MINISTÉRIO DA EDUCAÇÃO
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PROGRAMA DE PÓS-GRADUAÇÃO EM AGRONOMIA

CAMILLE FERREIRA MAIA

BRASSINOSTEROIDS POSITIVELY MODULATE GROWTH: PHYSIOLOGICAL, BIOCHEMICAL AND ANATOMICAL EVIDENCES USING TWO TOMATO GENOTYPES CONTRASTING TO DWARFISM

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Dissertation submitted to Universidade Federal Rural da Amazônia, as part of the requirements for obtaining the *Magister Scientiae* degree in Agronomy.
Advisor: Prof. Dr. Allan Klynger da Silva Lobato

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Dissertation presented to the Universidade Federal Rural da Amazônia, as part of the requirements of the Master Course in Agrarian Sciences: area of concentration in Agronomy, to obtain the title of Master.
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LIST OF ABBREVIATIONS

APX	Ascorbate peroxidase
BR	Brassinosteroid
CAT	Catalase
EL	Electrolyte leakage
F_0	Minimal fluorescence yield of the dark-adapted state
F_m	Maximal fluorescence yield of the dark-adapted state
F_v	Variable fluorescence
F_v/F_m	Maximal quantum yield of PSII photochemistry
H_2O_2	Hydrogen peroxide
LDM	Leaf dry matter
MDA	Malondialdehyde
MT	Micro-Tom
O_2^-	Superoxide
POX	Peroxidase
RDM	Root dry matter
ROS	Reactive oxygen species
SDM	Stem dry matter
SOD	Superoxide dismutase
TDM	Total dry matter

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SUMMARY

RESUMO

Nossa hipótese considerou que a utilização de genótipos com diferentes níveis de biossíntese para BR pode explicar os papéis desse esteróide em relação ao crescimento e ao comportamento metabólico. O objetivo desta pesquisa foi investigar as possíveis interferências ocasionadas pela aplicação exógena de BR sobre crescimento e metabolismo, utilizando dois genótipos contrastantes com o gene *DWARF* (MT-d e MT-D), que são BR-deficiente e BR-eficientes respectivamente. O experimento teve quatro tratamentos, sendo dois genótipos (eficiente e deficiente em BR) e dois níveis de brassinosteróides (0 e 100 nM BR, aqui descritos abaixo como BR e + BR, respectivamente). Esta pesquisa revelou que a aplicação exógena de BR promoveu melhoria no crescimento, induzindo aumentos em plantas defecientes para BR de 120%, 469%, 219%, e 203% em LDM, RDM, SDM e TDM, respectivamente. Os efeitos positivos nas trocas gasosas e fluorescência de clorofila confirmam os benefícios deste esteróide no aparelho fotossintético. As mudanças nas características anatômicas da folha estão relacionadas à contribuição do BR no influxo e consequente fixação de CO_2. Além disso, as modificações relacionadas à anatomia da raiz ocorreram pela ação do BR com objetivo aumentar a barreira contra estresses bióticos e abióticos, e a eficiência na absorção de água e nutrientes. As melhorias nos pigmentos fotossintéticos, verificadas nos aumentos de 16%, 35%, 20% e 67% em Chl a, Chl b, Total Chl e Car, respectivamente, para plantas deficientes em BR, sugeriram que o papel do BR deve estar ligado à rota de biossíntese de clorofila e manutenção da integridade do cloroplasto, sendo este resultado intrinsecamente associado aos incrementos encontrados nas atividades de enzimas antioxidantes que modulam o acúmulo de ROS.

PALAVRAS-CHAVE: Fluorescência de clorofila. Gene *DWARF*. Trocas gasosas. Anatomia foliar. *Solanum lycopersicum*. 24-epibrassinolide

ABSTRACT

Our hypothesis considered that the utilization of genotypes with different levels of biosynthesis to BR can explain the roles of this steroid in relation to growth and metabolic behavior. The aim of this research was to investigate the possible interferences occasioned by the exogenous application of BR on growth and metabolism, using two genotypes contrasting to the DWARF gene (MT-d and MT-D), that are BR-deficient and BR-efficient, respectively. The experiment had four treatments, being two genotypes (BR-efficient and BR-deficient) and two levels of brassinosteroids (0 and 100 nM BR, here after described as – BR and + BR, respectively). This research revealed that the exogenous application of BR promoted improvement on growth, inducing increases in BR-deficient plants of 120%, 469%, 219%, and 203% in LDM, RDM, SDM and TDM, respectively. The positive effects on gas exchange and chlorophyll fluorescence confirm the benefits of this steroid on photosynthetic apparatus. The changes in the anatomical characteristics of the leaf are related to contribution of the BR on influx and consequent fixation of CO_2. In addition, modifications related to root anatomy occurred by the BR action with objective to increase the barrier against biotic and abiotic stresses and the efficiency in the absorption of water and nutrients. The improvements in photosynthetic pigments, observed in the increases of 16%, 35%, 20% and 67% in Chl *a*, Chl *b*, Total Chl and Car, respectively, to BR-deficient plants, suggested that the role of BR must be linked to the chlorophyll biosynthesis route and maintenance of chloroplast integrity, this result being intrinsically associated with the increments found in the activities of antioxidant enzymes that modulate the accumulation of ROS.

KEYWORDS: Chlorophyll fluorescence. *DWARF* gene. Gas exchange. Leaf anatomy. *Solanum lycopersicum*. 24-epibrassinolide

1. CONTEXTUALIZATION

Besides being a species of world-wide economic relevance, tomato (*Solanum lycopersicum* L.) has been widely used as a suitable model to study several physiological phenomena. This fact is due to the presence of characteristics such as good climatic adaptation, relatively short life cycle, easy control of pollination and hybridization, and asexual propagation ability by grafting, which promoted the use of this species as a convenient model for basic and applied research (BAI; LINDHOUT, 2007; GERSZVERG et al., 2015).

However, due to the requirement of considerable growth spaces (> 1 m height) and longer production time (~ 4 months), the dwarf tomato MT has been increasingly used as a model system (CAMPOS et al., 2010), whose main characteristics are the short life cycle, unique genetic background, besides being able to grow in space limited to a high density in greenhouses with controlled conditions (FLORES et al., 2015).

The collection of MT mutants available constitutes a community genetic resource (SHIKATA et al., 2015), suitable for studies in different fields, from the interaction with environmental factors, such as heavy metals (GRATÃO et al., 2009) and herbivory (CAMPOS et al., 2009), to the behavior of hormones in plant responses (CARVALHO et al., 2010), including compounds such as brassinosteroids (CARVALHO et al., 2013).

BRs are a group of steroids originally isolated from pollen as substances involved in processes associated with growth (FRIDMAN; SAVALDI-GOLDSTEIN, 2013). BRs occurs in almost all parts of plants, such as pollen, flower buds, fruits, seeds, vascular exchange, leaves and roots (BAJGUZ; HAYAT, 2009). These compounds have the ability to promote the growth and development of plants (BARTWAL et al., 2013).

The height of the plant is an important agronomic characteristic interconnected to architecture with effects on lodging stability, harvest index and yield (ZANKE et al., 2014). Numerous exogenously applied substances have a considerable influence on the regulation of plant growth, which is evidenced by the dwarf or semi-dwarf phenotypes in several mutants unable to synthesize or perceive a particular hormone (ZHANG et al., 2014), and can be restored to a normal phenotype by the exogenous application of the hormone whose biosynthesis has been disturbed (BISHOP et al., 1999).

In several plant species, including tomato, mutants deficient or insensitive to BR were identified (WANG et al., 2014) which express numerous growth defects, including dwarfism, dark green leaves, late flowering, male sterility and dark photomorphogenesis. Several dwarf mutants, isolated for physiological processes apparently unrelated to BRs, had shown indeed to be lesions in genes encoding BR biosynthetic enzymes (CLOUSE; SASSE, 1998; LI et al., 2016).

Singh and Savaldi-Goldstein (2015) reported that BR activity intrinsically affects the growth and development of aerial and subterranean organs and is confirmed by the dwarf phenotype in mutants deficient and insensitive in BR. The mutant BR species exhibiting dwarfism have two types of mutations, BR-deficient mutant, which has the impaired BR biosynthesis gene, causing deficiency, but which can be reversed by the exogenous application of this hormone; and BR-insensitive mutant that possesses the affected BR receptor gene, inducing insensitivity, which can not be rescued by the BR supply (NOGUCHI et al., 1999; FERNANDEZ et al., 2009; NIE et al., 2017).

