



PHENOTYPING SOYBEANS FOR DROUGHT RESPONSES USING REMOTE SENSING TECHNIQUES AND NON-DESTRUCTIVE PHYSIOLOGICAL ANALYSIS

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ABSTRACT: Water deficit is the major abiotic factor that limits crop productivity. Climate changes are likely to exacerbate drought stresses in the future. In the present work, we investigated the feasibility of using the Normalized Difference Vegetation Index (NDVI) combined with the canopy temperature and other physiological characteristics, such as chlorophyll content and gas exchange, to monitor soybean (*Glycine max* L. Merrill) plants differing in their drought response under glasshouse conditions. Additionally, the drought responses of the cultivars Embrapa 48 and BR 16 were assessed under conditions of natural drought, water deficit simulated by sheltering the plants from rain at the vegetative and reproductive periods and irrigation at field conditions. Remote sensing techniques could be used to initially assess the drought responses of soybean plants under controlled conditions. Additionally, we observed the relationship between the NDVI and several physiological characteristics, such as chlorophyll content, photosynthesis, stomatal conductance and transpiration. Therefore, the combination between remote sensing techniques and the assessment of physiological traits of plant materials at the same developmental stage and leaf areas is useful to accurately monitor cultivars presenting different drought responses.

Key-words: *Glycine max* L. Merrill, NDVI, water deficit.

FENOTIPAGEM DE SOJA PARA RESPOSTAS À SECA USANDO TÉCNICAS DE SENSORIAMENTO REMOTO E ANÁLISES FISIOLÓGICAS NÃO-DESTRUTIVAS

RESUMO: O déficit hídrico é o maior fator abiótico que limita a produtividade das culturas. As mudanças climáticas provavelmente agravarão os estresses hídricos no futuro. No presente trabalho, nós investigamos a viabilidade de uso do Índice de Vegetação por Diferença Normalizada (NDVI) combinado à temperatura do dossel e a outras características fisiológicas, tais como teor de clorofila e trocas gasosas, para monitorar plantas de soja (*Glycine max* L. Merrill) com respostas diferenciais à seca, sob condições de casa de vegetação. Adicionalmente, as respostas à seca das cultivares Embrapa 48 e BR 16 foram avaliadas sob condições de seca natural, déficit hídrico simulado abrigando-se as plantas da chuva nos períodos vegetativo e reprodutivo e irrigação sob condições de campo. Tecnologias de sensoriamento remoto puderam ser usadas para inicialmente avaliar as respostas à seca de plantas de soja sob condições controladas. Além disso, nós observamos a relação entre o NDVI e diversas características fisiológicas, tais como teor de clorofila, fotossíntese, condutância estomática e transpiração. Portanto, a combinação entre técnicas de sensoriamento remoto e a avaliação de características fisiológicas de materiais vegetais no mesmo estágio de desenvolvimento e áreas foliares é útil para monitorar precisamente cultivares apresentando diferentes respostas à seca.

Palavras-chave: *Glycine max* L. Merrill, NDVI, déficit hídrico.

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INTRODUCTION

Water deficit is the major abiotic factor that limits crop productivity, and climate change is likely to exacerbate drought stresses in the future (STOKSTAD, 2004). Therefore, several studies have attempted to improve plant drought tolerance through conventional breeding or biotechnology. One major challenge in such research is the choice of suitable phenotyping methods, as most contemporary methods are too time consuming, expensive, or technically demanding for large-scale use (PASSIOURA; ANGUS, 2010; PASSIOURA, 2012; SETTER, 2012). Although a wide range of physiological and morphological measurements that can be assessed in plants growing in pots has been described (PASSIOURA, 2012), it is not known which traits are most relevant to differentiate genotypes with contrasting responses to drought.

Recently, several biophysical and physiological plant characteristics emerged as valuable tools for high-throughput phenotyping of plants due to their versatility and the rapid and non-destructive nature of the methodology. Among the spectral reflectance indices, NDVI (Normalized Difference Vegetation Index) has been correlated to several plant characteristics, such as chlorophyll (JONES et al., 2007), biomass (MARTI et al., 2007), ground cover (MULLAN; REYNOLDS, 2010), nitrogen status (WRIGHT et al., 2005), yield (ROYO et al., 2003) and drought stress (YUHAS; SCUDERI, 2009). The infrared thermometer can be used to easily measure canopy temperatures at all levels of water stress. The use of the canopy temperature to detect water stress is based on the principle that water lost through transpiration cools the leaves below the temperature of the surrounding air under well-watered conditions. If transpiration is greatly reduced or ceases, the leaf temperature will be greater than the air temperature because of the radiation absorbed by the leaf (JACKSON, 1982).

In our study, we assessed the behavior of two Brazilian soybean (*Glycine max* L. Merrill) cultivars with contrasting drought response using remote sensing techniques (NDVI and Infrared thermometry) and physiological and soil measurements in glasshouse and/or field conditions. We checked if remote sensing techniques could be used for initial assessment of the soybean drought responses. Additionally, we assessed the relationship between the NDVI and chlorophyll, photosynthesis, stomatal conductance and transpiration.

MATERIALS AND METHODS

Response of two drought-contrasting soybean cultivars to water deficit simulated under glasshouse conditions

Two soybean genotypes that display contrasting responses to water deficit (BR16, more sensitive versus Embrapa 48, less sensitive) were selected for the study based on previous experiments (OYA et al., 2004).

The experiment was performed in a glasshouse in Londrina, PR, Brazil, where the temperature and relative humidity were monitored by a thermohygrograph, model U14-002, manufactured by Hobo (Bourne, Massachusetts, USA). To prevent early flowering, plants were maintained under a photoperiod of 15h/8h daily. The vapor pressure deficit (VPD) was calculated using the atmospheric temperature and relative humidity (RH) according to the following formula: $VPD = (100 - RH) / 100 \times PV_{sat}$ (kPa). PV_{sat} (saturation vapor pressure) was calculated using the psychrometric chart available at <http://physics.holsoft.nl/physics/ocmain.htm>.

