher genetic diversity, which would explain the results of the analysis in the distribution of values for the parameters of the model (Figure 5). The correspondence analysis between HCPC genotypic and HPC phenotypic groups showed the following correspondence: for the $t_{0.5}$ parameter and in the biparental population; tq3 and g2; and tq1 and g3. The relation between tq2 and g1 is not clear. In the case of the breeding population, the correspondence indicated the relation between tq1 and g3; tq2 and g2; and g6, tq3, and g4. It was not possible to find a correspondence for g1 and g5 (Figure 6c,d). When the same correspondence analysis was performed between the genetic groups and the G_w parameters, the results showed correspondences between G_{wq3} and g2, and between G_{wq2} and g1 in the biparental population. It was not possible to find a relation between G_{wq1} and g3 (Figure 6e). In the case of the breeding population, correspondences between G_{wq3}, g3 and g6; G_{wq2} and g1; and g2 and g5 were found (Figure 6f). It was not possible to find a relation between G_{wq1} and g4. This type of analysis indicates that the changes in water consumption under hydric conditions, quantified by the parameters of the model, are related to the genetic components in the genotype.

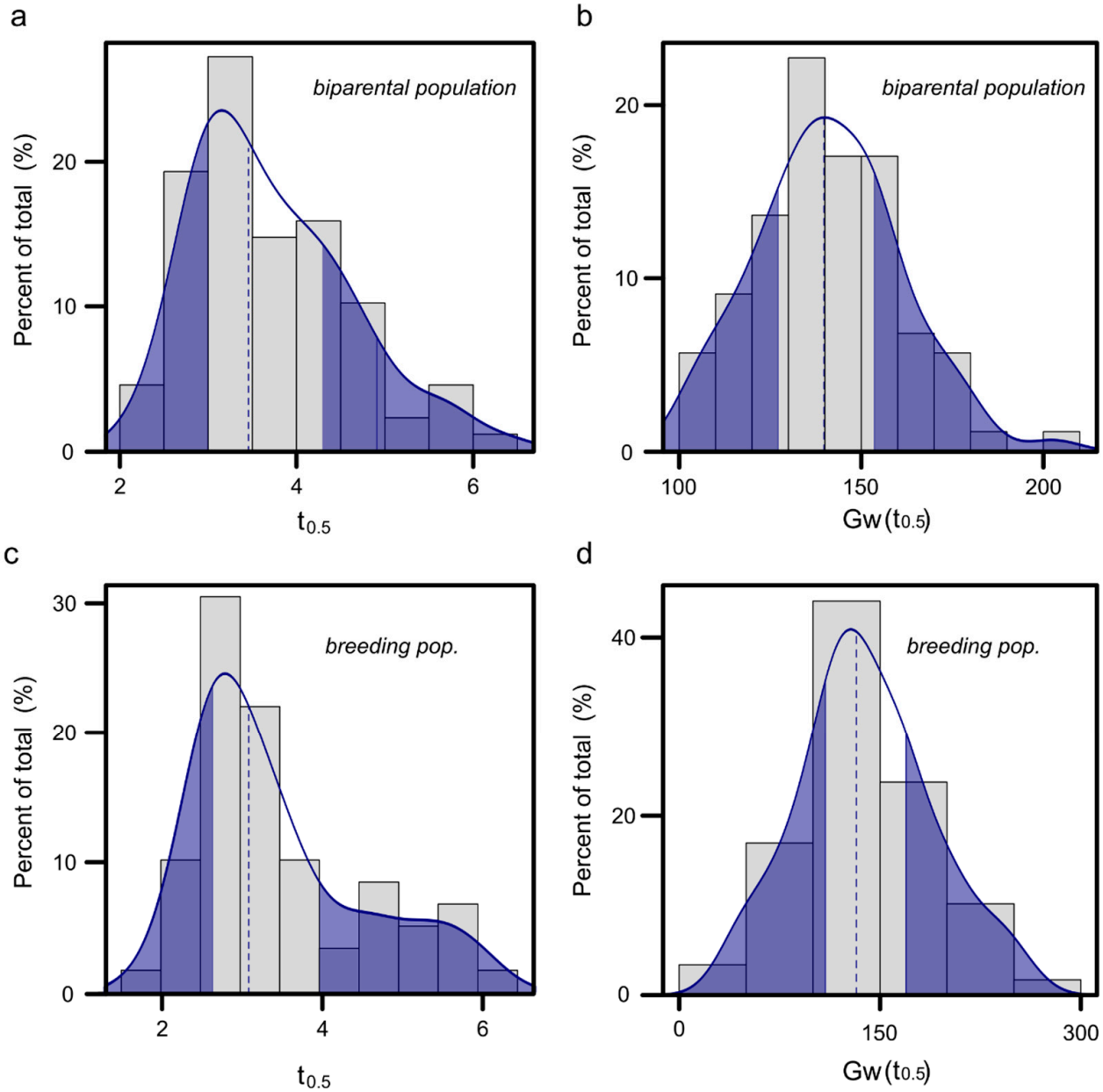

Figure 5. Graphic distribution of $t_{0.5}$ and $G_w(t_{0.5})$ values obtained from two soybean populations. $t_{0.5}$ is expressed in days and $G_w(t_{0.5})$ in mmol H_2O m^2 s^{-1}. A frequency analysis was performed in 177 and 89 genotypes of a biparental and breeding population, respectively. The darker regions represents the 25% of the genotypes with superior and inferior $t_{0.5}$ and $G_w(t_{0.5})$ values of the populations. (**a**,**b**) biparental population; (**c**,**d**) breeding population.

