UNIVERSIDADE FEDERAL DE ALFENAS

JOICE APARECIDA DE NOVAIS PORTUGAL

DERIVADOS DE TREALOSE NO SISTEMA ANTIOXIDANTE E FOTOSSINTÉTICO DE PLANTAS DE MILHO EXPOSTAS AO DÉFICIT HÍDRICO

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Orientador: Prof. Dr. Thiago Corrêa de Souza
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JOICE APARECIDA DE NOVAIS PORTUGAL

"Derivados da trealose no sistema antioxidante e fotossintético de plantas de milho expostas ao déficit hídrico"

A Banca julgadora, abaixo assinada, aprova a Dissertação apresentada como parte dos requisitos para a obtenção do título de Mestre em Ciências Ambientais pela Universidade Federal de Alfenas. Área de Concentração: Ciências Ambientais.

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RESUMO

Na agricultura um dos fatores limitantes do desenvolvimento das plantas e que afeta diretamente no resultado final da produção é a disponibilidade de água. Com as constantes mudanças climáticas, faz-se necessário a realização de estudos para a descoberta de novas tecnologias visando minimizar o estresse hídrico causado nas plantas, principalmente na fase de germinação e crescimento, floração e enchimento dos grãos. Este trabalho objetivou-se a analisar a aplicação de novos dissacarídeos derivados de trealose em plantas de milho verificando a capacidade de indução a tolerância à seca. O experimento foi conduzido em casa de vegetação, utilizando o milho híbrido BRS 1030 sensível ao déficit hídrico. O estresse hídrico foi aplicado quando as plantas atingiram o estádio vegetativo V6 e foi imposto por 12 dias, com aplicação foliar da mistura dos derivados (30mM) no primeiro e décimo dia de imposição do déficit hídrico. As análises da eficiência fotossintética da clorofila a (ETR_{max}, Ik e α), foram realizadas no primeiro e último dia de imposição do déficit hídrico e 12 horas após a reidratação, obtendo-se curvas rápidas de luz através de um fluorímetro. As coletas foliares para análise bioquímica foram realizadas no início e fim do déficit hídrico e 12 horas após a reidratação das plantas. Foram avaliados a peroxidação lipídica através dos teores de MDA e a atividade enzimática antioxidante SOD, APX, CAT e POD. Ao final do experimento a parte aérea e raízes foram coletadas para análises das concentrações de açúcares redutores, açúcares solúveis totais, amido, proteína, prolina e compostos fenólicos. Foi possível concluir que, a aplicação exógena da mistura dos derivados da trealose mostrou-se eficiente na mitigação de danos causados pelo déficit hídrico através da ativação das enzimas antioxidantes (SOD, APX e POD), além de promover o acúmulo de açúcares, prolina e compostos fenólicos e melhorando a eficiência fotossintética no híbrido de milho BRS 1030.

Palavras-chave: Milho. Secas. Fluorescência. Clorofila. Prolina. Estimulantes.

ABSTRACT

In agriculture one of the limiting factors of plant development that directly affects the final production result is water availability. With the constant climate changes, it is necessary to carry out studies to discover new technologies to minimize water stress caused on plants, especially in the germination and growth, flowering and fruit filling phase. This work aimed to analyze the application of new trehalose-derived disaccharides in maize plants by checking the drought tolerance induction capacity. The experiment was carried out in a greenhouse using BRS 1030 hybrid corn sensitive to water deficit. Water stress was applied when the plants reached vegetative stage V6 and was imposed for 12 days, with foliar application of the mixture of derivatives (30mM) on the first and tenth day of water deficit imposition. The photosynthetic efficiency analyzes of chlorophyll a (ETRmax, Ik and α) were performed on the first and last day of water deficit imposition and 12 hours after rehydration, obtaining fast light curves through a fluorimeter. Leaf collections for biochemical analysis were performed at the beginning and end of water deficit and 12 hours after plant rehydration. Lipid peroxidation through MDA contents and antioxidant enzymatic activity SOD, APX, CAT and POD were evaluated. At the end of the experiment the leaves and roots were collected to analyze the concentrations of reducing sugars, total soluble sugars, starch, protein, proline and phenolic compounds. It was concluded that the exogenous application of the trehalose derivatives mixture proved to be efficient in mitigating damage caused by water deficit through the activation of antioxidant enzymes (SOD, APX and POD), besides promoting the accumulation of sugars, proline and phenolic compounds and improving photosynthetic efficiency in the BRS 1030 maize hybrid.

Keywords: Zea mays L.. Drought. Fluorescence. Chlorophyll. Proline. Stimulants.

SUMÁRIO

1 INTRODUÇÃO

O milho (*Zea mays L.*) possui alto valor agregado a agricultura mundial e no território nacional ocupa o segundo lugar dentre os grãos mais produzidos atualmente (CONAB, 2018). As grandes variações climáticas afetam diretamente a produção de grãos e juntamente com a baixa disponibilidade de água e o aumento populacional impactam diretamente no resultado final das safras e na disponibilidade de recursos e bens de consumo (MCQUEEN, 2000; ZIPPER; QIU; KUCHARIK, 2016). Portanto a busca por alimentos com maior teor de nutrientes e plantas mais tolerantes são uma das vertentes de estudos na tentativa de balancear a oferta de recursos e o crescente aumento populacional, buscando soluções para a fome no mundo.

Neste contexto é primordial a promoção de estudos relacionados a tolerância à seca ou déficit hídrico, levando em consideração que este estresse abiótico é o que mais compromete os períodos de desenvolvimento e a morfologia das plantas, intensificando a atividade metabólica e gasto energético para a manutenção do desenvolvimento da planta (SARMENTO et al., 2019, ULLAH et al., 2018). A baixa disponibilidade de água no solo, juntamente com as altas temperaturas acionam mecanismos fisiológicos na tentativa de melhorar o controle da temperatura e transpiração, mas esta alteração fisiológica pode resultar em um desenvolvimento inferior ao esperado e consequentemente redução na produção final (RABÊLO et al., 2019).

Espécies mais tolerantes aos danos causados pelo déficit hídrico e com maior rendimento por área de plantio pode ser a resposta para o aumento da produção sem a necessidade do aumento da área utilizada para o plantio (WU et al., 2018). Em espécies vegetais mais tolerantes alguns mecanismos de defesas são observados, como a maior relação entre parte aérea e raiz, células com menor diâmetro, maior espessura da cutícula foliar e aumento na cerosidade, ativação do sistema de defesa antioxidante, alterações no tamanho e frequência estomática, ajuste osmótico, maior eficiência fotossintética entre outros (SOUZA et al., 2013; DE OLLAS; DODD, 2016).

Pesquisas relacionadas ao déficit hídrico ganham notoriedade no cenário nacional e internacional, abrangendo conhecimentos multidisciplinares. Para o desenvolvimento de genótipos tolerantes ao déficit hídrico faz-se necessário o uso de estudos fisiológicos e biotecnológicos. A aplicação de bioestimulantes é um exemplo e tem o intuito de induzir o mecanismo de defesa das plantas, interferindo diretamente na tolerância ao estresse causado pelo déficit hídrico (MULEY et al., 2019). Em milho, observa-se a aplicação de diversos

bioestimulantes como o ácido abscísico (SOUZA et al., 2013b), ácido fúlvico (YANG et al., 2019), ácido ascórbico (ZHANG et al., 2019), quitosana (RABÊLO et al., 2019), trealose (ZHOU et al., 2014).

Estudos como o realizado por Ali e Ashraf (2011) mostram que a aplicação exógena de trealose em milho induziu uma resposta satisfatória em relação aos danos causados pelo déficit hídrico, melhorando o sistema de defesa antioxidante e aumentando a biomassa, o que posteriormente resultou em aumento na produção de grãos. A trealose é um dissacarídeo amplamente encontrado na natureza e que não apresenta toxicidade. Diversas literaturas exploram a relação entre a trealose e a tolerância ao déficit hídrico, mas ainda se faz necessário uma maior contribuição científica sobre o assunto, principalmente envolvendo novos derivados, contribuindo para o desenvolvimento e maior produção de cereais como o milho.

2 REVISÃO BIBLIOGRÁFICA

2.1 EFEITO DO DÉFICIT HÍDRICO EM PLANTAS DE MILHO

No ambiente natural dificilmente uma planta encontrará todas as condições consideradas ideais para sua reprodução. A falta de alguns nutrientes (macro e micronutrientes), exposição excessiva a radiações, frio, excesso de sal e inclusive estresse hídrico moderado, seja por alagamento ou seca, é comum ao longo do desenvolvimento de toda espécie vegetal (ZHU, 2016; HOU; UFER; BARTELS, 2016). Porém, apenas níveis mais severos de faltas nutricionais e de estresses são prejudiciais causando danos permanentes no desenvolvimento e alterações na estrutura morfológica e fisiológica das plantas (ZHU, 2016).

No milho, segundo Magalhães e colaboradores (2009), a disponibilidade hídrica no solo faz com que ocorram mudanças no gradiente de potencial hídrico no sistema solo-planta-atmosfera, culminando na desidratação das células e tecidos fazendo com que a planta entre em período de estresse. Respostas fisiológicas e químicas, como a regulação estomática para otimizar a assimilação de CO_2 e reduzir a perda de água, diminuição do crescimento celular e limitação da fotossíntese, indução de sistema antioxidante enzimático e não enzimático são induzidas nas plantas para minimizar os danos causados pelo estresse (TOMBESI et al., 2015; SOUZA et al., 2013b; SOUZA et al., 2014).