The experimental design was completely randomized, with 10 replicates per treatment that consisted of the cultivars BR16 and Embrapa 48. Five extra pots were kept at the same experimental conditions as the 10 replicates and used for analyzing the soil water status.

Soybean seeds of the cultivars BR 16 and Embrapa 48 were inoculated with a

liquid inoculant (1.5×10^6 bacterial cells of *Rhizobium japonicum*, SEMIA 5,079 per seed) and sown in 1L pots containing a mixture of soil:sand:organic compound (1:3:1, 26% holding capacity). The pots contained one plant each and were well watered until reaching the V₂ developmental stage (FEHR; CAVINESS, 1977).

At this stage, the pots were saturated with water and allowed to drain overnight. The next morning, they were bagged in plastic bags that were wrapped around the stem to prevent water evaporation directly from the soil surface. Irrigation was thereafter suspended, and the pot weight was measured daily at 09h00 a.m. Brazilian Standard Time. Plants were re-watered ten days after suspension of irrigation for recovery determination.

The initial (H1) and final (H2) plant heights were determined on the day the pots were bagged and at the end of the experimental period, respectively. From this data, the relative shoot growth rate (RSGR) was calculated according to the following formula: $RSGR = (H2 - H1) / H1 \times 100$.

Using the pots' daily weights, transpiration (T) was calculated as the difference in the pot weights on successive days. The total transpiration (TT) was calculated as the sum of the daily transpiration from the initial day when the plants were bagged to the day when the plants were harvested.

Gas exchange (stomatal conductance, g_s ; photosynthesis, A) was determined using a portable photosynthesis meter (LI-6400, LI-COR Biosciences) under a flux density of $1,000 \mu\text{mol m}^{-2} \text{s}^{-1}$. The measurements were ascertained from the middle leaflet of the third fully expanded trifoliate leaf.

NDVI measurements were performed with a GreenSeeker 505 handheld sensor, Ntech Industries, Inc (Ukiah, California, USA), at a height of 80 cm from the canopy following the manufacturer's recommendations. To avoid interference

caused by the reflectance of the adjacent areas, the maximum NDVI readouts were used instead of the averaged values, thus only readings corresponding to the plant reflectance were considered.

The readout NDVI was automatically calculated by the equipment according to the following equation: $NDVI = (pivp - pv) / (pivp + pv)$, where $pivp$ and pv are the near infrared and red reflectance, respectively.

The canopy temperature was measured using a thermal infrared *InfraPro* manufactured by Oakton (Vernon Hills, Illinois, USA).

Chlorophyll was determined from the right and left sides of the leaf adaxial surface using a chlorophyll meter (SPAD-502) (Osaka-shi, Osaka, Japan). The final chlorophyll concentration was the average of both readings.

At the end of the experiment, the soil water potential was determined by means of using a WP4C (Decagon) (Pullman, Washington, USA) in the five extra pots previously mentioned. The gravimetric humidity (GH) was determined by weighing a moist soil sample, oven drying at 105°C for 24-48h, reweighing, and calculating the mass of the water lost as a percentage of the mass of the dried soil.

Response of two drought-contrasting soybean cultivars submitted to drought under field conditions

This study was conducted in the experimental fields (23°11'44"S, 51°10'35"W) during the 2012/2013 crop season. The temperature, relative humidity and rainfall at the site were monitored by the weather station installed in the experimental area. With data of rainfall and air temperature the water balance was calculated according to Thornthwaite and Mather (1955) (Figure 1).

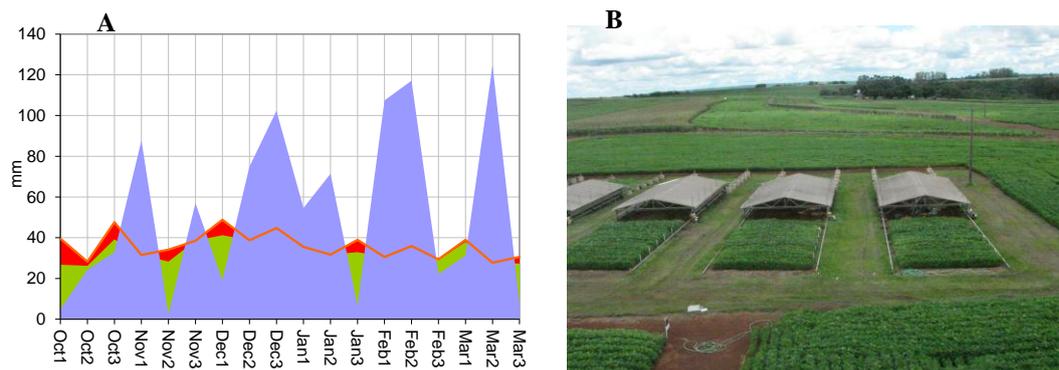


Figure 1. (A) Water balance according to Thornthwaite and Mather (1955). Red areas represent water-deficits in the 2012/2013 crop season (B) View of the field experiment showing the rain out shelters.

The experimental design was in completely randomized blocks, with treatments arranged in split plots and four replicates. The main plots received three different water regimes consisting of irrigated (IRR, matric soil-water potential was maintained between -0.03 and -0.05 MPa), non-irrigated (NIRR, natural rainfall) and plants artificially drought stressed at the reproductive or vegetative periods (DSR or DSV, respectively). The treatments in the sub-plots were the soybean cultivars BR 16 and Embrapa 48 regarded as more sensitive and less sensitive to drought respectively. To simulate drought stress, the plants were sheltered from rain using rain-out shelters programmed to automatically close at the first incidence of rainfall and open as soon as the rain stopped (Supplementary Figure S1B). The soil humidity was daily monitored by tensiometers placed 30 cm deep in the soil and weekly by the gravimetric method and a neutron probe.