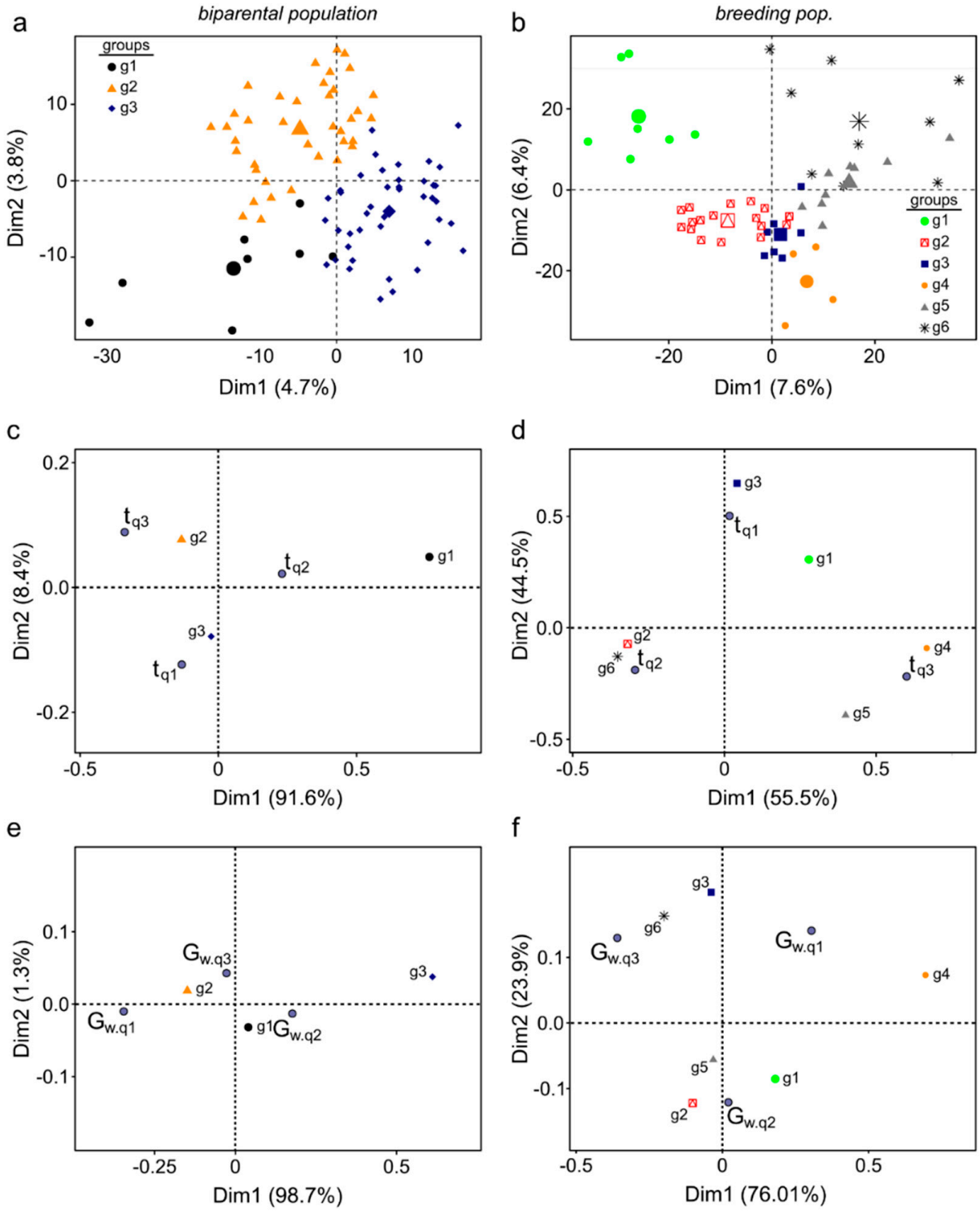

Figure 6. Population structure calculated from genotypic data (molecular markers), phenotypic data (parameters of the model), and the correspondence analysis. The groups were obtained by hierarchical clustering on the principal component (HCPCgenotypic). (**a**) Biparental population: g1, g2, and g3 groups. (**b**) Breeding population: g1, g2, g3, g4, g5, and g6 groups. Correspondence analysis of the contingency table between groups based on the parameters of the models and groups according to genotype. (**c**,**e**) Biparental population. (**d**,**f**) Breeding population. Population structure calculated from parameters $t_{0.5}$ and G_w of the model. tqn and G_{wqn} were obtained by hierarchical clustering on the principal component (HCPCphenotypic).

4. Discussion

In the current climate change scenario, soybean breeders must use effective strategies to develop varieties with a better ability to cope with periods of water shortage. The complexity of drought-tolerance traits has prevented the development of successful and accessible phenotyping strategies for selection, especially in small-scale breeding programs. This situation motivated our work to develop an effective phenotyping strategy. The simplification of phenotyping strategies becomes necessary when a high number of plants must be evaluated at the same time. In this regard, considerable efforts were made to reach this objective. In our case, we set the focus on the development of a model able to characterize and predict the water-consumption curve with low sampling requirements. Since the plant growth system minimizes water losses by evaporation, the water-consumption curve could be related to the transpiration curve, while also including the stomatal response as a parameter of the model. As demonstrated in several studies, water consumption using gravimetric methods correlates with the measurement of the transpiration rate under specific VPD conditions [37], so a fairly high throughput analysis based on that concept could be applied.