A intensificação do déficit hídrico altera a turgescência celular, reduzindo o desenvolvimento sistêmico da planta, o que resulta em alterações morfológicas como o enrolamento das folhas, mudança na angulação da disposição foliar, alterações na disposição e tamanhos dos estômatos (SOUZA et al., 2013a). O déficit hídrico reduz consideravelmente a área foliar, consequentemente a taxa fotossintética é diminuída. Essas alterações limitam a assimilação de CO_2, em fases iniciais do desenvolvimento pode resultar em plantas de milho com menor altura devido ao encurtamento dos entrenós e consequentemente a fase de produção e enchimento dos grãos será prejudicada, diminuindo a deposição de matéria seca, resultando em grãos menores do que o esperado (MAGALHÃES; DURÃES, 2006; MAGALHÃES et al., 2016).

A nível celular, o déficit hídrico pode facilitar o acúmulo de espécies reativas de oxigênio (EROs) que, em excesso, ativam reações peroxidativas danificando as células das plantas, pigmentos fotossintéticos, as proteínas e lipídios. As EROs são mais evidentes nas folhas por

causa dos pigmentos fotossintéticos e podem ser entendidas pelas plantas como um sinal para ativar as respostas de defesa quando em pequena quantidade (MITTLER et al., 2011).

2.2 ESPÉCIES REATIVAS DE OXIGÊNIO

As espécies reativas de oxigênio (EROs) são compostos formados a partir da forma reduzida ou ativada do oxigênio (O_2) e como subprodutos do metabolismo anaeróbico. As modificações na concentração de oxigênio em organismos aeróbicos alteram os processos naturais de ativação e inibição de enzimas, aumentando a concentração de EROs que em pequenas quantidades atuam como sinalizadoras de algum distúrbio dentro das células, ativando mecanismos de defesa e de resistência aos diferentes estresses enfrentados pelas plantas ao longo de sua vida (MITTLER et al., 2011).

As EROs podem ser geradas em diferentes compartimentos celulares, mitocôndrias, apoplastos, cloroplastos, vacúolos, núcleos, peroxissomos (CHOUDHURY et al., 2017), formando uma assinatura específica de produção e remoção de EROs, dependendo do estado estacionário ou redox dos compartimentos celulares, e podem ser encontradas desde bactérias até células de mamíferos (QI et al., 2017; ZANDALINAS; MITTLER, 2018). As principais fontes de produção de EROs estão relacionadas como resultado do próprio metabolismo e como sinalização de algum estresse sofrido, geralmente são formadas pela transferência de elétrons do O_2 (DEMIDCHIK, 2015). Estes compostos são energeticamente mais ativos e reagem com facilidade com outras moléculas gerando reações em cascata (KOHLI et al., 2017) .

As formas mais comuns de espécies reativas encontradas nas células são representadas pelos grupos de oxigênio singleto ($^1O_2$), radicais superóxidos (O_2^-), peróxido de hidrogênio (H_2O_2) e hidroxila (OH-) (CHOUDHURY et al., 2017; JAJIC; SARNA; STRZALKA, 2015). O radical superóxido (O_2^-) é uma das formas menos reativas e não possui a capacidade de ser transportado pelas membranas celulares, por isso rapidamente é dismutado em H_2O_2 (NIU; LIAO, 2016). Por sua vez o H_2O_2 pode ser gerado a partir da dismutação de radicais superóxidos ou a partir de vias oxidases, NADPH-oxidases, amina oxidases, (WASZCZAK; CARMODY; KANGASJÄRVI, 2018). Embora seja muito estável, é de fácil transporte entre as membranas, sendo relatado como um importante sinalizador de estresse (JAJIC; SARNA; STRZALKA, 2015).

A OH- e a espécie mais reativa, podendo ser formada a partir da dismutação de O_2^- ou H_2O_2 na reação de Haber-Weiss e Fenton (decomposição de peróxido de hidrogênio em radicais

hidroxila altamente reativos na presença de ferro) (FISCHBACHER; VON SONNTAG; SCHMIDT, 2017). A hidroxila reage com todos os compostos celulares e o acúmulo desta espécie reativa pode levar a morte celular. A produção do oxigênio singleto causadas por estresse, assim como O_2^-, pode afetar a distribuição de energia entre os fotossistemas I e II (PSI e PSII, devido ao excesso de energia absorvida, além de causar alterações na expressão de genes nucleares nos cloroplastos, causando a clorose e consequentemente a morte celular (TAKAGI et al., 2016).

2.3 SISTEMA ANTIOXIDANTE: ENZIMAS ANTIOXIDANTES E COMPOSTOS FENÓLICOS

Como resposta ao excesso de EROs, as plantas ativam mecanismos de defesa para manter a homeostase celular e controlar a desintoxicação celular evitando assim, a peroxidação lipídica e os danos oxidativos (HUSSAIN et al., 2019). Um dos mecanismos de defesa é o sistema de desintoxicação composto por enzimas antioxidantes superóxido dismutase (SOD), catalase (CAT), peroxidase do ascorbato (APX) e do guaiacol (POD) (MITTLER et al., 2004; ALI; ASHRAF, 2011; SANTOS et al., 2018).

2.3.1 SUPERÓXIDO DISMUTASE (SOD EC 1.15.1.1)

A SOD é a primeira enzima da linha de defesa contra EROs e atua realizando a dismutação do radical superóxido em H_2O_2. Esta enzima está presente em todos os compartimentos celulares suscetíveis ao estresse oxidativo (GILL et al., 2015).

Classificada como uma metaloenzima, depende do seu componente metal de reação e sua localização para ser caracterizada. A Cu/Zn-SOD está localizada no citosol, cloroplastos e peroxissomos, Fe-SOD localizada principalmente nos cloroplastos e podendo ser encontrada nos apoplastos e peroxissomos e Mn-SOD na matriz das mitocôndrias (HASANUZZAMAN et al., 2012) .

2.3.2 CATALASE (CAT EC 1.11.1.6)

A CAT é uma enzima que possui diferentes isoformas e que usa o H_2O_2 como substrato para a conversão em H_2O e O_2 (SOFO et al., 2015). Está presente peroxissomos, glioxissomos e organelas onde H_2O_2 é gerado (IANNONE; GROPPA; BENAVIDES, 2015). As catalases podem ser divididas em três classes, onde CAT1 é responsável pela remoção de H_2O_2 gerado no processo de fotorrespiração em tecidos fotossintéticos; CAT2 é encontrada em tecidos vasculares e suas funções biológicas não são bem definidas, supõe-se que esta classe de CATs seja expressa durante a lignificação (ANJUM et al., 2016); e CAT3 sendo expressa em sementes e órgãos jovens, responsável pela remoção de H_2O_2 resultante da degradação de ácidos graxos no ciclo glioxalato (ANJUM et al., 2016).

2.3.3 ASCORBATO PEROXIDASE (APX EC 1.1.11.1)

A enzima APX atua em diferentes compartimentos celulares regulando os níveis de EROs, considerada uma enzima de grande importância no controle das espécies reativas e proteção contra os estresses ambientais (HUSEYNOVA; ALIYEVA; ALIYEV, 2013). Eficiente na eliminação de H_2O_2 no citosol e nos cloroplastos, a APX é uma enzima importante no ciclo ascorbato-glutationae depende do ácido ascórbico (ASA) como doador de elétrons para reduzir em água o H_2O_2 formado pela SOD.

A APX apresenta várias isoformas, caracterizadas de acordo com a localização celular. Isoformas solúveis encontradas no citosol (cAPX), mitocôndrias (mitAPX) e estroma de cloroplastos (sAPX), isoformas ligadas a membrana (mAPX) e tilacóides de cloroplastos (tAPX) e cloroplastos (chlAPX) (CAVERZAN et al., 2012). A tAPX e sAPX estão envolvidas na eliminação de H_2O_2 produzido na fotossíntese e a mAPX e mitAPX eliminam o H_2O_2 da fotorrespiração e respiração, enquanto a cAPX está relacionada a proteção contra os danos causados pelo estresse (PANG; WANG, 2010).

2.3.4 PEROXIDASE DO GUAIACOL (POD EC 1.11.1.7)

A peroxidase (POD) possui grande afinidade com o guaiacol e utiliza-o como doador de elétrons para catalisar o H_2O_2. Amplamente distribuída entre as plantas, a POD está presente em diversos tecidos, nos vegetais é encontrada na forma solúvel. Além de ser uma importante enzima no controle de EROs, regulando o H_2O_2 e oxidando diversos substratos, as PODs ainda estão envolvidas em diversos processos fisiológicos, como a síntese de lignina e outros

polifenóis, alongamento celular e cicatrização de danos sofridos pelo estresse (MARCHAND; GREBENSHCHYKOVA; MENCH, 2016; ASTHIR, 2015).