The MT phenotype presents characteristics such as the reduction of internodes length and the production of smaller, rough and dark leaves (GONZALEZ et al., 2015), similar to those of BR deficiency in tomatoes, revealing that this cultivar contains a mutation related to biosynthesis of BR (CAMPOS et al., 2010).

The small size of MT is associated with the presence of the *dwarf* gene, and this mutation has been used for a long time to create dwarf tomato varieties with different degrees of dwarfism (MARTI et al., 2006), being able to carry the allele *d* that confers the dwarf size and also the allele of the wild type *D* that presents normal size for this variety (CARVALHO et al., 2011).

This research is related to the possibility that the use of genotypes with different levels of biosynthesis for BR explain the roles of this steroid in relation to development and metabolic behavior. Therefore, the objective was to investigate the possible interferences induced by the exogenous application of BR on growth and metabolism using two dwarf contrasting genotypes (MT-d and MT-D), which are deficient and efficient in BR, respectively.

1.1. Literture Review

1.1.1 General aspects of *Solanum Lycopersicum*

Tomato (*Solanum lycopersicum* L.) is one of the world's most important cultivated plants, with production and consumption increasing continuously (THEURL et al., 2013; SONG et al., 2014). This species belongs to the family Solanaceae, genus Solanum L., section Lycopersiconum, representing one of the major genera of angiosperms and is the largest genus in Solanaceae (PERALTA et al., 2008).

The wild species of cultivated tomato are native to western South America, with records found in countries such as Ecuador, Peru, northern Bolivia and Chile, including the Galapagos Islands. They are scattered throughout diverse habitats that include the desert of the Pacific coast at sea level, Andean regions, and even from arid to rainy climates (BERGOUGNOUX, 2013).

The tomato is a perennial plant, presenting hairy stem, bipinnate leaves, flowers usually with 5 petals and fleshy fruits (BLANCA et al., 2012). To Carvalho et al. (2011) photoperiod-independent sympodial flowering, with consequent seed production in any condition of day duration, the formation of fleshy climacteric fruits, compound leaves, mycorrhizal roots and glandular trichomes are characteristics that allow the tomato to be an alternative model of dicotyledons ideal to investigate several physiological phenomena not possible in other model plants such as *Arabidopsis thaliana*.

Gonzalez et al. (2015) observed several studies were elaborated on several important areas for agriculture such as tomato genetics, functions and hormonal interactions (CAMPOS et al., 2010), arbuscular mycorrhizal symbiosis (PARK et al., 2007), genomics of Solanaceae (AOKI et al., 2010). Including on the use and characterization of several natural mutants discovered in tomato (BAUCHET; CAUSSE, 2012).

The use of mutants has been shown to be an essential resource in research related to plant physiology and genetics that aim to understand the multiple mechanisms of action of hormones during the various stages of development of plants (FUJINO et al., 1988; KISSOUDIS et al., 2017).

The MT, which is a mutant tomato cultivar that was initially produced for ornamental purposes (MORA-ROMERO et al., 2015), presents several characteristics that make it an excellent model plant for basic and applied research, such as small size, measuring about 10-20 cm in height , short life cycle, with 70 to 90 days, from sowing to fruit maturity (FLORES et al., 2016) and relatively small genome (TOMATO GENOME CONSORTIUM, 2012).

The MT phenotype occurs by at least three mutations: *self-pruning* (producing a specific phenotype), *dwarf* (reducing the internode length and producing smaller, rough and dark green leaves) and *miniature* (probably associated with gibberellin signaling) (MARTÍ et al., 2015).

Mutant individuals, such as Micro-Tom, have been used as tools in biological research, making possible studies related to the functions of genes and compounds that participate in specific steps in the metabolic behavior of plants (CAMPOS et al., 2009). In this sense, MT acts as an important instrument of support in research aimed at understanding the applicability of steroidal compounds during plant development (CAMPOS et al., 2010).

1.1.2 Brassinosteroids: promoter of growth and development

BRs constitute a class of approximately 70 polyhydroxy steroid derivatives which appear to be distributed throughout the plant kingdom (CLOUSE, 2011). The identification of endogenous steroid compounds of plants resulted from the effort of almost 30 years of research with the aim of identifying new growth-favoring substances present in pollen extracts from different plant species (FARIDUDDIN et al., 2013).

The first reports of BRs were identified in a study using Brassica napus pollen extract, being the most active growth promoter discovered, showing increases in stem elongation and cell division in bean internodes (OKLESTKOVA et al., 2015). With the results of this study by Mitchell et al. (1970) it was prematurely concluded that BRs are specific translocable organic compounds isolated from a plant that allowed a measurable growth control when applied in minimal amounts in another plant (CLOUSE, 2011).

One of the most biologically active forms of BRs of natural occurrence is the brassinolide (JOO et al., 2015; AZHAR et al., 2017). Classes and Sausse (1998) affirmed that campesterol was predicted as the plant steroidal progenitor of the brassinolide as a function of its lateral chain structure and also that the brassinolide was synthesized from campesterol suggested by relative biological activities, co-occurrence and molecular structure of the intermediates inteasterone, tifasterol and castasterone.

With these facts, numerous studies have been carried out, consequently the BRs have become known substances for being involved in processes related mainly to the

promotion of growth of vegetative organs through the combined effect on expansion and division of cells (GUDESBLAT; RUSSINOVA, 2011).

Oh et al (2012) asserted that BRs when exogenously applied, at nanomolar to micromolar levels, presents a broad spectrum of physiological effect. In aim to verify the possibility of BRs acting to promote the regulation of a wide range of biological responses, several studies with a significant focus on agriculture are being carried out (DIVI; KRISHNA, 2009).

Zhiponova et al. (2013) with a study carried out using the Arabidopsis BR-deficient mutant *constitutive photomorphogenesis and dwarfism* to examine the role of BR in leaf growth, reported that this compound is essential for proliferation, expansion and cell division and that the balance between proliferation and differentiation in a temporal way depends on BR levels.

For Pereira-Netto et al. (2006), it is possible to affirm that BR is related to the shooting growth, considering that in a study with grafted apple plants it was observed that the brassinolide differentially affected elongation and the formation of main and primary lateral shoots, consequently reduced apical domain.

Another example of BR research was produced by Wei and Li (2016) which stated that recent studies have deepen the understanding of the behave of this steroid in root growth and development, since BRs regulate root meristem size and root development lateral, and still function in a cell-type-specific manner during root growth and development.

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Page title

Brassinosteroids positively modulate growth: Physiological, biochemical and anatomical evidences using two tomato genotypes contrasting to dwarfism

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Author contribution statement
AKS Lobato was advisor of this project, planning all phases of this research. CF Maia conducted the experiment in the greenhouse and performed physiological, biochemical and morphological determinations, while BRS Silva measured anatomical parameters.

Brassinosteroids positively modulate growth: Physiological, biochemical and anatomical evidences using two tomato genotypes contrasting to dwarfism

Abstract

Growth and development are vital processes in the life cycles of plants. Brassinosteroids (BRs) are steroids that when exogenously applied can regulate several biological responses. The aim of this research was to investigate the possible interferences caused by the exogenous application of BR on growth and metabolism using two genotypes of the *DWARF* gene, MT-*d* and MT-*D*, that are BR-deficient and BR-efficient, respectively. The experiment had four treatments with two genotypes (BR-efficient and BR-deficient) and two levels of brassinosteroids (0 and 100 nM BR, described as – BR and + BR, respectively). This study revealed that the exogenous application of BR promoted improvement in growth, inducing increases in all variables. The positive effects on gas exchange and chlorophyll fluorescence confirm the benefits of this steroid on the photosynthetic apparatus. The changes in the anatomical characteristics of the leaf are related to the contribution of BR on the influx and consequent fixation of CO_2. In addition, modifications related to root anatomy occurred as a result of the BR action with the purpose of increase the root protection and absorption of water and nutrients. Increases in photosynthetic pigments suggest that the role of BR is linked with chlorophyll biosynthesis and the maintenance of chloroplast integrity, resulting from associations with the increases found in the activities of antioxidant enzymes that modulate the accumulation of reactive oxygen species.