The data confirms the strong relation between the kinetics of water consumption and stomatal conductance, which means that the water transpired from the PPS defined in the study is regulated by stomatal conductance, and this last variable is regulated by the water availability in the pot. The model generated in this work contemplates the time variable, therefore it considers the changes in the parameters of water consumption. These types of models included dynamic variables which are scarce and would allow more precise characterization of complex biological phenomena such as plant adaptation to changes in the levels of available water. In addition, the data input of the model is based on a simple variable and easily quantifiable as the weight of PPS.

Moreover, the model can predict the value of water when the conductance is 0 and define the limit of water extraction. This trait has been identified as an important factor, because genotypes with high values of water thresholds begin to partially close their stomata at a relatively high water content, therefore saving soil water [9]. A study using data from different regions and years of the USA has shown, through simulation tools, that this trait would lead to a significant increase in soybean yield, especially in crop seasons classified as dry [6]. An early and accurate screening of the genotypes with specific responses in the water-consumption curve under water deficit seems to be an interesting advantage to be explored in plant breeding programs.

How drought episodes are established in field conditions varies depending on the agro-climatic region, the rain patterns, the soil characteristics, and the atmospheric demand. Under alternating drought conditions, in which there is a frequent period of stress alleviation, genotypes with high evapotranspiration capacity and water extraction (higher B and lower A_R) could be more interesting than those with a contrary response (lower B and high A_R). However, in a drought situation with a low probability of soil water recovery, the selection of genotypes with low B and high A_R could be the main objective for breeding.