2.3.5 COMPOSTOS FENÓLICOS

Os compostos fenólicos possuem em sua estrutura anéis aromáticos ou grupos hidroxilas e desempenham importante papel no combate ao estresse oxidativo (VAN HUNG, 2016). Este grupo de compostos possui variadas estruturas moleculares, as principais são ácidos fenólicos, flavonoides e derivados da cumarina.

As características antioxidantes dos compostos fenólicos e as diferentes propriedades químicas, atuam de maneira sinérgica contribuindo para a proteção celular no sequestro de radicais livres (BLOMHOFF et al., 2006).

2.4 METABOLISMO PRIMÁRIO

O metabolismo primário das plantas está associado aos processos fotossintéticos responsáveis pela assimilação de carboidratos e estão envolvidos nos processos comuns e vitais das plantas. Através da assimilação do carbono e em resposta a estresses abióticos, há a formação e acúmulo de amido, proteínas, prolina, açúcar solúveis e açúcares redutores a fim de evitar danos a planta (KOSAR et al., 2018).

2.4.1 AÇÚCARES SOLÚVEIS TOTAIS E AÇÚCARES REDUTORES

Os açúcares acumulados em altas concentrações são sacarose, frutose e glicose. Os açúcares estão presentes no metabolismo primário das plantas, na germinação de sementes e na fotossíntese onde o carbono fixado é convertido em açúcares através de células fotossintéticas especializadas (KOSAR et al., 2019).

O açúcar fotossintetizado imediatamente está disponível para transporte e alocação em vacúolos e tecidos de armazenamento de diversos órgãos da planta, servindo como reservatório para períodos onde a fotossíntese é reduzida ou comprometida. O transporte ocorre por meio do floema e a forma mais comum do açúcar transportado é a sacarose(PAGLIARANI et al., 2019).

O metabolismo, transporte e armazenamento de açúcares é constante e controlado por diversos processos de regulação. Durante períodos de estresse a reserva de amido pode ser hidrolisada em açúcares solúveis e ser translocada para o apoplasto mantendo o funcionamento de atividades essenciais para a sobrevivência celular (SECCHI; ZWIENIECKI, 2012).

O transporte de açúcares entre as células e o apoplasto é mediado pelos cotransportadores de açúcar/prótons da membrana plasmática, esta regulação evita o colapso celular por falta de nutrientes e controla o fluxo de açúcares através da membrana plasmática. Plantas expostas a longos períodos de seca podem alterar o pH e a concentração de solutos na seiva do floema. O acúmulo de fontes de energia como os açúcares é fundamental para o enfrentamento de danos causados pelo déficit hídrico, como a manutenção do turgor celular, baixas pressões hidrostáticas são mantidas durante o acúmulo de açúcares (KOSAR et al., 2018; SECCHI; ZWIENIECKI, 2012).

Os monossacarídeos também conhecidos como açúcares redutores (AR) possuem em sua estrutura um grupo cetona ou aldeído e são capazes de reduzir íons oxidantes. Em cana de açúcar constatou-se que condições de estresse podem reduzir a concentração de amido e sacarose nas folhas e aumentar a concentração de açúcares redutores (GARCIA et al., 2020), portanto pode-se dizer que a concentração de açúcares redutores ajudam no controle osmótico de plantas submetidas ao déficit hídrico.

2.4.2 AMIDO

O amido é a principal polissacarídeo de reserva na maioria das plantas, nas folhas é considerado transitório e em raízes, tubérculos, frutos e sementes, como amido de reserva. O amido é formado por cadeias α-D-glucose, composto por cadeias lineares conhecidas como amilose e cadeias ramificadas como amilopectinas (LIN et al., 2016).

Durante estresse osmóticos e em resposta a altas temperaturas, as plantas mobilizam amido, resultando no acúmulo de maltose. A hidrólise de maltose por β-amilase é a principal via de degradação do amido (THALMANN et al., 2016).

O amido é sintetizado nos plastídeos/cloroplastos nas folhas e em amiloplastos especializados nos órgãos e tecidos de armazenamento. A síntese de amido envolve três etapas, sendo a primeira o alongamento das extremidades não redutoras das cadeias de glicose pela ADPglucose (adenosina 5’-difosfato-glicose), segunda etapa ramificações das cadeias existentes através das reações de glucanotransferase e a terceira etapa enzimas de degradação

hidrolisam novamente alguns ramos das cadeias existentes. Há uma complexidade na síntese do amido, conferindo características especificas aos grânulos de amido (PFISTER; ZEEMAN, 2016).

2.4.3 PROLINA E PROTEÍNAS

O acúmulo de solutos osmoprotetores, ativação de vias alternativas que estimulam os mecanismos de defesa celular, o fechamento estomático, o controle fotossintético, na tentativa de regular a atividade metabólica e a cadeia de transporte de elétrons, são alguns dos recursos utilizados na tentativa de minimizar os danos causados pelo déficit hídrico (SELLO et al., 2019).

A presença da prolina em células vegetais está associada ao mecanismos de ajuste osmótico (NOUNJAN et al., 2018), o que pode favorecer a manutenção do tugor celular, estabelecendo o controle de atividades celulares. A prolina atua diretamente no controle osmótico em plantas submetidas ao déficit hídrico, contribuindo para a estabilidade de membranas celulares e mantendo adequando as concentrações de $NADP^+$/NADPH (HAYAT et al., 2012).

Em seus estudos Laloum et al. (2018) e Farooq et al. (2018) mostram que diante de situações de estresse vias alternativas de codificação de proteínas são estimuladas, facilitando o acúmulo de grupos de proteínas osmoprotetoras responsáveis por proteger as membranas, evitando a desnaturação de proteínas e facilitando a tolerância ao estresse causado pelo déficit hídrico.

2.5 EFICIÊNCIA FOTOSSINTÉTICA

Sob déficit hídrico as plantas ativam diferentes mecanismos para manter o equilíbrio de todo o aparato fotossintético. Entre estes mecanismos a fotoaclimatação e o fechamento estomático, regulam a cadeia de transporte de elétrons entre os fotossistemas (PSI e PSII) permitindo que danos causados pelo desequilíbrio de energia não afetem o metabolismo vegetal (SELLO et al., 2019).

Os efeitos do déficit hídrico no aparato fotossintético podem ser investigados com o auxílio de curvas rápidas de luz, onde parâmetros como a máxima eficiência do uso da luz (α), taxa máxima de transporte de elétrons (ETR_{max}) e irradiância mínima de saturação (Ik) são

mensuradas (WU et al., 2013). Trabalhos apresentados por Wu et al. (2013) e Lobos et al. (2019) indicam que durante períodos de estresse as plantas tendem a reduzir a taxa de transporte de elétrons passando do PSII para o PSI alterando a quantidade de luz que satura os fotossistemas, e a eficiência fotossintética da clorofila a, reduzindo assim, a eficiência do uso da luz, contribuindo para baixo rendimento fotossintético e assimilação de carbono.

2.6 TREALOSE

A aplicação de bioestimulantes em plantas (substâncias capazes de promover a tolerância frente a estresses bióticos e abióticos) é relatada em diversos trabalhos mostrando a eficiência na promoção a tolerância à seca em diversas culturas como milho (SOUZA et al., 2014), arroz (GARG et al., 2002), batata (MULEY et al., 2019) e rabanete (SHAFIQ; AKRAM; ASHRAF, 2015a).

A trealose é um dissacarídeo não redutor constituído por duas moléculas de D-glicose numa ligação α-α, (α-D-glicopiranosil-[1,1]-α-D-glicopiranosídeo). É amplamente distribuída na natureza, fonte de energia na maioria dos organismos vivos, encontrada em insetos, fungos, invertebrados e plantas (HIGASHIYAMA, 2002; SATOH-NAGASAWA et al., 2006). Está associada à capacidade de proteção e estabilização de diferentes organismos a estresses bióticos e abióticos. Nas plantas a trealose desempenha um importante papel como bioestimulante na tolerância ao déficit hídrico por ser considerada por alguns autores como osmoprotetora, quando acumulada (AVONCE et al., 2006).

São registradas cinco diferentes rotas metabólicas da trealose em fungos, bactérias e leveduras. Nas plantas a biossíntese da trealose ocorre a partir de moléculas de UDP- glicose e glicose-6-fosfato que pela ação de duas enzimas principais, trealose-6-fosfato sintase (TPS) e trealose-6-fosfato fosfatase (TPP), que atuam sobre a trealose-6-fosfato (T6P), desfosforilando em trealose (AVONCE et al., 2006; FERNANDEZ et al., 2010).

2.7 EFEITOS DA APLICAÇÃO DA TREALOSE E DERIVADOS EM PLANTAS

A trealose constantemente é associada à capacidade de conferir aos vegetais tolerância a diferentes condições de estresse, devido a sua ação osmoprotetora (ZHOU et al., 2014). Os mecanismos de ação mostrando a eficácia da trealose podem incluir a vitrificação e formação

de cristais de anidro, que após períodos de seca são capazes de reabsorver água (STREETER, 2003). Podendo ainda gerar ligações de hidrogênio estabilizando as membranas celulares em condição de déficit hídrico (ITURRIAGA et al., 2009).