Keywords Chlorophyll fluorescence ● *DWARF* gene ● Gas exchange ● Leaf anatomy ● *Solanum lycopersicum* ● 24-epibrassinolide

Abbreviations

APX	Ascorbate peroxidase
BR	Brassinosteroid
CAR	Carotenoids
CAT	Catalase
Chl *a*	Chlorophyll a
Chl *b*	Chlorophyll b
C_i	Intercellular CO_2 concentration
CO_2	Carbon dioxide
E	Transpiration rate
EBR	24-epibrassinolide
EDS	Equatorial diameter of the stomata
EL	Electrolyte leakage
ETAb	Epidermis thickness from abaxial side
ETAd	Epidermis thickness from adaxial side
ETR	Electron transport rate
ETR/P_N	Ratio between the apparent electron transport rate and net photosynthetic rate
EXC	Relative energy excess at the PSII level
F_o	Minimal fluorescence yield of the dark-adapted state
F_m	Maximal fluorescence yield of the dark-adapted state
F_v	Variable fluorescence
F_v/F_m	Maximal quantum yield of PSII photochemistry
g_s	Stomatal conductance
H_2O_2	Hydrogen peroxide
LDM	Leaf dry matter
MDA	Malondialdehyde
MT	Micro-Tom
NPQ	Nonphotochemical quenching
O_2^-	Superoxide
PDS	Polar diameter of the stomata
P_N	Net photosynthetic rate
P_N/C_i	Instantaneous carboxylation efficiency
POX	Peroxidase
PPT	Palisade parenchyma thickness
PSII	Photosystem II
q_P	Photochemical quenching
RCD	Root cortex diameter
RET	Root epidermis thickness
RDM	Root dry matter
RMD	Root metaxylem diameter

ROS	Reactive oxygen species
RuBisCO	Ribulose-1,5-bisphosphate carboxylase/oxygenase
RXT	Root exodermis thickness
SD	Stomatal density
SF	Stomatal functionality
SI	Stomatal index
SPT	Spongy parenchyma thickness
SDM	Stem dry matter
SOD	Superoxide dismutase
TDM	Total dry matter
Total Chl	Total chlorophyll
VCD	Vascular cylinder diameter
WUE	Water-use efficiency
Φ_{PSII}	Effective quantum yield of PSII photochemistry

Introduction

The tomato is considered an excellent model for basic and applied research in agriculture (Suresh et al. 2014) due to its adaptability to all climatic conditions (Gerszberg and Hnatuszko-Konka 2017), relatively short life cycle, high capacity for seed production, easy control of pollination and ability to regenerate plants by different explants (Bai and Lindhout 2007; Gerszberg et al. 2015). In this context, this species has been used in research related to the growth and development of plants (Carvalho et al. 2011), fruit ripening (Li et al. 2017), tolerance to water deficiency (Shi et al. 2014), nutrient uptake and transport, (Rady and Rehman 2016) and disease resistance (Ercolano et al. 2012).

Growth and development are vital processes in the life cycles of plants (Krouk et al. 2011), both of which use signalling routes that are frequently modified due to environment conditions such as water, light, CO_2, temperature, and nutrients (Smeekens et al. 2010; Kocsy et al. 2013). Several substances can interfere with the process of growth regulation in plants, as described in the literature (Zhang et al. 2014), including brassinosteroids (BRs) that when exogenously applied can enhance plant growth and improve metabolism (Alabadí and Blázquez 2009; Lima and Lobato 2017).

The BRs are a group of steroid compounds that when applied exogenously in nanomolar concentrations can regulate various biological responses (Divi and Krishna 2009). The 24-epibrassinolide (EBR) is one of the BRs most bioactive forms occurring naturally (Joo et al. 2015; Azhar et al. 2017), presenting a broad spectrum of action on metabolism (Oh et al. 2012) such as proliferation, expansion and cell division (Zhiponova et al. 2013), root development (Wei and Li 2016) and shooting growth (Pereira-Netto et al. 2009).

Singh and Savaldi-Goldstein (2015) reported that BR action intrinsically regulates the growth and development of aerial and subterranean organs, being confirmed by the dwarf phenotype in mutants either deficient in or insensitive to BR. In the BR-deficient mutant, one of two types of mutants related to dwarfism, the gene of BR biosynthesis is impaired, causing deficiency; however, this can be reversed by the exogenous application of this hormone. In the BR-insensitive mutant, the BR receptor gene is affected, inducing insensitivity that cannot be recovered by the BR supply (Noguchi et al. 1999; Salas Fernandez et al. 2009; Nie et al. 2017).

The Micro-Tom (MT) cultivar has several favourable characteristics for biological assays, such as a small size (10-20 cm in height), a short life cycle (70 to 90 days), and a broad genetic background that is available (Flores et al. 2016), including the BR-deficient mutant that is used to create dwarf tomatoes (Marti et al. 2006). This genotype can carry the *d* or *D* alleles linked to the dwarf gene, generating dwarf or normal phenotypes, respectively (Carvalho et al. 2011).

We hypothesize that the utilization of genotypes with different levels of biosynthesis to BR must explain the roles of this steroid in relation to development and metabolic behaviour (Verhoef et al. 2013). The aim of this research was to investigate the possible interferences caused by the exogenous application of BR on growth and metabolism using two contrasting genotypes of the *DWARF* gene (MT-*d* and MT-*D*) that are BR-deficient and BR-efficient, respectively.

Materials and Methods

Location and growth conditions

The experiment was performed at the Campus of Paragominas of the Universidade Federal Rural da Amazônia, Paragominas, Brazil (2°55′ S, 47°34′ W). The study was conducted in a greenhouse with controlled temperature and humidity. The minimum, maximum, and median temperatures were 21 °C, 30 °C, and 25.5 °C, respectively. The relative humidity during the experimental period varied between 60% to 80%.

Plants, containers and acclimation

Solanum lycopersicum L. cv. Micro-Tom (MT), being the MT cultivar used to assemble a tomato collection into a unique background, was used in this study (Carvalho et al. 2011). Seeds of two genotypes linked to the *DWARF* gene (MT-*D* and MT-*d*, described as BR-efficient and BR-deficient, respectively) (Marti et al. 2006) were used. These materials were obtained from the Laboratory of Hormonal Control of Plant Development of the Universidade de São Paulo (USP/Brazil). Seeds were germinated using Plantmax™ substrate and transplanted on the 13th day into 1 L pots (0.12 m in height and 0.11 m in diameter) filled with a mixture of substrate (Plantmax™), NPK (4 g L^{-1}), and dolomitic limestone (8 g L^{-1}).

Experimental design

The experiment was randomized with four treatments, including two genotypes (BR-efficient and BR-deficient) and two levels of brassinosteroid applications (0 and 100 nM BR, described as – BR and + BR, respectively). Five replicates for each one of the four treatments were conducted yielding a total of 20 experimental units used in the experiment with one plant in each unit.

Plant conduction and treatments with BR

For the BR treatment, 15-day-old plants were sprayed with 24-epibrassinolide (EBR) or Milli-Q water (containing a proportion of ethanol that was equal to that used to prepare the EBR solution) at 5-day intervals until day 35. The 100 nM EBR (Sigma-Aldrich, USA) solutions were prepared by dissolving the solute in ethanol followed by dilution with Milli-Q water [ethanol: water (v/v) = 1:10,000] (Ahammed et al. 2013b). On day 40 of the experiment, the physiological and morphological parameters were measured for all plants, and leaf tissues were harvested for biochemical and anatomical analyses.

Measurement of chlorophyll fluorescence

The minimal fluorescence yield of the dark-adapted state (F_0), maximal fluorescence yield of the dark-adapted state (F_m), variable fluorescence (F_v), maximal quantum yield of PSII photochemistry (F_v/F_m), effective quantum yield of PSII photochemistry (Φ_{PSII}), photochemical quenching coefficient (q_P), nonphotochemical quenching (NPQ), electron transport rate (ETR), relative energy excess at the PSII level (EXC), and ratio between the electron transport rate and net photosynthetic rate (ETR/P_N) were determined using a modulated chlorophyll fluorometer (model OS5p; Opti-Sciences). The chlorophyll fluorescence was measured in fully expanded leaves under light. Preliminary tests determined that the acropetal third of leaves in the middle third of the plant and that adapted to the dark for 30 min yielded

the greatest F_v/F_m ratio. Therefore, this part of the plant was used for measurements. The intensity and duration of the saturation light pulse were 7,500 μmol $m^{-2}.s^{-1}$ and 0.7 s, respectively.

Evaluation of gas exchange

The net photosynthetic rate (P_N), transpiration rate (E), stomatal conductance (g_s), and intercellular CO_2 concentration (C_i) were evaluated using an infrared gas analyser (model LCPro$^+$; ADC BioScientific). These parameters were measured at the adaxial surface of fully expanded leaves that were collected from the middle region of the plant. The water-use efficiency (WUE) was estimated according to Ma et al. (2004) and the instantaneous carboxylation efficiency (P_N/C_i) was calculated using the formula described by Aragão et al. (2012). Gas exchange was evaluated in all plants under a constant CO_2 concentration (360 μmol mol^{-1} CO_2), photosynthetically active radiation (800 μmol photons m^{-2} s^{-1}), air-flow rate (300 μmol s^{-1}), and temperature (28 ºC) in the test chamber between 10:00 and 12:00 h.