The sowing date for both cultivars and treatments was 05 Nov 2012. Water deficit during the vegetative period (DSV) started in 05 Dec 2012 (V4) and ended at 27 Dec 2012 (R2), when plants were in the reproductive period and then they were allowed to receive rainfall water. On this date, another group of plots that had received water of precipitation during vegetative period were subjected to water deficit in the reproductive period (DSR).

The phenological stage of the plants was evaluated three times a week from the date of germination, which started five days after sowing (DAS), according to the procedures established by Embrapa Soybean.

The NDVI was measured using the device/methodology described above. The distance and angle of the sensor positioning followed the manufacturer's recommendations. When both cultivars reached the same developmental stage (R5.5), NDVI was measured at 09h00 am and the leaf area index (LAI) was calculated as the ratio between leaf area and the area of land occupied by the plant.

The plot grain yields (at 13% humidity) were calculated through the following equation: $\text{yield (kg ha}^{-1}\text{)} = (100 - \text{grain humidity at harvest, \%}) \times (\text{harvested grain weight, kg} \times 10000) / (\text{plot harvested area, m}^2\text{)}$.

Statistical analysis

The data were statistically analyzed using an exploratory diagnostic that tested for assumptions of normality, the independence of the residue, the additivity of the model, and the homogeneity of treatment variances, followed by an analysis of variance (ANOVA). After these analyses were performed and when the F test indicated statistical significance, Duncan's test for multiple comparisons among treatment means was applied ($p \leq 0.05$).

RESULTS AND DISCUSSION

In our study, we assessed the different behaviors of two Brazilian soybean cultivars, one of which is considered more sensitive and the other less sensitive to drought, using remote sensing techniques (NDVI and Infrared thermometry), non-destructive physiological (gravimetric transpiration, chlorophyll and gas exchange) analysis and soil measurements (water potential and gravimetric humidity) in glasshouse conditions. Our major aims were to verify the different responses to drought of these cultivars in field conditions and to check that remote sensing techniques could be used for assessment of the soybean drought responses.

In the glasshouse experiment, infrared thermometry was used to identify the day on which the plants started to experience water stress within the water deficit period. Based on the results in Figure 1, the plants started to experience water stress two days after suspension of irrigation, at the hottest hours of the day (11h00 a.m. – 02h00 p.m.) as the leaf temperature was higher than the air temperature, especially at the adaxial surface. Generally, plants under water stress possess higher leaf temperatures, and in soybeans it can be elevated up to 8°C (RAO, 1985). The higher temperature peaks in the adaxial surface were most likely caused by a higher exposure of this surface to the sunlight and a lower stomata density.

NDVI analysis demonstrated that before the water stress started (SI- the day when irrigation was suspended and ISI-the first day after suspension of irrigation), NDVI values were higher for BR 16 plants most likely due to their higher leaf area. However, as the water deficit progressed, the NDVI of both cultivars tended to decrease until 10 days after suspension of irrigation-DASI (Figure 2A); however, at the 10th DASI, when the plants were re-irrigated, the NDVI values of the cultivar Embrapa 48 increased whereas those of the BR 16 plants remained low.

The higher NDVI of the cultivar Embrapa 48 after the 8th day is attributable to

its lower rate of chlorophyll degradation (Figure 2B), which must have allowed for the recovery of the photosynthesis after re-irrigation (Figure 2C). According to Liu et al. (2012), chlorophyll is the major component that influences the NDVI value, as the error margin of NDVI readings increased or decreased with alterations in the leaf area index (LAI). The decreased chlorophyll content under drought stress has been considered a typical symptom of pigment photo-oxidation and chlorophyll degradation (ANJUM et al., 2011). Because the production of reactive oxygen species is mainly driven by excess energy absorption in the photosynthetic apparatus, its production might be avoided by degrading the absorbing pigments (MAFAKHERI et al., 2010). A decreased chlorophyll content in plants such as *Paulownia imperialis* (AYALA-ASTORGA; ALCARAZ-MELENDZ, 2010) and *Carthamus tinctorius* (SIDDIQI et al., 2009) has been reported under drought stress.

Higher stomatal conductance values (Figure 2D) were also observed for the Embrapa 48 plants after re-irrigation, which indicates stomatal opening. The g_s data indicated that both BR 16 and Embrapa 48 cultivars were under control conditions ($g_s > 0.2 \text{ mol m}^{-2} \text{ s}^{-1}$) in the first DASI; under moderate water stress ($g_s = 0.1 \text{ to } 0.2 \text{ mol m}^{-2} \text{ s}^{-1}$) from the second to the fourth DASI and under severe water stress ($g_s < 0.1 \text{ mol m}^{-2} \text{ s}^{-1}$) at least from the seventh to the tenth DAS (FLEXAS et al., 2004).

According to Lawlor (2013), differences in plant development under water stress e.g. decreased A and increased non-photochemical chlorophyll a fluorescence for instance (WANG et al., 2008; WOO et al., 2008) may be a consequence of water saving mechanisms due to lower transpiration rates. Plants with lower transpiration rates caused by lower g_s and/or leaf areas dry the soil more slowly than plants with higher transpiration rates, and thus the drought symptoms (decreased leaf water potential (ψ_P), relative water content (RWC), g_s , A , etc.) are more rapidly observed in plants with

higher transpiration rates. Consequently, the metabolism of plants with higher transpiration rates is not initially affected, whereas the metabolism of plants with lower transpiration rates is greatly impaired.