Em plantas superiores a presença da trealose era questionada, até a identificação em espécies como *Arabidopsis thaliana* (WINGLER et al., 2000). No cultivo de arroz a aplicação de trealose elevou os teores de açúcares solúveis aumentando a tolerância ao déficit hídrico e ao estresse salino (FERNANDEZ et al., 2010; GARG et al., 2002). A trealose ainda pode influenciar na formação da inflorescência do milho, e aumentar a tolerância a estresses como salino e ao déficit hídrico (SATOH-NAGASAWA et al., 2006; ZHOU et al., 2014). Em nabo forrageiro a aplicação com sprays de trealose nas folhas aumentou a biomassa (AKRAM et al., 2015). A trealose em trigo aumentou a tolerância a seca e reduziu o acúmulo de EROs (LUO et al., 2018).

Diversas pesquisas demonstram que algumas culturas têm obtido ganhos significativos com a aplicação da trealose. A manipulação das vias bioquímicas e as enzimas envolvidas no processo de metabolização da trealose, aliado ao melhoramento genético, trouxe ganhos na área da produção mundial, aumentando a resistência e a tolerância dos cultivares a diversos tipos de estresse (LUNN et al., 2014; PAUL et al., 2015; ZHAO et al., 2019).

A maioria dos trabalhos citados ao longo deste estudo envolvem a aplicação da mistura de trealose e outros reguladores de crescimento em espécies como milho, soja, cana-de-açúcar, nabo (ALI; ASHRAF, 2011; AVONCE et al., 2006; SHAFIQ; AKRAM; ASHRAF, 2015), porém não foi encontrado na literatura o uso de derivados da trealose na tentativa de inibir estresses bióticos e abióticos, ou como estimulantes no desenvolvimento de espécies vegetais. Neste estudo analisamos o uso da trealose e uma mistura de seus derivados trealose tosilada (6,6’didesoxi-6,6’-di-O-(p-toluenossulfonil)-α,α-trealose) e trealose azídico (6,6’diazido-6,6’-didesoxi-α,α-trealose).

3 OBJETIVOS

3.1 OBJETIVOS GERAIS

Comparar o efeito mitigador da trealose e da mistura de seus derivados (tosila e azídica) sobre o déficit hídrico em plantas de milho.

3.2 OBJETIVOS ESPECÍFICOS

a) Verificar os mecanismos de defesa antioxidante de plantas de milho sob estresse hídrico, em casa de vegetação, com e sem aplicação foliar de trealose e seus derivados, pela avaliação do sistema antioxidante enzimático e não enzimático (compostos fenólicos);
b) avaliar as modificações no metabolismo primário e na eficiência fotossintética após a aplicação da trealose e dos seus derivados de em milho submetido ao estresse hídrico.

4 JUSTIFICATIVA

O milho é um dos cereais mais produzidos no mundo, servindo como base de alimentação para grande parte da população mundial e inclusive para alimentação de muitos animais. De acordo com a Companhia Nacional de Abastecimento (CONAB), a produção de grãos na primeira safra de 2018/2019 sofreu decréscimo em torno de 10% em relação à safra anterior. Podemos atribuir esta baixa na produção a dois fatores, o primeiro é o baixo preço praticado do produto e o segundo fator está relacionado às alterações climáticas dos últimos anos, fazendo com que o excesso ou falta de chuvas interfira diretamente no cultivo das plantas (CONAB, 2019).

No Brasil a plantação de milho estende-se em quase todo o território nacional, sendo a região centro-oeste onde as lavouras estão mais concentradas. As condições climáticas, disponibilidade de nutrientes e de água no solo são características que devem ser observadas para o plantio, porém as variações climáticas tornam-se um desafio para a produção da cultura (SANS *et al.*, 2001).

A descoberta de novas tecnologias possibilitando a indução de tolerância ao déficit hídrico, como o proposto com a aplicação de uma mistura de derivados de trealose que nunca foram testados em plantas traz novas possibilidades de produção e estudos de bioestimulantes baseados em trealose para o mercado brasileiro.

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ANEXO A

Mixture of trehalose derivatives stimulates the antioxidant system and improves photosynthetic efficiency in maize under water deficit.

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Abstract

So far, studies on synthesis of trehalose derivatives and their effects on plants have not been explored. Therefore, the purpose of this study has been to evaluate antioxidant activity, primary methabolism and photosynthetic efficiency in maize plants under water deficit sprayed with a mixture of trehalose derivatives (tosyl and azidic). The experiment was conducted in greenhouses, using a maize hybrid sensitive to water deficit. Water deficit was imposed for 12 days, with foliar application of the mixture of derivatives (30mM) on the first day and on the fifth day of stress imposition.The analysys of photosynthetic efficiency of the antioxidant enzymatic system and of lipid peroxidation through rapid light curves were conducted on the first and on the last day of water deficit imposition and 12 hours after rehydration. Reducing sugars, total soluble sugars, starch, protein, proline, and phenolic compounds were analyzed at the end of the stress. Both trehalose and the mixture of trehalose derivatives influenced the response in the plants and the effects and possible reasons were analyzed. It can be concluded that the mixture of trehalose derivatives can contribute to increase tolerance to water deficit in maize through the stimulus of superoxide dismutase enzyme, ascorbate peroxidase, guaiacol peroxidase, through the accumulation of sugars, proteins, proline and phenolic compounds and through the improvement in the maximum electron transport rate.

Key words: *Zea mays* L.; oxidative stress; dissaccharide; natural substance; proline; electron transport rate; phenolic compounds.

1. Introduction

The constant climatic changes have been causing substantial variations on temperature, altering the seasons and sometimes increasing the period of drought [1]. Thereby, one of the environmental factors which are most limiting to development and to plant production is the poor availability of water, or water deficit [2].

Water deficit can enable the maize plant to enter a period of stress and cause cell dehydration. Thereby, changes such as osmotic control and photosynthesis reduction occur, altering the harvesting and dissipation of light energy, causing modifications to the whole photosynthetic apparatus [3, 4].

Once photosynthesis (carboxylation) is reduced, an excess of energy in the leaves occurs, excess that can be transferred to O_2 and that, consequently, increases the formation of oxygen reactive (EROs) [5, 6, 7, 4, 8]. The accumulation of EROs may cause enzymatic and DNA degradation, modifications to the plasma membrane, such as lipid peroxidation, and even cause cellular apoptosi [9].

Maize is demanding with regard to the amount of water available and studies have shown that water deficit can cause irreversible damage to the cultivation, when it occurs: (1) at the vegetative stage , mainly at V6 stage, in which the production point is fixed and the result of the final production may be affected; (2) in flowering and in (3) grain filling, affecting floral synchrony and grain formation and filling [10, 11].

Water-deficit tolerant maize plants can activate the antioxidant protective system by increasing enzymatic activity as dismutase superoxide (SOD), and peroxidases such as ascorbate peroxidase (APX), catalase (CAT), guaiacol peroxidase (POD) [12, 13, 7, 14].

In the fight against the oxidative stress caused by the excess of EROs, maize plants can activate the non-enzymatic antioxidant system as polyphenols in an attempt to combat the damage [12]. In addition, plants can mitigate water stress by activating the primary methabolism, altering sugar content, proline, or even by controlling photosynthesis and improving its efficiency through biochemical ways (enzymatic activity) and through biophysical ways (photosynthetic apparatus and electron transport rate control) [15, 16].

Trehalose (αD-glucopyranosyl-[1,1]-α-D-glucopyranose) is a natural substance, a non-reducing dissaccharide with osmoprotective properties [17], formed by a catalyzed reaction through trehalose phosphate synthase (TPS), obtaining trehalose-6-phosphate (T6P) and uridine diphosphate (UDP). Subsequently, the trehalose phosphate phosphatase enzyme (TPP) converts T6P in free trehalose [18]. Trehalose is widely found in insects, fungi, bacteria and plants, in

which there are reports of its functions as osmoprotector and osmoregulator toward stress conditions [19, 20, 21]. In agriculture, trehalose is used as a biostimulator, acting on tolerance increase to abiotic stress in plants, including water deficit, being a sustainable alternative to synthetic products [22].

Trehalose has been identified as a growth and development (productivity) promoter and also associated with osmotic, photosynthetic and antioxidant regulation in plants such as radish [23, 24], rice [25], cassava [26], maize [5, 27], wheat [28] and sunflower [28] cultivated under stress conditions. However, it is still necessary to explore the technologies that contribute to the mitigation of the effects of water deficit on vegetables, reducing losses, mainly in productivity [29, 30, 31]. Although many studies have widely shown the efficiency of trehalose usage in agriculture (including maize under water stress), there are no reports in the literature of studies showing results for the synthesis and use of trehalose derivatives, thus justifying the importance of researches that analyze the effect of trehalose derivatives on cultivations like maize when submitted to water deficit. Therefore, the goal of this study has been to verify the potential effect of the application of a mixture of trehalose derivatives on maize under water deficit, on the antioxidant system activation,on primary methabolism and on photosynthetic efficiency.