Measurements of anatomical parameters

Samples were collected from the middle region of the leaf limb of fully expanded leaves of the third node and roots 5 cm from the root apex. Subsequently, all collected botanical material was fixed in FAA 70 for 24 hours and dehydrated in ethanol and butanol for inclusion in histological paraffin (Johansen 1940). Transverse sections with a thickness of 12 μm were obtained with a rotating microtome (model Leica RM 2245, Leica Biosystems), stained with Safranin and Astra Blue (Gerlach 1977), and mounted on synthetic resin (Merck). For stomatal characterization, the epidermal impression method was used according to Segatto et al. (2004). The slides were observed and photomicrographed under an optical microscope (Motic BA 310, Motic Group Co. LTD.) coupled to a digital camera (Motic 2500, Motic Group Co., LTD.). The images were analysed with Moticplus 2.0 previously calibrated with a micrometre slide of the manufacturer. The anatomical parameters evaluated were polar diameter of the stomata (PDS), equatorial diameter of the stomata (EDS), epidermis thickness from adaxial leaf side (ETAd), epidermis thickness from abaxial leaf side (ETAb), Palisade parenchyma thickness (PPT), spongy parenchyma thickness (SPT), and the ratio PPT/SPT. In both leaf faces, the stomatal density (SD) was calculated as the number of stomata per unit area and the stomatal functionality (SF) as the ratio PDS/EDS according to Castro et al. (2009). The stomatal index (SI %) was calculated as the percentage of stomata in relation to total epidermal cells by area. In root samples, the root epidermis thickness (RET), root exodermis thickness (RXT), root cortex diameter (RCD), vascular cylinder diameter (VCD) and root metaxylem diameter (RMD) were measured.

Extraction of antioxidant enzymes, superoxide and soluble proteins

Antioxidant enzymes (SOD, CAT, APX, and POX), superoxide, and soluble proteins were extracted from leaf tissues according to the method of Badawi et al. (2004). The extraction mixture was prepared by homogenizing 500 mg of fresh plant material in 5 ml of extraction buffer, which consisted of 50 mM phosphate buffer (pH 7.6), 1.0 mM ascorbate, and 1.0 mM EDTA. Samples were centrifuged at 14,000 × *g* for 4 min at 3 °C, and the supernatant was collected. Quantification of the total soluble proteins was

performed using the method described by Bradford (1976). Absorbance was measured at 595 nm, using bovine albumin as a standard.

Superoxide dismutase assay

For the SOD assay (EC 1.15.1.1), 2.8 ml of a reaction mixture containing 50 mM phosphate buffer (pH 7.6), 0.1 mM EDTA, 13 mM methionine (pH 7.6), 75 µM NBT, and 4 µM riboflavin was mixed with 0.2 ml of supernatant. The absorbance was then measured at 560 nm (Giannopolitis and Ries 1977). One SOD unit was defined as the amount of enzyme required to inhibit 50% of the NBT photoreduction. The SOD activity was expressed in unit mg^{-1} protein.

Catalase assay

For the CAT assay (EC 1.11.1.6), 0.2 ml of supernatant and 1.8 ml of a reaction mixture containing 50 mM phosphate buffer (pH 7.0) and 12.5 mM hydrogen peroxide were mixed, and the absorbance was measured at 240 nm (Havir and McHale 1987).The CAT activity was expressed in µmol H_2O_2 mg^{-1} protein min^{-1}.

Ascorbate peroxidase assay

For the APX assay (EC 1.11.1.11), 1.8 ml of a reaction mixture containing 50 mM phosphate buffer (pH 7.0), 0.5 mM ascorbate, 0.1 mM EDTA, and 1.0 mM hydrogen peroxide was mixed with 0.2 ml of supernatant and the absorbance was measured at 290 nm (Nakano and Asada 1981). The APX activity was expressed in µmol $AsAmg^{-1}$ protein min^{-1}.

Peroxidase assay

For the POX assay (EC 1.11.1.7), 1.78 ml of a reaction mixture containing 50 mM phosphate buffer (pH 7.0) and 0.05% guaiacol was mixed with 0.2 ml of supernatant, followed by the addition of 20 µL of 10 mM hydrogen peroxide. The absorbance was then measured at 470 nm (Cakmak and Marschner 1992). The POX activity was expressed in µmol tetraguaiacol mg^{-1} protein min^{-1}.

Determination of superoxide concentration

For the determination of O_2^-, 1 ml of extract was incubated with 30 mM phosphate buffer [pH 7.6] and 0.51 mM hydroxylamine hydrochloride for 20 min at 25 °C. Seventeen mM sulphanilamide and 7 mM α-naphthylamine were then added to the incubation mixture for 20 min at 25 °C. After the reaction, ethyl ether was added in the identical volume and centrifuged at 3,000 × *g* for 5 min. The absorbance was measured at 530 nm (Elstner and Heupel 1976).

Extraction of nonenzymatic compounds

Nonenzymatic compounds (H_2O_2 and MDA) were extracted as described by Wu et al. (2006). Briefly, a mixture designed to extract H_2O_2 and MDA was prepared by homogenizing 500 mg of fresh leaf material in 5 mL of 5% (w/v) trichloroacetic acid. Samples were then centrifuged at 15,000 × *g* for 15 min at 3 °C to collect the supernatant.

Determination of hydrogen peroxide concentration

To measure H_2O_2, 200 µL of supernatant and 1800 µL of reaction mixture (2.5 mM potassium phosphate buffer [pH 7.0] and 500 mM potassium iodide) were mixed and the absorbance was measured at 390 nm (Velikova et al. 2000).

Quantification of malondialdehyde concentration

MDA was determined by mixing 500 µL of supernatant with 1 mL of the reaction mixture, which contained 0.5% (w/v) thiobarbituric acid in 20% trichloroacetic acid. The mixture was incubated in boiling water at 95 °C for 20 min with the reaction terminated by placing the reaction container in an ice bath. The samples were centrifuged at 10,000 × *g* for 10 min and the absorbance was measured at 532 nm. The nonspecific absorption at 600 nm was subtracted from the absorbance data. The amount of MDA–TBA complex (red pigment) was calculated based on the method of Cakmak and Horst (1991), with minor modifications and using an extinction coefficient of 155 mM^{-1} cm^{-1}.

Determination of electrolyte leakage

Electrolyte leakage was measured according to the method of Gong et al. (1998) with minor modifications. Fresh tissue (200 mg) was cut into pieces 1 cm in length and placed in containers with 8 mL of distilled deionized water. The containers were incubated in a water bath at 40 °C for 30 min and the initial electrical conductivity of the medium (EC_1) was measured. The samples were then boiled at 95 °C for 20 min to release the electrolytes. After cooling, the final electrical conductivity (EC_2) was measured Gong et al. (1998). The percentage of electrolyte leakage was calculated using the formula EL (%) = (EC_1/EC_2) × 100.

Determination of photosynthetic pigments

Determinations of the chlorophyll and carotenoid levels were performed with 40 mg of leaf tissue. The samples were homogenized in the dark with 8 mL of 90% methanol (Sigma-Aldrich™). The homogenate was centrifuged at 6,000 × *g* for 10 min at 5 ºC. The supernatant was removed and the chlorophyll *a* (Chl*a*) and *b* (Chl*b*), carotenoid (Car), and total chlorophyll (total Chl) levels were quantified using a spectrophotometer (model UV-M51; Bel Photonics) according to the methodology of Lichtenthaler and Buschmann (2001).

Measurements of morphological parameters

The growth of roots, stems, and leaves was measured as constant dry weights (g) after drying in a forced-air ventilation oven at 65 °C.

Data analysis

The data were subjected to analysis of variance, and significant differences between the means were determined by the Scott–Knott test at a probability level of 5% (Steel et al. 2006). Standard deviations were calculated for each treatment. The statistical analyses were performed with Assistat software.

Results

Interferences on chlorophyll fluorescence

BR-efficient plants presented higher values of F_m and F_v (Fig. 1). BR-deficient plants with BR expressed a significant (P<0.05) increase in F_v/F_m compared to plants of the same genotype without BR. F_0 was not significantly altered. For q_P, BR-deficient plants + BR had an increase of 30% in relation to BR-deficient without BR (Table 1). In NPQ, BR-deficient plants with BR presented a reduction of 13%. In relation to ETR/P_N, both genotypes treated with BR suffered significant decreases in comparison to plants of the same genotypes without BR. For Φ_{PSII}, ETR, and EXC, the treatments resulted in non-significant changes.