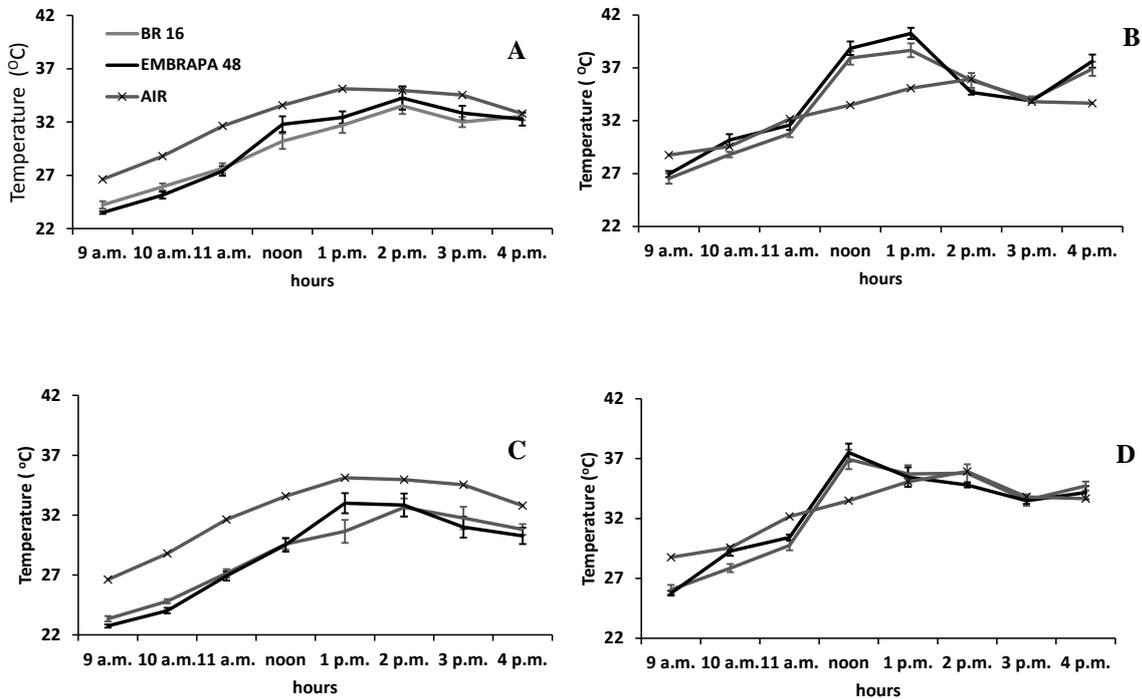


Figure 1. Air and leaf temperature of the cultivars BR 16 and Embrapa 48 on the day that irrigation was suspended (A/C) and on the second day after suspension of the irrigation (DASI) (B/D). Measurements were made at the adaxial (A-B) and abaxial surfaces (C-D). Line with x marker=air temperature, solid black line=Embrapa 48 and solid grey line= BR 16 (n=10 ± standard error).