2. Results

2.1 Quantification of lipid peroxidation (MDA) and antioxidant system

Comparing the treatments both on a water deficit day (1d) and after 12 days (12d) it was noted that the level of MDA did not show significant difference between Water Deficit (WD), Water Deficit and Trehalose (WD+TRE) and Water Deficit and Mixture of Trehalose Derivatives (WD+ DER) (Fig. 1). However, throughout water deficit (harvest season 1 d e 12 d) it was observed that the level of MDA decreased more considerably during (WD+TRE) treatment. And after 12 hours from rehydration, there was a decrease in the concentration of MDA during all the treatments, with values similar to the ones for irrigated plants (Fig. 1).

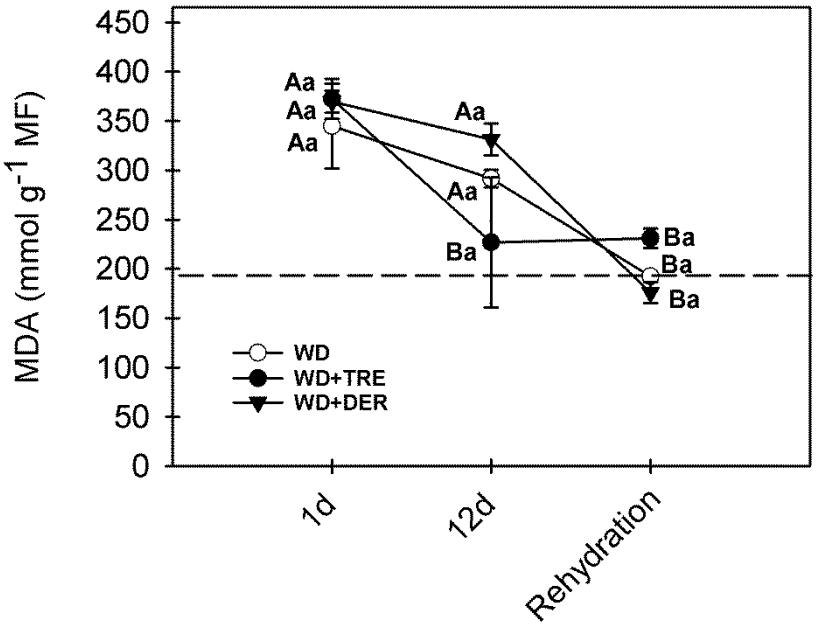

Figure 1. Lipid peroxidation (MDA) in maize leaves sprayed with trehalose and its derivatives during the imposition of water deficit and rehydration. Means followed by the same uppercase letter between the harvest seasons and lowercase letter between treatments do not differ by Scott-Knott test at 5% of probabillity (P≤0.05). Treatments: WD = water deficit; WD+TRE= water deficit with foliar application of trehalose; and WD+DER= water deficit with foliar application of a mixture of trehalose derivatives. Harvest seasons: 1d = one day of water deficit; 12d= 12 days of water deficit; and Rehydration = 12 hours after rehydration. The bars correspond to standard error of the mean (four repetitions). The dotted line represents the average activity of irrigated control enzymes during the experiment.

In the evaluation of the antioxidant system it was observed that during water deficit (1d and 12d) there was a decrease in the superoxide dismutase enzymatic activity (SOD) (Fig. 2A). However, during rehydration there was an increase in the enzymatic activity in all the tested treatments. Among the treatments, it should be stressed that, during rehydration, WD+DER enzymatic activity was the highest and the closest to the value found for irrigated control (dotted line).

Throughout the water deficit, the ascorbate peroxidase enzyme (APX) showed greater activity after 12 days of stress for the three treatments (Fig. 2B). In assessing the treatments, we could highlight greater activity in the plants that were sprayed with the mixture of derivatives (WD+DER) after 12d and that were sprayed with trehalose (WD+TRE) on rehydration.

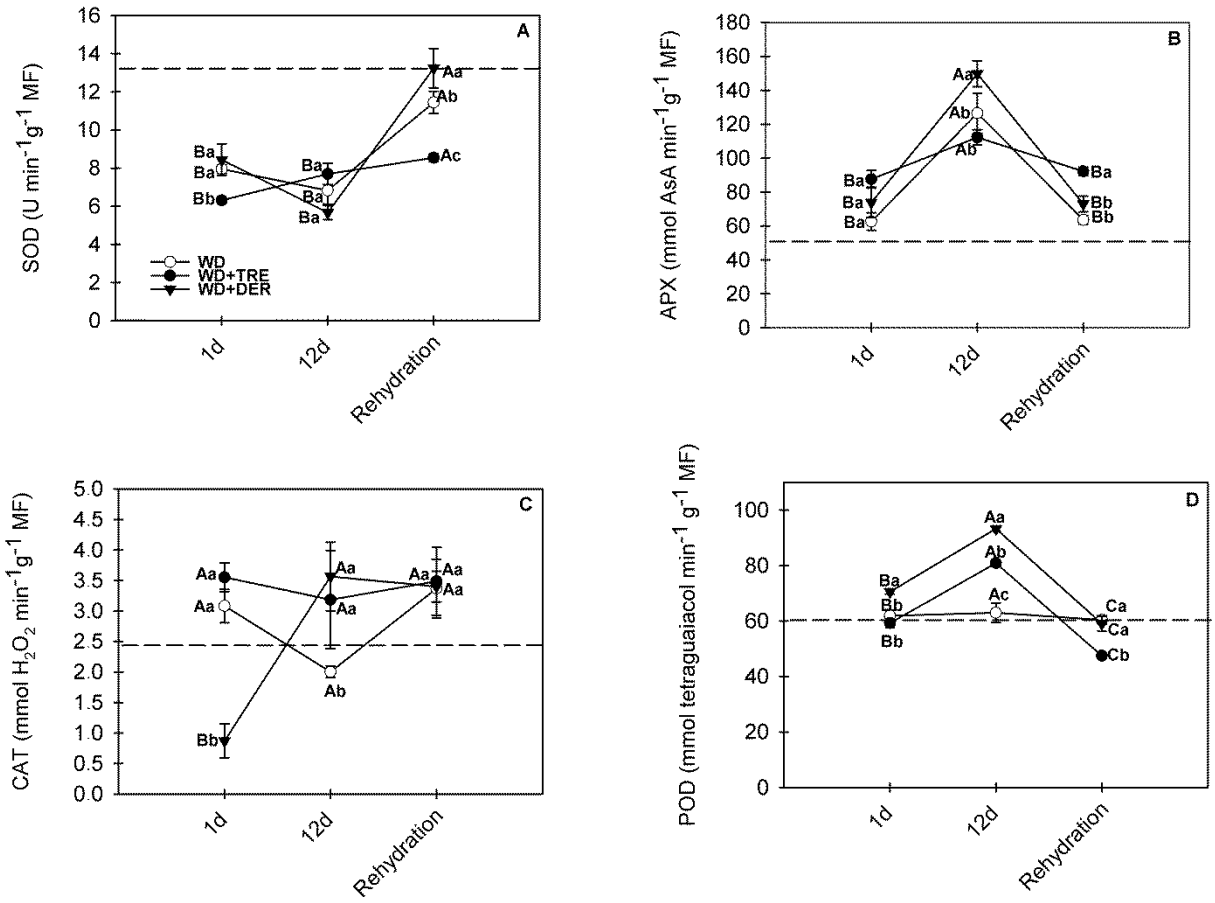

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Figure 2. Superoxide dismutase enzymatic activity (SOD) (A), ascorbate peroxidase enzyme (APX) (B), catalase (CAT) (C) and guaiacol peroxidase (POD) (D) in maize leaves sprayed with trehalose and its derivatives during the imposition of water deficit and rehydration. Means followed by the same uppercase letter between harvest seasons and lowercase between the treatments do not differ by Scott-Knott test at 5% of probability (P≤0.05). Treatments: WD= water deficit; WD+TRE= water deficit with foliar application of trehalose; and WD+DER= water deficit with foliar application of a mixture of treahlose derivatives. Harvest seasons: 1d = one day of water deficit; 12d= 12 days of water deficit; and Rehydration= 12 hours after rehydration. The bars correspond to standard error of the mean (four repetitions). The dotted line represents the activity of irrigated control enzymes during the experiment.

For Catalase enzyme (CAT) no difference between the harvest seasons was observed (1d, 12d, rehydration) (Fig. 2C). However, comparing the treatments, on the first day of water deficit there was greater activity in WD and WD+TRE and, on the twelfth day of water deficit, both the plants that received trehalose and the plants that received the mixture of derivatives increased their activity. On the other hand, on rehydration, there was no difference between the treatments.

At 1 day and 12 days of water deficit we could notice, in all the treatments, an increase in the activity of guaicol peroxidase enzyme (POD) followed by a reduction on Rehydration (Fig. 2D). Comparing the treatments, it was observed that POD activity was higher in the plants that received the mixture of derivatives (WD+DER), both on the first and on the twelfth day of

water deficit.

The quantification of Phenolic Compounds showed a difference only in the leaves, proving to be higher in WD+TRE and WD+DER treatments (Fig. 3A).

2.2 Quantification of Primary Metabolism

In analyzing reducing sugars level (Fig. 3B), it became apparent that the values found in WD, WD+TRE, WD+DER treatments were similar and lower than those found in the irrigated treatment, both in the leaves and in the maize roots.