Benefits promoted by the BR on gas exchange

BR-efficient plants with BR expressed increases of 19%, 16%, 40% and 37% in the variables P_N, E, g_s, and P_N/C_i, respectively, compared with plants of the same genotype without BR (Table 2). BR-deficient + BR presented increases in P_N, g_s, WUE, and P_N/C_i of 25%, 11%, 15%, and 48%, respectively, when compared BR-deficient – BR. Plants of both genotypes sprayed with BR presented decreases in C_i.

BR increased the stomatal density

In both the adaxial and abaxial sides of the leaf, the treatment with BR resulted in increases (P<0.05) in SD, and SI and reductions in PDS and EDS (Table 3). On the abaxial side, BR-deficient plants with BR had increases of 9%, 5%, and 10% in SD, SF, and SI, respectively, compared to BR-deficient plants without BR. In relation to PDS and EDS (abaxial side), plants sprayed with BR suffered reductions of 14% and 17%, respectively.

BR-efficient plants presented higher values in relation to leaf anatomy

For ETAd, ETAb, and PPT, BR-deficient plants with BR expressed increases of 10%, 33%, and 5%, respectively, when compared to plants of the same genotype without BR (Table 4). For SPT, the treatments did not indicate significant alterations.

BR improved the root structure

BR-deficient plants had lower values of root characteristics (Table 5). For RET, RXT, RCD, VCD, and RMD, the BR-deficient + BR obtained increases of 9%, 14%, 12%, 7%, and 17%, respectively, when compared with BR-deficient plants without BR.

Modulation promoted by BR linked to antioxidant system

The treatment with BR improved enzymatic activities (SOD, CAT, and APX). The activities of CAT and APX were increased by 17% and 19%, respectively, in BR-deficient plants with BR when compared to plants of the same genotype without BR (Fig. 2). On the other hand, BR-efficient plants sprayed with BR had a non-significant increase in CAT, but in APX a significant increase of 53% was observed in relation to BR-efficient plants without BR. The activity of SOD increased in plants under BR spraying. The activity of POX did not suffer significant interference.

BR attenuated the detrimental effects of ROS

Plants sprayed with BR exhibited reductions in oxidant compounds. BR-efficient plants with BR presented reductions of 20% in O_2^- (Fig. 3). For H_2O_2, BR-deficient plants with BR had a significant decrease by 20% compared to plants of the same genotype without BR. In MDA, the treatments did not have significant modifications. BR application induced decreases in EL ($P \geq 0.05$).

Photosynthetic pigments presented increases after treatment with BR

The BR application maximized the levels of photosynthetic pigments in both genotypes. BR-deficient plants presented increases of 16%, 35%, 20%, and 67% in Chl *a*, Chl *b*, Total Chl, and Car, respectively, in comparison to BR-deficient plants without BR (Table 6). In BR-efficient plants + BR, increases of 16%, 29%, 19%, and 164% were detected for Chl *a*, Chl *b*, Total Chl, and Car, respectively. For the ratio Chl *a*/Chl *b*, BR-deficient and BR-efficient plants treated with BR expressed reductions of 14% and 10%, respectively. For the ratio of Total Chl/Car, BR-efficient and BR-deficient plants with BR showed decreases of 55% and 28%, respectively.

BR application caused maximization on plant growth

BR-efficient plants presented higher values to growth. In addition, BR-efficient plants + BR had increases of 146%, 123%, 102%, and 122% in LDM, RDM, SDM, and TDM, respectively, compared to plants of the same genotype without BR (Fig. 4). BR-deficient plants + BR expressed increases of 120%, 469%, 219%, and 203% for variables LDM, RDM, SDM, and TDM, respectively.

Discussion

Plants sprayed with BR showed increased levels of F_v and F_v/F_m. The increase in F_v in BR-efficient plants + BR can be explained by the maximization of F_m. Plants treated with BR presented increases in F_v/F_m, showing improvement in the conversion efficiency and capture of light energy at the PSII reaction centre (Qiu et al. 2013). Li et al. (2015) confirmed in a study with *Capsicum annuum* under cold stress that the BR spray attenuated the damages on F_v/F_m, improving the activity at the PSII.

The BR application promoted benefits in Φ_{PSII}, q_P, and ETR. The increase obtained in q_p after the treatment with BR stimulated the separation of the charge of electrons in the reaction centres, promoting increases in Φ_{PSII} and ETR. These results confirm the improvements related to chlorophyll fluorescence such as an increase in the photochemical dissipation and efficiency in the capture of energy due to reaction centres remaining open (Guo et al. 2006; Zhang et al. 2013; Thussagunpanit et al. 2015a). A study conducted by Dobrikova et al. (2014) with *Pisum sativum* described that the application of BR maximized Φ_{PSII}, q_p, and ETR, increasing the capacity of the photosynthetic apparatus to maintain oxidized Q_A and the transport of electrons through the PSII.

The treatment with BR promoted decreases in NPQ, EXC, and ETR/P_N. In regards to NPQ, the reduction observed in plants sprayed with BR induced a reduction of the heat dissipation and maintenance of the transfer of excitation energy from the antenna system to the PSII reaction centres (Calatayud and Barreno 2004; Thussagunpanit et al. 2015b). The decrease in ETR/P_N in plants under application of BR is

intrinsically related to the decrease of EXC, suggesting that the excess of electrons were used less for secondary processes, such as photorespiration and were potentially available for the primary processes, such as $NADP^+$ reduction during CO_2 fixation (Silva et al. 2012). Ogweno et al. (2008) verified that the BR application in *Lycopersicon esculentum* under high temperature stress significantly reduced NPQ, promoting the protection of PSII against possible damages due to the excess of excitation, thereby improving the integrity of the thylakoid membranes.

Positive effects on P_N, E, and g_s were detected in plants treated with BR. These increases are related to the benefits promoted by the BR on the photosynthetic apparatus, as demonstrated in the increments reached in q_P in this study. The increment found in P_N/C_i can be explained by the action of BR increasing the RuBisCO activity (reduction in C_i) and the fixation of CO_2 (increase in P_N) during photosynthesis (Farooq et al. 2009; Xia et al. 2009; Shu et al. 2016). Under cold stress, Hu et al.(2010) observed that the application of BR in *Cucumis sativus* promoted positive effects in P_N and g_s, alleviating the effects caused by stress in these variables.

Increases detected in SD, SF, and SI revealed that the BR improved the stomatal performance, corroborated by the increase in g_s obtained in this study. Concomitantly, this fact demonstrates that the BR can maximize the gas flow, increasing the opportunity of CO_2 absorption. SD and SI are variables intrinsically connected with quantity, dimension, and functionality of the stomata, respectively (Franks and Beerling 2009). The reductions in PDS and EDS promoted by the action of BR suggests that the stomata have a more elliptical form, a characteristic attributed to functional stomata considered normal (Sha Valli Khan et al. 2003). Asmar et al. (2013) working with plantlets of *Musa acuminata* under different silicon sources reported reduction in PD and increase in ED, which correspond with PDS and EDS described in this study, respectively.

Plants treated with BR had beneficial effects on leaf anatomy (ETAd, ETAb, PPT. and SPT); the increases in PPT and SPT are associated with maximization in P_N and P_N/C_i, as was verified in the study. PPT and SPT are tissues that contribute to the influx and consequent fixation of CO_2; specifically, PPT is a tissue frequently composed of a higher amount of chloroplasts, the organelles responsible for the photosynthetic process, while SPT is related with an intense formation of intercellular spaces involved with gas exchanges (Sorin et al. 2015). In addition, increases in ETAd and ETAb in plants sprayed with BR can be explained by the higher values in E and WUE as the epidermis is a coating tissue, clearly contributing to the water utilization and avoidance of an excessive loss of water during the transpiration process (Javelle et al. 2011). Pereira et al. (2016) working with young *Schinus molle* exposed to the Cd found that P_N exercises influence on PPT and SPT.

BR had positive effects on root characteristics (RET, RXT, RCD, VCD, and RMD). The increases in RET, RXT, and RCD suggest that plants treated with BR presented a higher protection to this organ. The epidermis, exodermis, and cortex are tissues that act on the protection and selectivity of the root, and increases in the thicknesses of these tissues will work as barrier, protecting the root against abiotic and biotic stresses. In relation to VCD and RMD, the BR promoted increases in the diameters of the vascular cylinder and the metaxylem, suggesting that the higher thicknesses of these tissues can facilitate the flux of water and nutrients via simplastic (Hameed et al. 2009; Meyer et al. 2011).