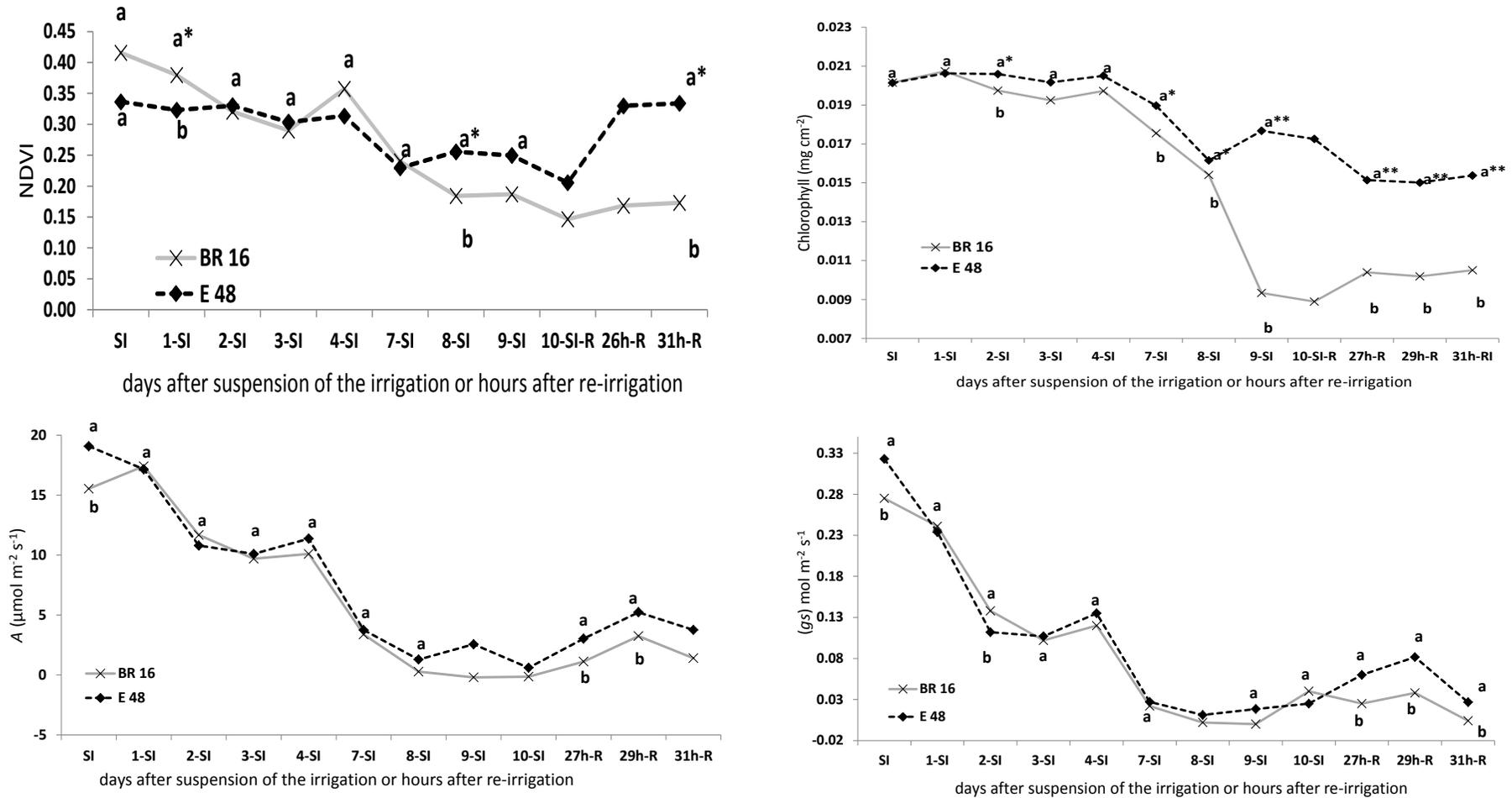


Figure 2. A) NDVI B) chlorophyll content C) photosynthesis and D) stomatal conductance of the cultivars BR16 and Embrapa 48 after suspension of the irrigation (SI) or re-irrigation (R) at glasshouse conditions. Means followed by the same letter are not significantly different according to the Duncan's test ($p \leq 0.05$).

In our study, the transpiration rate of both cultivars diminished with the development of water stress (Figure 3A). However, until the 4th DASI, transpiration was higher for BR 16 plants. At the end of the experimental period, the BR 16 plants exhibited observably higher total transpiration values (Figure 3B). To verify that the higher transpiration of BR 16 plants caused an earlier depletion of water of the substrate, the percentage of water related to its field capacity (Figure 3C), soil water potential (Figure 3D) and gravimetric humidity (Figure 3E) were calculated/measured before the plants were re-irrigated. A higher percentage of water related to its field capacity, soil water potential and gravimetric humidity was observed in the pots with Embrapa 48 plants, thus confirming that BR 16 plants depleted the soil moisture more rapidly and that the Embrapa 48 plants displayed water saving mechanisms thus avoiding water stress.

Certain genotypes have been well-documented to maintain transpiration rates until the soil becomes dry, whereas others display a decline in transpiration when the soil is still relatively moist. This was verified in maize (*Zea mays* L.) (RAY; SINCLAIR,

1997), soybeans (HUFSTETLER et al., 2007; VADEZ; SINCLAIR, 2001) and peanuts (*Arachis hypogaea* L.) (BHATNAGAR-MATHUR et al., 2007). Some drought tolerant soybean genotypes could limit an increase in transpiration when VPD was higher than 2 kPa (SADOK; SINCLAIR, 2009). Kholová et al. (2010a) suggested that other mechanisms might be related to the slow rate of water loss per unit of leaf area regardless of VPD or lower leaf area.

The control of the total water loss at the leaf level when water is available is one aspect of the water management that is often neglected (KHOLOVÁ et al., 2010a, b). A conservative use of water, even if soil moisture is sufficient to fully supply the plant's water demand, maintains water in the soil profile for a longer period of time. This could be advantageous under prolonged drought conditions or a terminal drought and in soils that possess textures/structures that favor evapotranspiration.

The data in Figure 4B-4D indicate that the higher moisture in the soil containing Embrapa 48 plants allowed them to wilt and yellow later than BR 16 plants, which resulted in a faster recovery of the former after re-irrigation (Figure 4E).