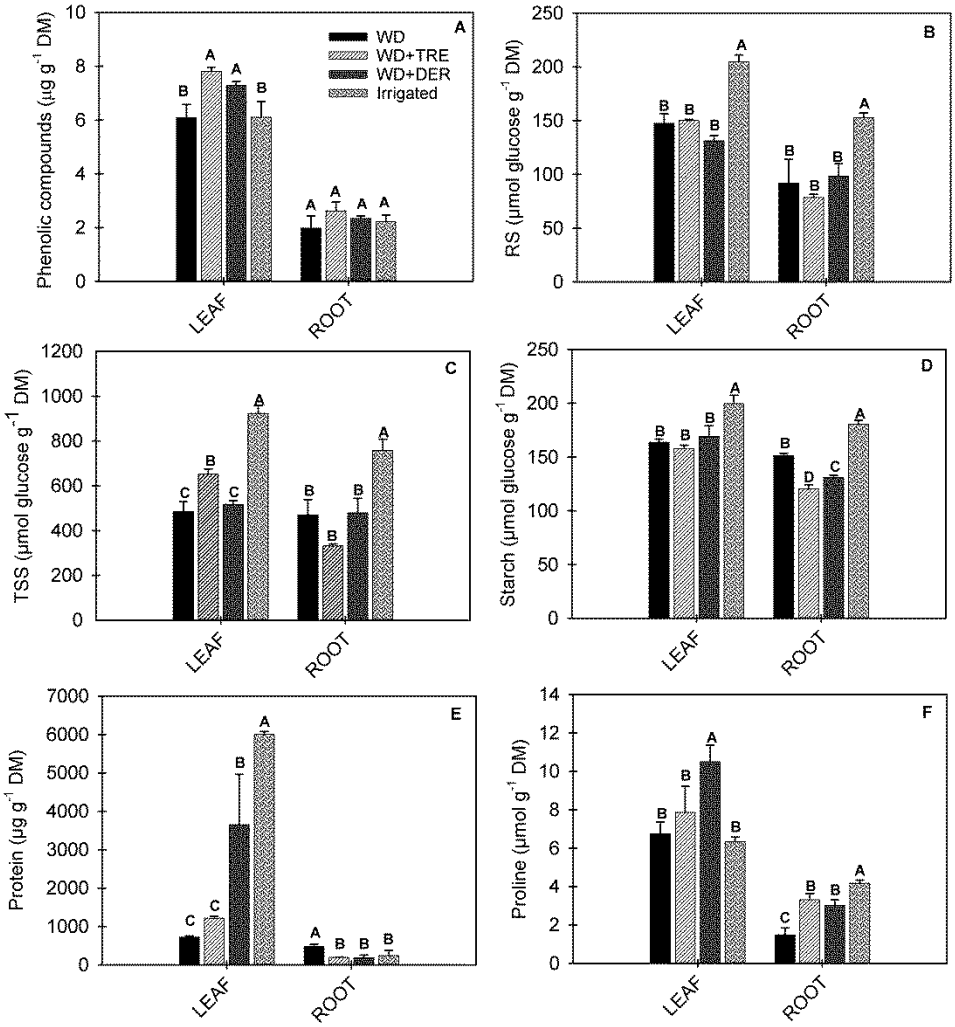

Figura 3. Phenolic compounds concentration (A), reducing sugars (RS) (B); total soluble sugars (TSS) (C); starch (D); protein (E); proline (F) in maize leaves and roots sprayed with trehalose and its derivatives after 12 days of water deficit imposition. Means followed by the same letter do not differ by *Skott-Knott* test at 5% of probability (P≤0.05). Treatments: WD= water deficit; WD+TRE= water deficit with foliar application of trehalose; WD+DER= water deficit with foliar application of a mixture of trehalose derivatives; and Irrigated= irrigated control. The bars correspond to standard error of the mean (four repetitions).

The total soluble sugars (Fig. 3C) showed higher concentration in the Irrigated treatment, followed by WD+TRE when analyzed in the leaves, and showed higher

concentration in the Irrigated treatment when analyzed in the roots. The starch level (Fig. 3D) in the leaves was higher in the irrigated treatment and in the roots was lower in WD+TRE and WD+DER treatments.

The protein concentration in the leaves was higher in the Irrigated treatment followed by WD+DER (Fig. 3E). And, in the roots, the WD treatment showed the highest concentration of proteins with respect to the other treatments which did not differ from each other. The proline level in the leaves (Fig. 3F) was higher in the WD+DER treatment, while in the other treatments the levels were lower and did not differ from each other. In the roots there was higher concentration of proline level in the Irrigated treatment, followed by the treatments with application of trehalose and derivatives (WD+TRE e WD+DER). In addition, the WD treatment showed lower average of proline levels.

2.3 Analysis of photosynthetic efficiency

Throughout the water deficit (1d e 12d) and on rehydration we could observe that the maximum light use efficiency (α) (Fig. 4A), maximum electron transport rate (ETR_{max}) (Fig. 4B) and the minimum saturating irradiance (Ik) (Fig.4C) parameters increased, with the exception of WD treatment which reported a decrease in these parameters on water stress days.

In comparing the treatments, it was observed that at 1d there was no difference in any of the parameters analyzed. However, at 12d it was observed that the trehalose application (WD+TRE) and the mixture of derivatives (WD+DER) increased α and ETR_{max}. This emphasized that the values of the treatment with application of the mixture of derivatives were much higher in these two parameters compared with the values of the treatment with trehalose.

On rehydration, and in all the three parameters analyzed, the WD+TRE and WD+DER treatments showed higher averages than the WD treatment, thus highlighting for α that the application of the mixture of derivatives resulted in higher averages compared with the application of only trehalose (FIG. 4A).

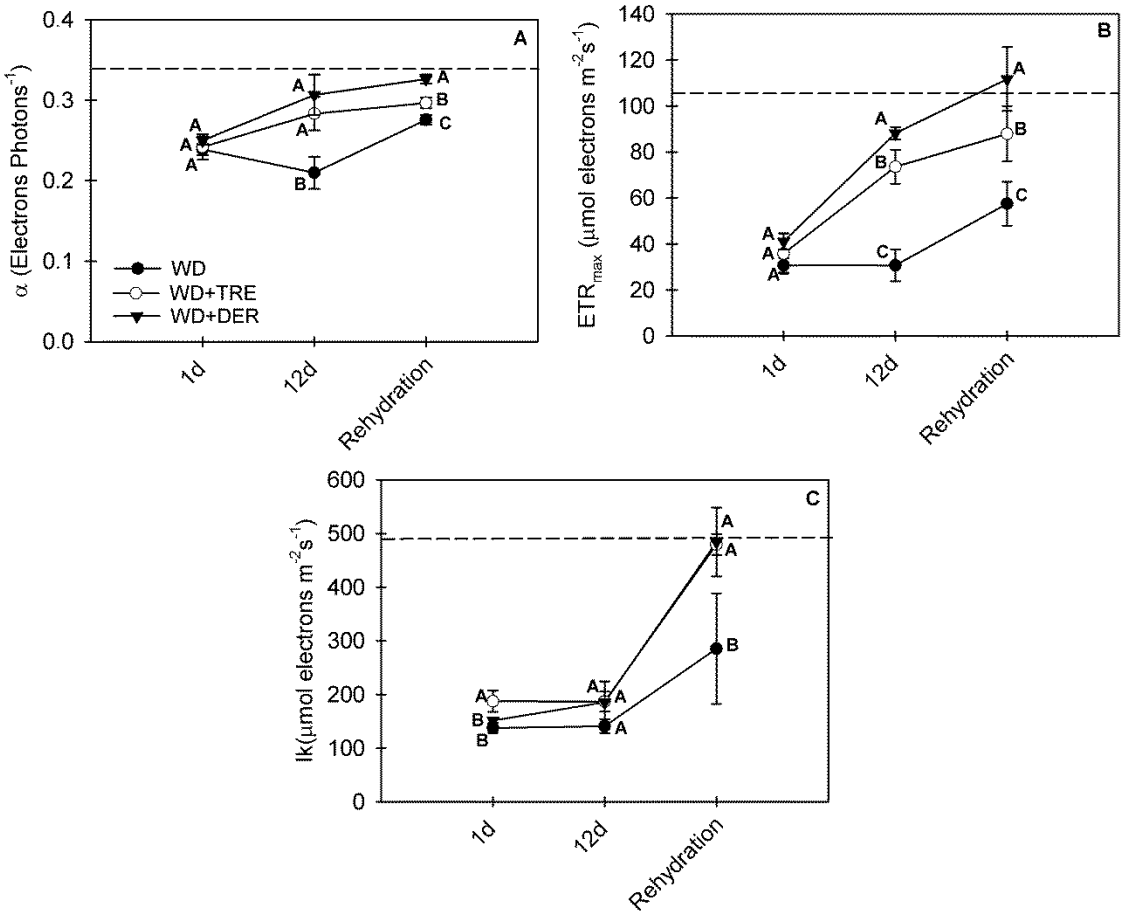

Figura 4. Analysis of photosynthetic efficiency through the measurement of clorophyll *a* fluorescence parameters through rapid light curves in maize leaves sprayed with trehalose and its derivatives during the imposition of water deficit and rehydration. (A) maximum light use efficiency (α), (B) maximum electron transport rate (ETR_{max}), (C) Minimum saturating irradiance (Ik). Means followed by the same letter do not differ by *Skott-Knott* test at 5% of probability ($P \leq 0.05$). Treatments: WD= water deficit; WD+TRE= water deficit with foliar application of trehalose; e WD+DER= water deficit with foliar application of a mixture of trehalose derivatives. Harvest seasons: 1d= one day of water deficit; 12d= 12 days of water deficit; and Rehydration= 12 hours after rehydration. The bars correspond to standard error of the mean (three repetitions). The dotted line represents the average activity of irrigated control enzymes during the experiment.