Plants sprayed with BR showed beneficial effects on SOD, CAT, and APX. Responses detected in this study suggest that these antioxidant enzymes are regulated by BR, contributing to the antioxidative mechanism due to the equilibrium between the formation and detoxification of reactive oxygen species (Liu et al. 2009). The BR application promoted increases in SOD, CAT, and APX activities in a study with *Lycopersicon esculentum* under water deficient conditions (Yuan et al. 2010) and in *Raphanus sativus* under stress caused by Zn (Ramakrishna and Rao 2015).

The leaf pulverization with BR promoted reductions in O_2^- and H_2O_2, indicating interferences of the BR action on antioxidant enzymes previously reported in this study. CAT and APX enzymes act in the H_2O_2 dismutation, with consequent formation of H_2O and O_2, reducing the amounts of oxidant compounds (Gill and Tuteja 2010). Similar to our research, other studies found that the use of BR attenuated the detrimental effects of ROS due to increases in antioxidant enzyme activities in *Solanum lycopersicum* stressed by the phenanthrene-cadmium co-contaminations (Ahammed et al. 2013a) and under stress occasioned by polychlorinated biphenyls (Ahammed et al. 2013b).

Plants treated with BR presented increases in photosynthetic pigments (Chl *a*, Chl *b*, Total Chl, and Car). These increases indicate that BR has a double effect, improving both the integrity of chloroplasts and the transport of electrons. The chloroplast impermeability can be explained by lower MDA and EL levels which are indicators of cell damages (Genisel et al. 2013; Wu et al. 2015). With respect to the electron transport, the light-harvesting complex (LCH) is composed by Chl a, Chl b, and Car molecules, which is involved in the absorption and transfer of light energy to the reaction centres. Therefore, these changes in pigments exercise influence also on the transport of electrons, being detected in the increases to ETR in this study (Wang et al. 2015; Akhtar et al. 2015). Ahammed et al. (2012) studying *Solanum lycopersicum* identified that plants treated with BR and under stress induced by phenanthrene and pyrene showed increases in photosynthetic pigments.

The BR application caused increase in plant growth (LDM, RDM, SDM, and TDM). These responses are intrinsically associated with the increases obtained in q_P and P_N showed in this study, indicating the improvements promoted by the BR on chlorophyll fluorescence and the gas exchange. The increase of biomass in plants treated with this substance is related to the increase of the photosynthetic rates stimulated by the higher energy absorption and CO_2 fixation (Shahbaz et al. 2008). Zheng et al. (2016) evaluating *Lycopersicon esculentum* under saline stress demonstrated that application of BR increased in biomass of leaf, root, stem and total.

Conclusions

This research revealed that the exogenous application of BR promoted improvements in growth and development, inducing increases in LDM, RDM, SDM, and TDM. The positive effects on gas exchange and chlorophyll fluorescence confirm the benefits of this steroid on photosynthetic apparatus. The changes in the anatomical characteristics of the leaf are related to contribution of the BR on the influx and consequent fixation of CO_2. In addition, modifications related to root anatomy occurred as a result of the BR action with the purpose of increase the root protection and absorption of water and nutrients. Increases in photosynthetic pigments suggest that the role of BR is linked to the route of chlorophyll

biosynthesis and the maintenance of chloroplast integrity. This result is intrinsically associated with the increases found in the activities of antioxidant enzymes that modulate the accumulation of ROS.

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Figures

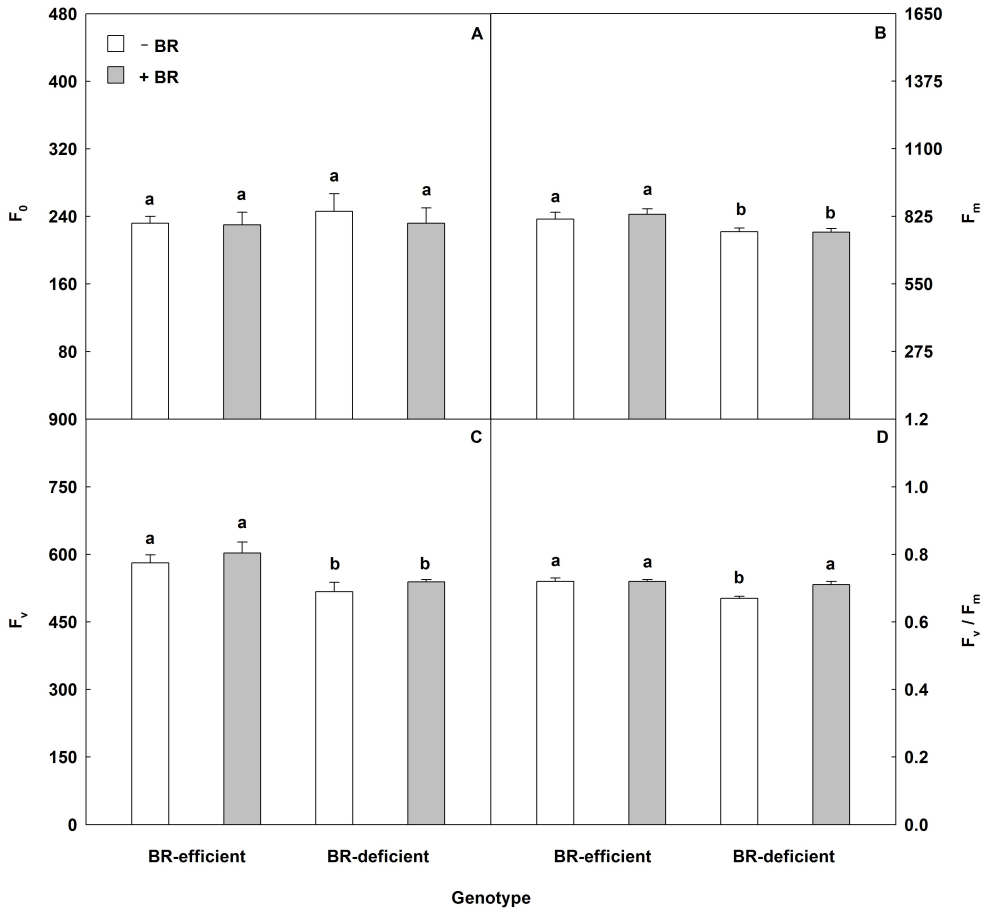

Fig. 1. Minimal fluorescence yield of the dark-adapted state (F_0), maximal fluorescence yield of the dark-adapted state (F_m), variable fluorescence (F_v), and maximal quantum yield of PSII photochemistry (F_v/F_m) in tomato genotypes sprayed with BR. Different letters indicate significant differences from the Scott-Knott test (P<0.05). Values correspond to means and standard deviations from five replicates.