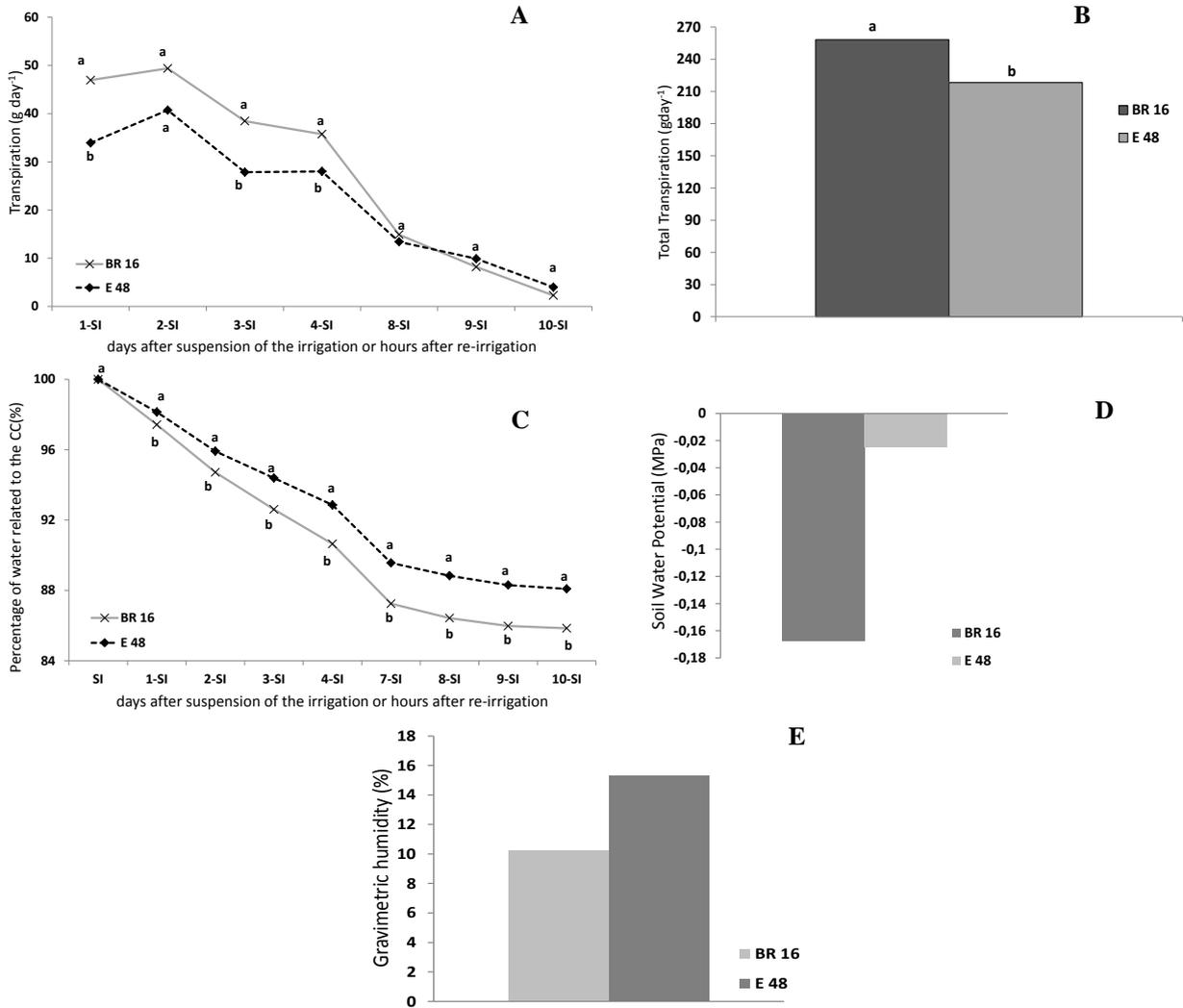


Figure 3. A) Daily transpiration and B) accumulated transpiration of the cultivars BR16 and Embrapa 48 after suspension of the irrigation (SI) or re-irrigation (R) at glasshouse conditions. (C) Percentage of decline in water related to field capacity over the experimental period (D-E) soil water potential and gravimetric humidity at the end of the experimental period. Means followed by the same letter are not significantly different according to the Duncan's test ($p \leq 0.05$).

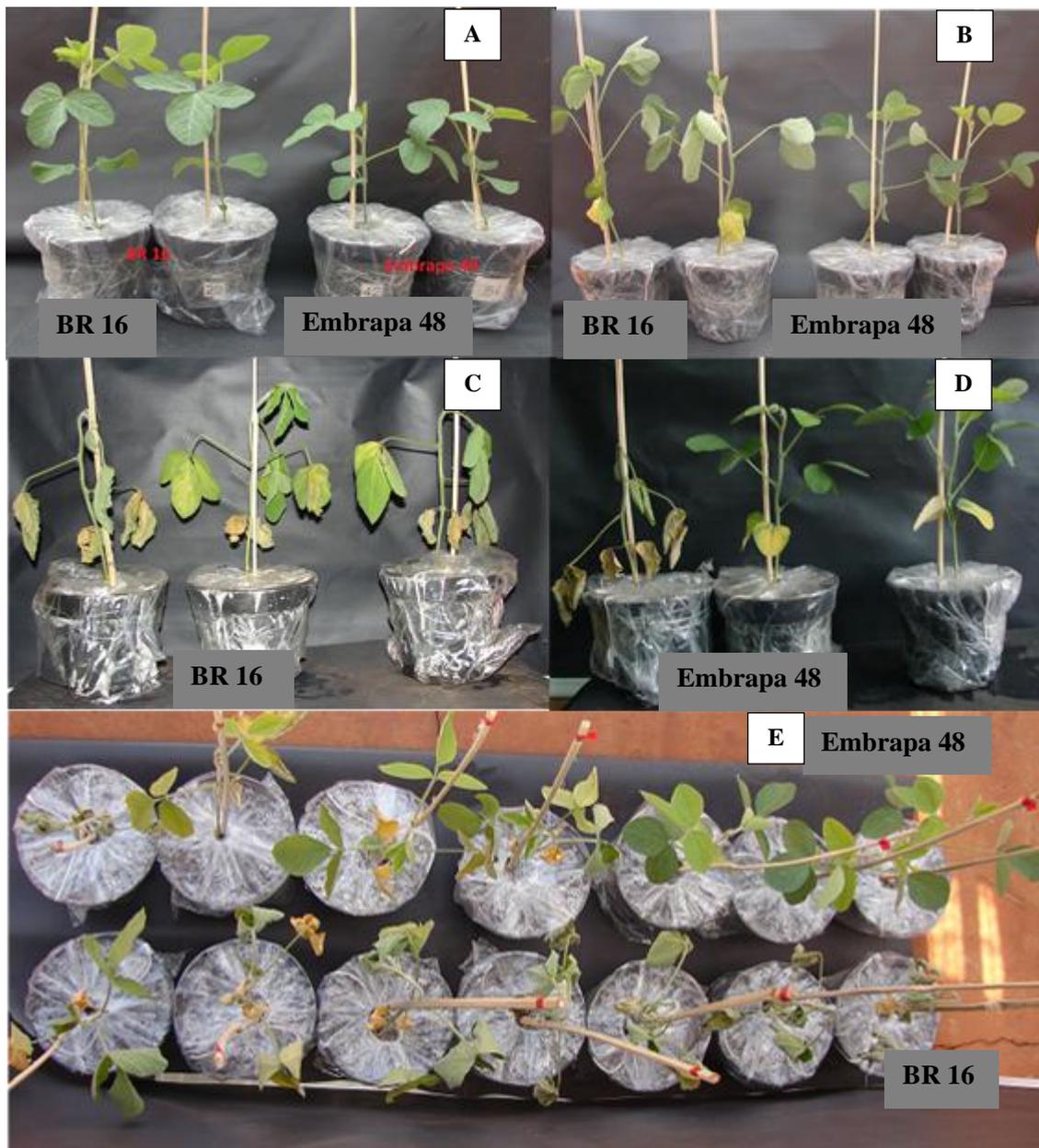


Figure 4. Plants of the cultivars Embrapa 48 and BR 16 under well watered conditions (A) 1st DASI (B), 7th DASI (C/D), 10th DASI and one day after re-irrigation (E).