3. DISCUSSION

Water deficit is one of the limiting factors to plant development. In maize, the increase in the production of oxygen reactive species (ERO) and the imbalance in the antioxidant defense system result in oxidative damage in the whole plant system [7, 32, 12]. The increase in EROs may cause changes in the cellular membrane structure leading to malonaldehyde increase (MDA), that is, lipid peroxidation, causing alterations in its functions [33].

Souza et al.[8] demonstrated that during water deficit maize presented higher levels of MDA, confirming the result found in this study with BRS 1030 genotype, which is sensitive to water deficit. The foliar application of trehalose and its derivatives did not interfere with the concentration of MDA, compared with the stressed treatment (WD), unlike the study presented

by Ali and Ashraf [5], in which, after foliar application of trehalose there was stimulation of the reduction in concentration of MDA in maize under water deficit. A possible explanation for obtaining no changes in MDA levels is that this mixture of trehalose derivatives, when applied on maize leaves, stimulates a regulated dissipation of energy in photosystems [34] that may reduce the excessive formation of EROs and, consequently, not change MDA levels significantly.

The foliar application of trehalose and its derivatives did not alter lipid peroxidation under water deficit, but modified the antioxidant enzymatic activity, raising the hypothesis that, although trehalose and its derivatives are not responsible for the reduction of cellular damage, they act as stress markers. Both trehalose and the mixture increased the antioxidant enzymatic activity. SOD, when activated, is responsible for O2•-free radicals sequestration, dismuting in H_2O_2 and O_2 [35], reducing the risk of OH- formation and controlling the accumulation of H_2O_2 in chloroplast, mitochondria, cytosol and peroxisomes [36]. Subsequently, the antioxidant enzymatic activity of APX, CAT and POD is stimulated for H_2O_2 elimination [32].

In some studies, the foliar application of trehalose has increased SOD [5]; APX [32], CAT [37] and POD activity [28]. This higher activity could be explained by the endogenous increase of trehalose in the cytoplasm of cells [38, 39, 20]. The foliar application of trehalose induces the endogenous accumulation of sugar, characterized by different studies as a compatible solute, that is, non-toxic, highly stable due to its high binding stability (α-α) and low binding energy spending (1 kcal mol^{-1}), which gives trehalose an important role in the maintenance and cellular protction of plants under different kinds of abiotic stress, as water deficit [40, 41].

After analyzing the mixture of derivatives, the latter was more effcient in stimulating antioxidant activity than trehalose itself (there was an increase in SOD on rehydration, APX and POD during water deficit), which might demonstrate higher influence of these new derivatives in the induction of trehalose-6-phosphate synthase genes (TPS). These genes are associated with the production of enzymes that synthesize trehalose [42]. Maize plants tolerant to water deficit tend to have a higher antioxidant enzyme system activity [8, 12] and the mixture of derivatives seems to enhance this activity.

It is important to stress the considerable activity of guaiacol peroxidase (POD), both at the beginning (1d), and at the end of stress (12d), when the mixture of derivatives was applied. POD in plants under water deficit, besides acting on hydrogen peroxide sequestration, can be associated with other enzymes acting on diphenols and phenols for the production of other

phenolic compounds relevant to mitigate the damage caused by oxidative stress through the sweeping of free radicals [43, 44, 45]. This increase in POD could explain the higher level of phenolic compounds in maize treatments that received the disaccharides.

With regard to carbohydrates metabolism, several studies have shown its modulation by trehalose. Our study did not reveal great modifications, but the application of trehalose increased the levels of soluble sugars in the leaves. In rice transgenic plants that were added to the fusion of 2 trehalose biosynthetic genes, increases in the levels of soluble sugars were reported as well [46].

However, the mixture of derivatives, as well as trehalose, reduced the starch in the roots. This reduction can be associated with starch degradation and sugar transport via xylem corroborating the increase of soluble sugars in the leaves. These adjustments are important for plant tolerance, since soluble sugars can work as antioxidant compounds or as osmotically active solutes in plants under stress [47]. In contrast to this, in Arabdopis the application of trehalose in leaves stimulated starch biosynthesis, as the applied sugar induced an *ApL3* gene expression that codifies the great subunit of Adenosine Diphosphate (ADP)-glucose pyrophosphorylase [48, 49].

The exogenous application of trehalose and the mixture of its derivatives can activate a cascade of reactions in plants, in the attempt to mitigate the effect caused by water deficit . Among the processes activated, the accumulation of proline in plant cells is associated with the osmotic adjustment mechanism [50], which may favour the maintenance of cell turgor, establishing cellular activity control. Studies as presented by Kosar [21] corroborate the data found in this study, in which the application of trehalose was able to increase the concentration of proline. In our results, the application of tozyl and azidic trehalose derivatives proved to be more efficient for the accumulation of proline, mitigating water deficit to a greater extent.

The mixture of trehalose derivatives promoted the accumulation of proteins. Studies as the one presented by Laloum et al. [51] and Farooq et al. [52] show that, in the face of stress situations, alternative ways of protein coding are stimulated, facilitating the accumulation of osmoprotective protein groups responsible for protecting the membranes, avoiding protein desnaturation and facilitating tolerance to stress caused by water deficit. Since an increase in photosynthetic efficiency was also reported in this study, this increase in the total proteins of the leaves due to the mixture of trehalose derivatives could also be explained by the increase of Rubisco content (photosynthetic enzyme), one of the most plentiful proteins in the leaves. This because the exogenous application of trehalose induces genes involved in the production of

precursors such as trehalose-6-phosphate [53, 54] that increase Rubisco level [55, 49]. Thus, the mixture of trehalose derivatives could be more active in this genetic induction than trehalose alone.

With regard to photosynthetic efficiency in plants, tolerant genotypes can be discriminated with clorophyll fluorescence parameters *a* through rapid light curves (RLC) [56].

Maize plants sensitive to drought (and other species as well) tend, under water deficit, to reduce the electron transport rate (ETR) going from PSII to PSI, altering the amount of light that saturates the photosystems, thus also reducing light use efficiency (α) [57, 58, 59]. However, trehalose, and more intensively the mixture, seems to mitigate the stress and induce tolerance to drought, since these sprayed substances increased these parameters at 12d of stress and on rehydration. Moreover, this higher ETR_{max} in the mixture occurs because these derivatives increase leaf gas exchanges (photosynthesis) and photochemical dissipation (qP) in maize under water deficit [34], so that a higher electron flow in the photosystems is necessary in order to supply the production of photoassimilates at the biochemical stage.

High values of maximum light use efficiency (α) in maize plants that received the mixture of derivatives may indicate a stable apparatus and alliviation in photoinhibition, since trehalose itself was identified as photosystem II [54] D1 Protein protector.

The possible justification for the mixture of trehalose derivatives to increase photosynthetic efficiency more than trehalose is a better action on specific genes. The application of trehalose may lead to an overexpression of the *OTsA* gene involved in the production of trehalose-6-phosphate and to the increase of photosythetic efficiency [27, 55, 60].

4. Material and Methods

4.1 Synthesis of trehalose derivatives mixture

The trehalose derivatives used were tosylate trehalose, 6-*O*-[(4-methylphenyl) sulfonyl]-α-D-glucopyranoside of 6-*O*-[(4-methylphenyl) sulfonyl]-α-D-glucopyranosyl with molecular weight of 650,13 g mol^{-1} and azidic trehalose, 6-azide-6-deoxy-α-D-glucopyranoside of 6-azide-6-deoxy-D-glucopyranosyl with molecular weight of 392,13 g mol^{-1}. The molecular structures of trehalose, tosylate trehalose and azidic trehalose are presented in Figure 5. These trehalose derivatives used in the mixture were synthesized from pure trehalose (Sigma-Aldrich®). Trehalose reacted with tosyl chloride, pyridine, acetic anhydride resulting in the intermediate derivative 2,3,4-tri-*O*-acetyl-6-*O*-[(4-methylphenyl)sulfonyl]-α-D-

glucopyranoside of 2,3,4-tri-*O*-acetyl-6-*O*-[(4-methylphenyl) sulfonyl]-α-D-glucopyranosyl. Tosylate trehalose was then produced by basic hydrolysis of the intermediate derivative acetyl esters with NaOH in methanol. The intermediate derivative also reacted with sodium azide in pyridine, generating a new intermediate derivate that, by basic hydrolysis of acetyl esters with NaOH in methanol, produced azidic trehalose [61, 62]. The chemical characterization of the derivatives was detailed by [34].