The gravimetric measurements of the transpiration-curves trait performed under different VPD could lead to an increase in the adaptation capacity of several crop genotypes to different environments, using the proposed method with a high scale-up potential. Phenotyping protocols have been used in different ways to classify genotypes in response to drought. In this work, we propose the assignment of some parameters of the model as a specific trait of genotypes, so these parameters should be included in drought phenotyping methods. Moreover, associations among the variables of the model increase the possibility of identifying other more informative parameters related to plant-drought tolerance. For example, the model demonstrated that the stomatal conductance could be included as an explicative variable of plant response to progressive water deficit. It is clear that regulating the speed of stomatal closure is a key element in the drought response [9]. In this context, $G_w(t_{0.5})$ appears to be an effective measurement to explain the responsiveness of genotypes to changes in water availability.

Moreover, the phenotype of soybean plants in response to the hydric deficit from two breeding populations could be characterized by the proposed model. Since the phenotype of each genotype is determined by the genome-environment interaction [38,39], the variables generated by the model $t_{0.5}$ and $G_w(t_{0.5})$ were subjected to a correspondence analysis. The analysis confirmed a possible genetic association between the response of genotypes to water restriction and the parameter of the model. As expected, a clearer grouping association was observed in the biparental population than in the breeding population, where the genetic diversity of the germplasm was greater. However, the different number of genotypes included in both analyses could explain the results in the correspondence analysis. Moreover, a genome-wide association analysis could contribute to identifying the specific genomic region and genetic marker associated with the parameters defined by the model.

A recent report has classified the same genotype collection using indexes such as *drought susceptibility index* (DSI) and *yield stability index* (YSI) in relation to the crop cycle group [31], and the authors were able to identify QTLs associated with those traits. A preliminary but not confirmatory correspondence analysis between the parameter of the model $t_{0.5}$ and DSI showed a grouping (Figure S3), indicating that the phenotyping strategy discussed in this work could be useful for improving genotype selection in relation to the performance at field conditions. However, a specific validation assay of water-deficit response in field conditions of a set of genotypes previously characterized by the model represents the next challenge. This point is critical for proposing the phenotyping methodology as a tool to be included in crop breeding programs, especially in those with low-income support.

5. Conclusions

The developed model in the study characterizes and predicts, using gravimetric measurements and with low sampling requirements, the water-consumption curve of soybean plants when watering is withdrawn. The model confirms the strong relationship between the kinetics of water consumption and stomatal conductance. The correspondence analysis between model parameters and the response of the genotypes to water restriction in two different soybean breeding populations confirmed a possible genetic association between parameters of the model and genotypic identity. A preliminary approximation shows that the phenotyping methodology presented in this work could be included in crop breeding programs, especially in those with low-income support.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy12030575/s1, Figure S1: Experimental setup and plant growth system. Figure S2: Solution of the first-order linear homogeneous differential Equation (3). Figure S3: Cluster analysis based on drought susceptibility index (DSI) and the parameter of the model $t_{0.5}$.

Author Contributions: S.S. performed the theoretical mathematical model. O.B., V.B., E.C., G.Q. and S.C. were involved in the planning of the work. G.Q. conducted all data analyses of the experiments. E.C. and G.Q. performed the phenotypic evaluation. S.C. generated the breeding populations. O.B. and V.B. wrote the manuscript. All authors corrected the manuscript. All authors have read and agreed to the published version of the manuscript.

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3.1.1. MATERIAL SUPLEMENTARIO

El material suplementario del artículo precedente se encuentra en el siguiente enlace: https://www.mdpi.com/2073-4395/12/3/575#supplementary

4. DISCUSIÓN GENERAL

La tolerancia o capacidad de mantener el rendimiento que presentan diferentes genotipos de soja ante una situación de déficit hídrico está en gran medida condicionada por cómo responden a esa condición. El control de la pérdida de agua foliar es una de las respuestas más determinantes, dado que tanto la fijación de carbono como la pérdida de agua foliar están determinadas mayoritariamente por el control del cierre estomático. La respuesta más eficiente dependerá tanto de la duración como de la frecuencia del déficit hídrico, asumiendo como respuesta más eficiente la que redunda en una menor caída del rendimiento. El incremento del número de nuevos genotipos evaluados por los programas de mejoramiento hacen necesaria la generación de nuevas metodologías de fenotipado, más rápidas, sencillas y de bajo costo, que permitan cuantificar las respuestas al déficit hídrico de manera precisa. Es por ello que la presente tesis se centró en la búsqueda de herramientas aplicables a los programas de mejoramiento de soja que permitan la selección de genotipos con las respuestas más adecuadas a las características del déficit hídrico al que se encuentran sometidas.