The differential behavior between both drought-contrasting cultivars when water was available and subsequently after water stress reflected in the plant growth, as verified by the higher relative shoot growth rate (RSGR) of the cultivar Embrapa 48 compared to the cultivar BR 16 (Figure 5a and b). According to Hsiao (1973), plant

growth is one of the most water-deficit sensitive processes and is usually reduced before photosynthesis or stomatal conductance. Anjum et al. (2011) suggested that a permanent or temporary water deficit hampers plant growth and development more severely than any other environmental factor.

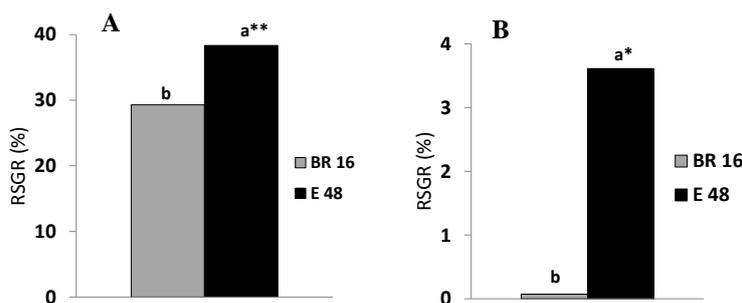


Figure 5. Relative Shoot Growth Rate (RSGR) of the cultivars BR16 and Embrapa 48 under control (A) and water stress conditions (B).

Our data established correlations between the NDVI and physiological traits, such as A , g_s , transpiration and chlorophyll (Figure 6A-6G). For the cultivar BR 16, the NDVI variation was better explained by the variation in the A ($r^2=0.91$). However, for the cultivar Embrapa 48, the NDVI variation was better explained by the variation in the transpiration ($r^2=0.96$).

Attempts to correlate the transpiration data of both cultivars with the atmospheric VPD revealed that, within a range of VPD from 1.0 to 2.0 kPa, transpiration was always higher for the cultivar BR 16. Above 2.0 kPa, both cultivars exhibited the same transpiration rate (Figure 7).

From the data above-mentioned, we could assume that remote sensing techniques and non-destructive physiological analysis could be used to assess the initial drought responses of soybean plants under controlled conditions. However, because leaf area of BR 16 plants seems to be visually higher than those of Embrapa 48 plants, the data obtained may be a consequence of delayed stress onset in the Embrapa 48 plants due to its lower transpiration rates and water use.

In the field, we verified that the cultivar Embrapa 48 showed higher NDVI, at the same developmental stage (R5.5) and leaf area (Figure 8a-c), than those of BR 16. Yield was also higher when water deficit was applied at the reproductive period. However, when water stress was applied at the vegetative period, BR16 plants outperformed those of the cultivar Embrapa 48. Oya et al. (2004) evaluated physiological characteristics of Embrapa 48 and BR 16 cultivars and

observed that they were moderately tolerant and highly sensitive to drought, respectively, when water stress was applied at the reproductive stage.

Among the three water regimes investigated in the current study, drought stress at the reproductive period greatly reduced the productivity of both soybean cultivars (Figure 8A) in the crop season. A water deficit during the reproductive period has been demonstrated to be a dominant environmental factor that accelerates the rate of abortion (KATO, 1964; PEDERSEN et al., 2005; WESTGATE; PETERSON, 1993) as it reduces photosynthesis and the amount of photoassimilates allocated to reproductive tissues (RAPER; KRAMER, 1987).

Based on our findings, we concluded that the combination of remote sensing techniques and analysis of non-destructive physiological traits can be used to phenotype soybean cultivars with contrasting responses to drought.