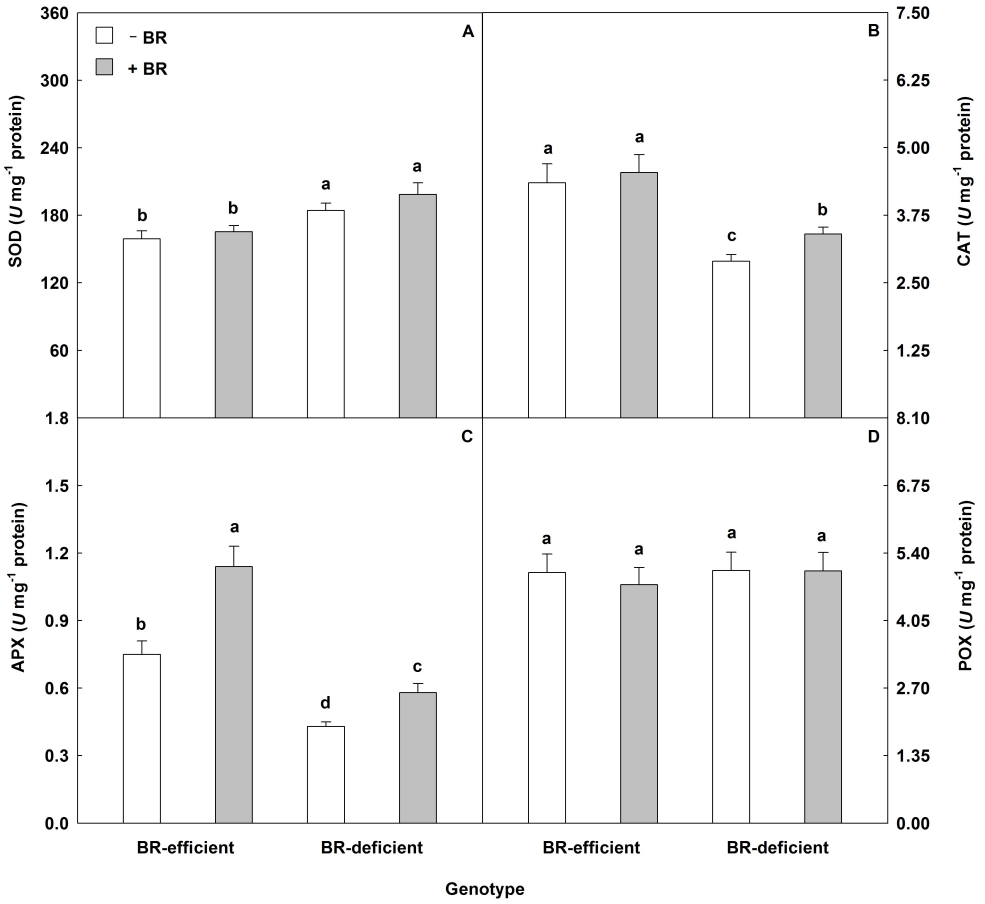

Fig. 2. Activities of superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), and peroxidase (POX) in tomato genotypes sprayed with BR. Different letters indicate significant differences from the Scott-Knott test (P<0.05). Values correspond to means and standard deviations from five replicates.

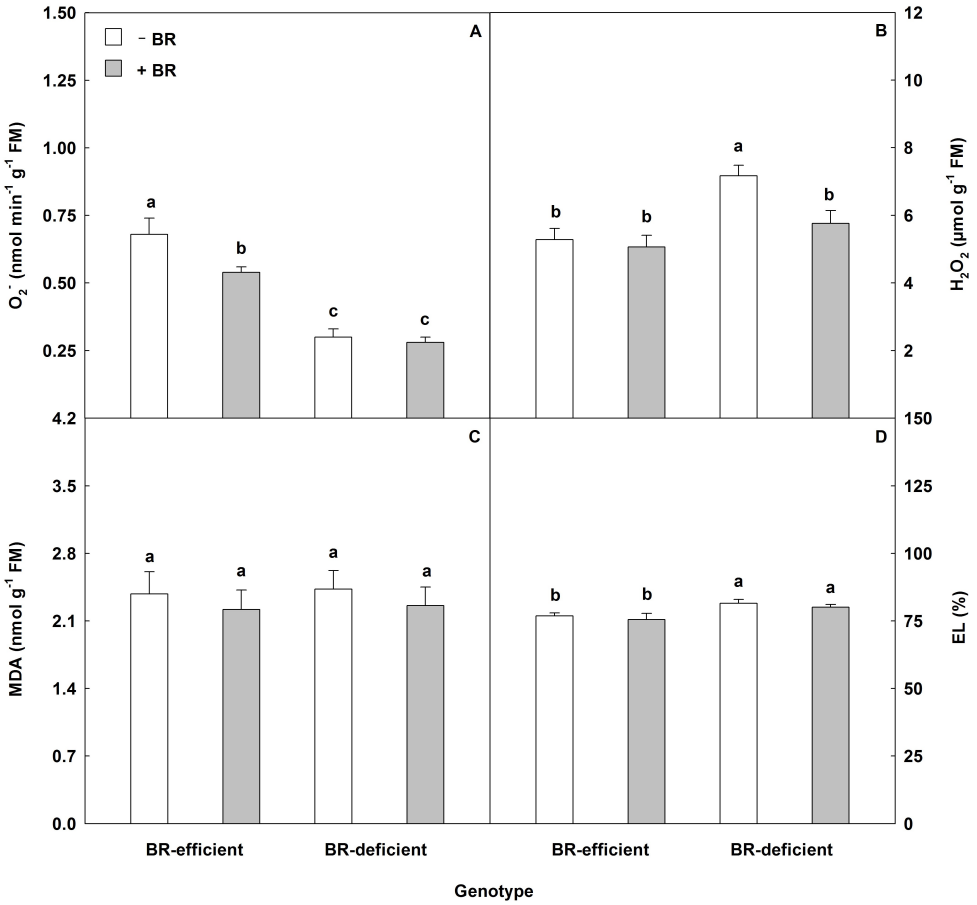

Fig. 3. Superoxide (O_2^-), hydrogen peroxide (H_2O_2), malondialdehyde (MDA), and electrolyte leakage (EL) in tomato genotypes sprayed with BR. Different letters indicate significant differences from the Scott-Knott test (P<0.05). Values correspond to means and standard deviations from five replicates.

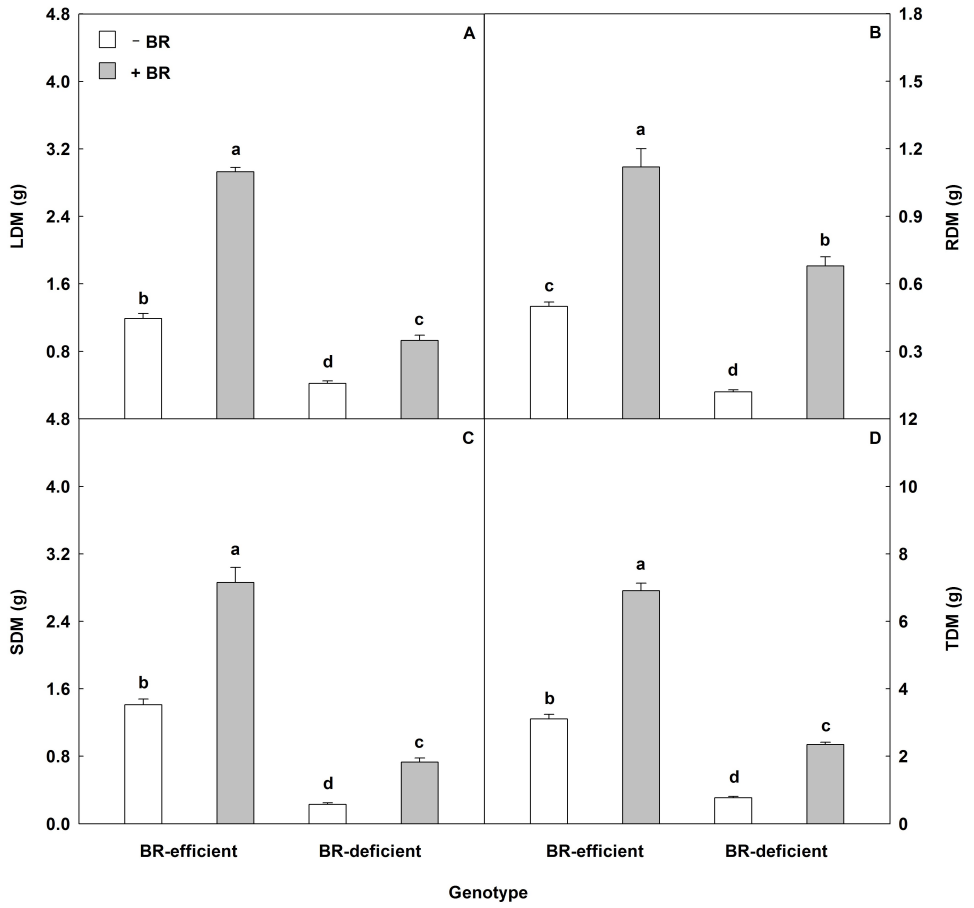

Fig. 4. Leaf dry matter (LDM), root dry matter (RDM), stem dry matter (SDM), and total dry matter (TDM) in tomato genotypes sprayed with BR. Different letters indicate significant differences from the Scott-Knott test (P<0.05). Values correspond to means and standard deviations from five replicates.

Tables

Table 1. Chlorophyll fluorescence in tomato genotypes sprayed with BR.