En la primer parte de la tesis profundizamos en la comprensión del control de la pérdida de agua foliar en un genotipo de soja, a través de la regulación del cierre estomático. En ese contexto, nuestro trabajo se enfocó en el estudio del óxido nítrico ($^{\bullet}$NO) y su rol en el control del cierre estomático bajo condiciones de déficit hídrico. Determinamos el patrón de acumulación de $^{\bullet}$NO endógeno en estomas a partir del establecimiento del déficit hídrico y su relación con el cierre estomático y otras respuestas bioquímicas y fisiológicas. Los resultados obtenidos demostraron que la acumulación de $^{\bullet}$NO endógeno en respuesta al déficit hídrico a nivel de estomas, podría actuar como un regulador negativo del mecanismo involucrado en el cierre de los estomas mediado por ABA.

En la segunda parte de la tesis se enfatiza en el estudio de la pérdida de agua a través de los estomas, para lo cual desarrollamos un modelo matemático que permite caracterizar y predecir la cinética de consumo de agua en soja a partir de la suspensión del riego, utilizando un bajo número de muestras.

5. CONCLUSIONES Y PERSPECTIVAS

Teniendo en cuenta que determinamos que el cierre estomático es una respuesta muy temprana en soja, la cual ocurre independientemente de otros cambios en parámetros bioquímicos y fisiológicos que se dan cuando el estrés por déficit hídrico es más severo, y que los resultados obtenidos por el modelo desarrollado confirman la fuerte relación entre la cinética del consumo de agua y la conductancia estomática, los resultados obtenidos en la tesis nos permiten proponer estrategias que apunten a modular el metabolismo del •NO para optimizar la producción de biomasa vegetal o aumentar la tolerancia a la sequía. Por ejemplo, los genotipos que se cultivan en zonas sin restricciones hídricas o con períodos cortos de déficit hídrico podrían manipularse o seleccionarse para producir más •NO en las células estomáticas, permitiendo que los estomas permanezcan abiertos por más tiempo y, de esa forma, incrementar la fijación de dióxido de carbono. Por el contrario, se podrían desarrollar genotipos que crecen en zonas sujetas a períodos prolongados de déficit hídrico, con menor acumulación de •NO en estomas, de forma tal de minimizar la inhibición del cierre estomático por •NO. Esto contribuiría a un cierre estomático más temprano y sincronizado, lo que redundaría en una mayor tolerancia a la sequía.

A partir de los resultados obtenidos podemos suponer que las diferentes respuestas encontradas entre los genotipos de soja en relación a la cinética de consumo de agua bajo condiciones de déficit hídrico podrían estar explicadas por diferencias en la cinética de acumulación de •NO en los estomas. Este hipótesis resta ser confirmada a través del estudio de genotipos identificados como contrastantes respecto a la cinética de consumo de agua.

Un análisis exhaustivo de las respuestas al estrés hídrico asociadas al consumo de agua en plantas requiere de un monitoreo continuo, tanto del agua consumida como de los parámetros de interés. Este tipo de análisis supone una manipulación excesiva para la toma de medidas, siendo esta la causa principal de posibles errores o alteraciones en los datos obtenidos de las respuestas en plantas. La evaluación manual también limita la cantidad de individuos estudiados y el volumen de información generada en cada experimento. Con el fin de automatizar la cuantificación del consumo y la reposición de agua en tiempo real, así como de los parámetros de interés, se diseñará y construirá una

plataforma de fenotipado de bajo costo a través de la modalidad open hardware. El uso de dicha plataforma nos dará mayor precisión, exactitud y capacidad de toma de datos, permitiendo identificar respuestas/comportamientos que solamente a través del monitoreo en tiempo real pueden ser cuantificados. Un ejemplo de las respuestas que pueden ser estudiadas a través de esta plataforma, es la determinación de los cambios en el comportamiento de consumo de agua en diferentes condiciones de déficit de presión de vapor.

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