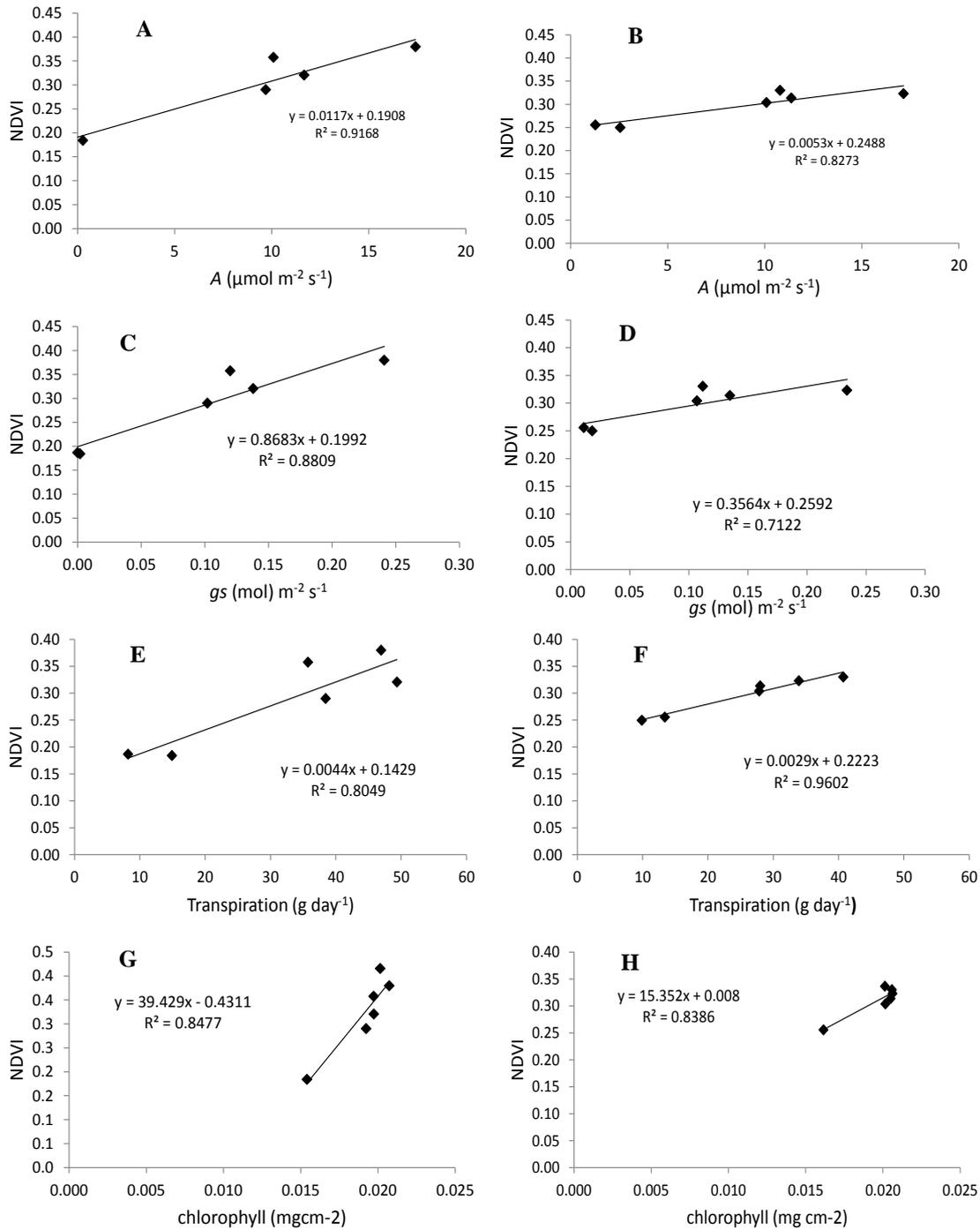


Figure 6. Correlations between NDVI and A, gs, T and chlorophyll for the cultivars BR 16 (A-C-E-G respectively) and Embrapa 48 (B-D-F-H respectively).

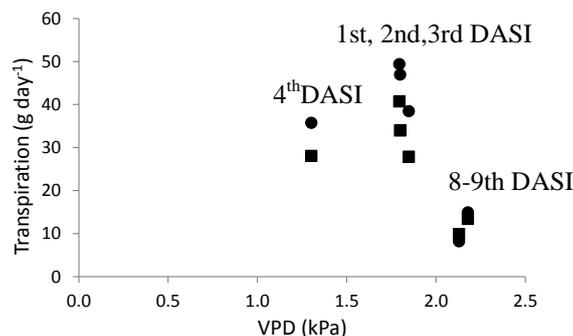


Figure 7. Transpiration versus VPD of the cultivars BR 16 (circle) and Embrapa 48 (square) over the experimental period in glasshouse conditions.

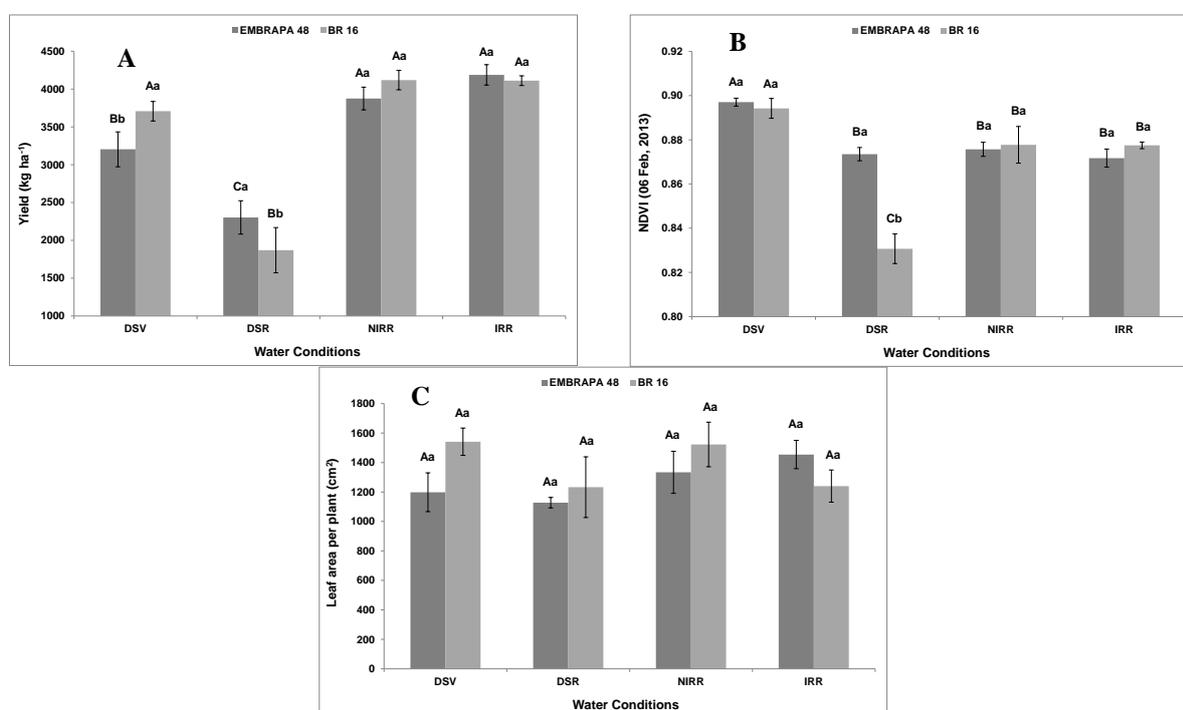


Figure 8. Yield, NDVI and leaf area in Embrapa 48 and BR 16 soybean cultivars subjected to drought stress in the vegetative (DSV) and reproductive (DSR) periods and kept under non irrigated (NIRR) and irrigated (IRR) conditions. Means \pm SE followed by the same uppercase letters (among water conditions) and same lowercase letters (between cultivars) do not differ by the Duncan's test ($p \leq 0.05$). $n=4$.

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