Genotype	Treatment	Φ_{PSII}	q_P	NPQ	ETR (µmol m^{-2} s^{-1})	EXC (µmol m^{-2} s^{-1})	ETR/P_N
BR-efficient	**- BR**	0.53 ± 0.03a	0.59 ± 0.03b	1.47 ± 0.11b	78.4 ± 3.8a	0.25 ± 0.02a	10.3 ± 0.5c
BR-efficient	**+ BR**	0.56 ± 0.05a	0.63 ± 0.02b	1.38 ± 0.09b	81.5 ± 6.8a	0.23 ± 0.02a	9.0 ± 0.4d
BR-deficient	**- BR**	0.51 ± 0.05a	0.51 ± 0.03c	1.72 ± 0.09a	75.5 ± 6.7a	0.24 ± 0.02a	14.3 ± 0.4a
BR-deficient	**+ BR**	0.55 ± 0.05a	0.67 ± 0.01a	1.49 ± 0.12b	81.2 ± 7.5a	0.21 ± 0.02a	12.5 ± 1.0b

Φ_{PSII} = Effective quantum yield of PSII photochemistry; q_P = Photochemical quenching coefficient; NPQ = Nonphotochemical quenching; ETR = Electron transport rate; EXC = Relative energy excess at the PSII level; ETR/P_N = Ratio between the electron transport rate and net photosynthetic rate. Columns with different letters indicate significant differences from the Scott-Knott test (P<0.05). Values correspond to means and standard deviations from five replicates.

Table 2. Gas exchange in tomato genotypes sprayed with BR.

Genotype	Treatment	P_N (µmol m^{-2} s^{-1})	E (mmol m^{-2} s^{-1})	g_s (mol m^{-2} s^{-1})	C_i (µmol mol^{-1})	WUE (µmol $mmol^{-1}$)	P_N/C_i (µmol m^{-2} $s^{-1}Pa^{-1}$)
BR-efficient	**- BR**	7.63 ± 0.42b	2.00 ± 0.08b	0.17 ± 0.008b	275 ± 10a	3.82 ± 0.10a	0.028 ± 0.002b
BR-efficient	**+ BR**	9.05 ± 0.56a	2.31 ± 0.14a	0.24 ± 0.016a	236 ± 14b	3.93 ± 0.21a	0.038 ± 0.001a
BR-deficient	**- BR**	5.27 ± 0.51d	1.61 ± 0.09c	0.15 ± 0.005c	294 ± 15a	3.28 ± 0.22b	0.018 ± 0.001c
BR-deficient	**+ BR**	6.59 ± 0.34c	1.74 ± 0.10c	0.17 ± 0.009b	256 ± 7b	3.78 ± 0.15a	0.026 ± 0.002b

P_N = Net photosynthetic rate; E = Transpiration rate; g_s = Stomatal conductance; C_i = Intercellular CO_2 concentration; WUE = Water-use efficiency; P_N/C_i = Carboxylation instantaneous efficiency. Columns with different letters indicate significant differences from the Scott-Knott test (P<0.05). Values correspond to means and standard deviations from five replicates.

Table 3. Stomatal characteristics in tomato genotypes sprayed with BR.

Genotype	Treatment	SD (stomata per mm^2)	PDS (µm)	EDS (µm)	SF	SI (%)
Adaxial side						
BR-efficient	**- BR**	160 ± 10a	18.2 ± 0.9c	22.8 ± 1.4c	0.80 ± 0.04a	17.6 ± 0.7a
BR-efficient	**+ BR**	164 ± 9a	17.7 ± 1.1c	22.1 ± 1.2c	0.80 ± 0.04a	18.2 ± 0.9a
BR-deficient	**- BR**	104 ± 9c	24.3 ± 0.6a	35.0 ± 1.1a	0.70 ± 0.03b	11.6 ± 0.6c
BR-deficient	**+ BR**	132 ± 8b	20.1 ± 0.7b	27.2 ± 1.2b	0.74 ± 0.01b	15.9 ± 0.4b
Abaxial side						
BR-efficient	**- BR**	268 ± 13a	17.9 ± 0.8c	20.8 ± 1.2c	0.87 ± 0.01a	29.7 ± 1.8b
BR-efficient	**+ BR**	271 ± 13a	17.6 ± 0.9c	20.3 ± 1.5c	0.87 ± 0.02a	31.3 ± 0.8a
BR-deficient	**- BR**	212 ± 7c	23.1 ± 1.1a	29.4 ± 1.9a	0.79 ± 0.03b	22.7 ± 0.8d
BR-deficient	**+ BR**	231 ± 9b	20.1 ± 0.7b	24.3 ± 1.1b	0.83 ± 0.01b	25.0 ± 0.9c

SD = Stomatal density; PDS = Polar diameter of the stomata; EDS = Equatorial diameter of the stomata; SF = Stomatal functionality; SI = Stomatal index. Columns with different letters indicate significant differences from the Scott-Knott test (P<0.05). Values correspond to means and standard deviations from five replicates.

Table 4. Leaf anatomy in tomato genotypes sprayed with BR.

Genotype	Treatment	ETAd (µm)	ETAb (µm)	PPT (µm)	SPT (µm)	Ratio PPT/SPT
BR-efficient	**- BR**	18.1 ± 0.5a	14.7 ± 0.6a	64.24 ± 1.9a	64.42 ± 5.2a	1.00 ± 0.04a
BR-efficient	**+ BR**	19.4 ± 1.1a	15.6 ± 0.9a	64.70 ± 2.4a	65.02 ± 4.0a	1.00 ± 0.05a
BR-deficient	**- BR**	15.0 ± 0.9b	9.6 ± 0.5c	51.04 ± 2.7b	58.60 ± 3.8a	0.87 ± 0.03b
BR-deficient	**+ BR**	16.6 ± 0.4b	12.7 ± 0.7b	53.78 ± 3.3b	63.54 ± 4.3a	0.85 ± 0.05b

ETAd = Epidermis thickness from adaxial leaf side; ETAb = Epidermis thickness from abaxial leaf side; PPT = Palisade parenchyma thickness; SPT = Spongy parenchyma thickness. Columns with different letters indicate significant differences from the Scott-Knott test ($P<0.05$). Values correspond to means and standard deviations from five replicates.

Table 5. Root anatomy in tomato genotypes sprayed with BR.

Genotype	Treatment	RET (µm)	RXT (µm)	RCD (µm)	VCD (µm)	RMD (µm)
BR-efficient	**- BR**	12.8 ± 0.8a	33.7 ± 2.2a	168 ± 5a	140 ± 5a	29.7 ± 1.2a
BR-efficient	**+ BR**	12.8 ± 0.8a	34.4 ± 2.2a	172 ± 9a	143 ± 4a	30.0 ± 1.4a
BR-deficient	**- BR**	10.1 ± 0.3c	26.9 ± 1.4c	143 ± 2b	128 ± 3b	22.3 ± 0.5c
BR-deficient	**+ BR**	11.0 ± 0.2b	30.7 ± 1.2b	160 ± 6a	137 ± 4a	26.0 ± 1.5b

RET = Root epidermis thickness; RXT = Root exodermis thickness; RCD = Root cortex diameter; VCD = Vascular cylinder diameter; RMD = Root metaxylem diameter. Columns with different letters indicate significant differences from the Scott-Knott test ($P<0.05$). Values correspond to means and standard deviations from five replicates.

Table 6. Photosynthetic pigments in tomato genotypes sprayed with BR.

Genotype	Treatment	Chl *a* (mg g^{-1} FM)	Chl *b* (mg g^{-1} FM)	Total Chl (mg g^{-1} FM)	Car (mg g^{-1} FM)	Ratio Chl *a*/Chl*b*	Ratio Total Chl/Car
BR-efficient	**- BR**	3.94 ± 0.45c	0.97 ± 0.09d	4.90 ± 0.35d	0.24 ± 0.02d	4.10 ± 0.11a	20.34 ± 1.99b
BR-efficient	**+ BR**	4.58 ± 0.28c	1.24 ± 0.06c	5.78 ± 0.12c	0.64 ± 0.02a	3.69 ± 0.10b	9.15 ± 0.81c
BR-deficient	**- BR**	7.61 ± 0.49b	1.92 ± 0.18b	9.53 ± 0.78b	0.34 ± 0.03c	3.98 ± 0.11a	27.86 ± 1.87a
BR-deficient	**+ BR**	8.83 ± 0.51a	2.59 ± 0.16a	11.42 ± 0.82a	0.57 ± 0.03b	3.42 ± 0.19b	20.03 ± 1.88b

Chl *a* = Chlorophyll *a*; Chl *b* = Chlorophyll *b*; Total Chl = Total chlorophyll; Car = Carotenoids. Columns with different letters indicate significant differences from the Scott-Knott test ($P<0.05$). Values correspond to means and standard deviations from five replicates.

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To whom it may concern

The paper "Brassinosteroids positively modulate growth: Physiological, biochemical and anatomical evidences using two tomato genotypes contrasting to dwarfism" by Camille Ferreira Maia ● Breno Ricardo Serrão da Silva ● AgustínZsögön ● Allan Klynger da Silva Lobatowas edited by Elsevier Language Editing Services.

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