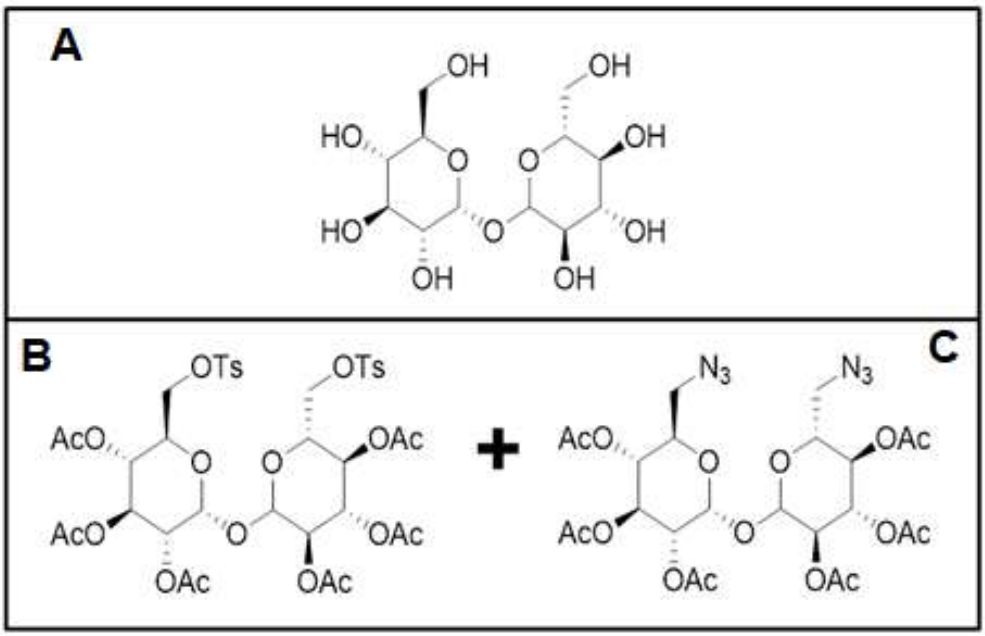

Figura 5 Molecular structure of trehalose (A), of the mixture of tosylate trehalose derivatives (B) and of azidic trehalose (C).

4.2 Plant material and growth conditions

The experiment was conducted in greenhouse at Unidade Educacional Santa Clara of Universidade Federal de Alfenas (UNIFAL-MG), located in the city of Alfenas, South of Minas Gerais state (21º25' S, 45°58'W, 818 meters above mean sea level). Air relative humidity and temperature were registered daily inside the greenhouse, being 30ºC the maximum average temperature, 25ºC the minimum average temperature and 74,4% the relative humidity average. The global radiation incident inside the greenhouse was measured by radiometer (Instrutherm/MES-100, São Paulo - SP) with values of 900 W m^{-2} at midday.

8-liter capacity pots filled with 6 kg of dry, pounded to break up clods, penerated top layer soil were used (0 – 20cm). The soil was classified as distrophyc Red Latosol soil, very

clayey. Subsequently, further fertilizations were made, according to the recommended for maize cultivation [63]. After the fertilization, four seeds of BRS 1030 sensitive to water deficit maize seeds, coming from Embrapa Maize and Sorghum Program, were sown in each pot [4].

Soil moisture was kept at about 70% of field capacity (CC) for all plants, through daily irrigation, until the plants reached V6 stage, with 6 fully-expanded leaves. Water replacement was made according to the weight of the pots (in the morning and in the afternoon), examined with the aid of a digital weight scale (Blackbull 200SS, São Paulo -SP).

4.3 Imposition of water deficit and application of trehalose and derivatives

Once the plants reached V6 stage, irrigation was suspended in order to reach 55% of field capacity (CC), considered to be the maximum allowable stress value for the execution of the experiment.

In addition, to typify the soil water deficit, the base water potential (pre-dawn, ψpd) of the plants, determined before dawn (5 a.m.), through a pressure chamber of Scholander type (Soil Moisture Equipment Corp., Modelo SEC-3015G2, Santa Barbara CA, USA) was used in four full-expanded by treatment leaves. The base water potential reflects the water level of the plants and of the soil (equilibrium) [64]. The plants under water deficit (55% CC) reached a water potential value of-1,7 MPa, while, under 70% CC, a water potential of -0,31 MPa was verified. In all, it was 12 days of water deficit; the stress imposition effectively started in plants an the V6 stage, one of the stages that report higher susceptibility to abiotic stresses, including water deficiency [10].

The experiment consisted of three treatments: water deficit (WD), water deficit with foliar application of trehalose (WD+TRE) in concentration of 30 mM and water deficit with foliar application of the mixture of (azide + tosyl) trehalose derivatives (WD+DER) in the concentration of 15 mM each, totaling 30 mM as recommended by Ali e Ashraf [5].

After obtaining 55% of field capacity in the pots, two foliar applications were made on the 1^{st} and 5^{th} day of water deficit, with trehalose and the mixture having a final concentration of 3,75 mmol $plant^{-1}$, using a mechanical hand-compression sprayer PCP1P with capacity of 1,5L (Guarany®). This sprayer allowed the application of a fine mist of treatment syrup on each plant. The molecules were diluted in ethylene glycol 20%. The applications were made at dusk, the treatments were disposed in straight lines and out of the greenhouse, in order to cover the entire abaxial and adaxial foliar surface (V1 Vídeo in Supplementary Material).

Three collections were made, the first on the 1st day of water deficit (1d), the second on the 12th day of water deficit (12d) and the third collection 12 hours after Rehydration, that is, when all treatments returned to 70% of soil CC. In each collection, samples of leaves for biochemical analyses (lipid peroxidation and antioxidant enzymes) were taken and photosynthetic efficiency parametres were measured (Clorophyll Fluorescence *a*). Instead, the quantification of phenolic compounds and primary metabolism was made at 12 days of water deficit in leaves and roots. Irrigated plants (under condition of 70% CC throughout the entire experiment) were also collected and/or analyzed and named as "Irrigated Control".

4.4 Extraction and quantification of Lipid Peroxidation (malonaldehyde - MDA)

200 mg of fresh plant material were macerated in liquid nitrogen with 50% PVPP until a fine powder was obtained. After that, 1500µL of trichloroacetic acid (TCA) at 0,1% were added. The material was centrifuged at 12.000rpm for 15 minutes at 4ºC, followed by the supernatant collection.

The quantification of lipid peroxidase (MDA level) was made according to the methodology proposed by Buege and Aust [65]. The level of lipid peroxidation was measured in values read in absorbance of 535 to 600 nm, the higher the color presented, the higher the level of MDA concentration, using the molar extinction coefficient of 1,56 $mM^{-1}cm^{-1.}$

4.5 Antioxidant system: Extraction and quantification of antioxidant enzymes and phenolic compounds

For enzyme extraction, 200 mg of fresh plant material, macerated in liquid nitrogen and 50% PVPP until a fine powder was obtained, were used. The extraction was made with an extraction buffer composed of a mixture of potassium phosphate at 400 mM, pH 7.8, 10 mM EDTA, 200 mM ascorbic acid and water, with a final volume of 1500µL/reaction. Subsequently, the enzymes were transferred to micro centrifuge tubes. The samples were centrifuged for 10 minutes at 13.000rpm, at the temperature of 4ºC and the supernatants collected for quantification, according to the methodology proposed by Biemelt et al. [66].

All the enzymes had their activity analyzed using Elisa spectrophotometer reader (anthos Zenyth 200rt, Austria). Superoxide dismutase activity (SOD, EC 1.15.1.1) was measured by the capacity of inhibition of nitrotetrazole blue (NBT) photoreduction, according to Giannopolitis and Ries methodology [67]; ascorbate peroxidase activity (APX, EC 1.11.1.11) was determined by kinetic characterization of the decrease in absorbance of ascorbate oxidation

at 290 nm for 3 minutes, with 2.8 mM^{-1} cm^{-1} extinction coefficient. The methodology used was the one proposed by Nakano, Y.; Asada [68]; catalase activity (CAT, EC 1.11.1.6) was determined by decrease in absorbance due to the consumption of H_2O_2 at 240 nm for 3 minutes, with molar extinction coefficient of 36 mM^{-1} cm^{-1} according to Havir and McHale [69]; guaiacol peroxidase activity (POD, EC 1.11.1.7) was determined by guaiacol oxidation with increase in absorbance at 470 nm, molar extinction coefficient of 26.6 mM^{-1} cm^{-1}. The methodology for the observation of POD activity followed the one proposed by Nakano, Y.; Asada [68] with García-Limones et al. [70] modifications.

For phenolic compounds extraction, 500 mg of dry plant material were macerated and 4 mL of ethanol were added. The material rested overnight and was subsequently centrifuged at 4.000 rpm for 30 minutes at the temperature of 20ºC. The supernatant was collected and the above described procedure repeated. The quantification of phenolic compounds followed Singleton et al. [71] methodology.

4.6 Primary methabolism: Extraction and quantification of reducing sugars, total soluble sugars, starch, protein, proline

For the extraction of reducing sugars, total soluble sugars, proteins and starch, 400 mg of dry plant material were macerated, adding the extractor solution of 3 mL of methanol, 1,25 mL of chloroform and 0,75ml of water. The samples were left to rest overnight and after 24 hours were centrifuged for 30 minutos at 1300 rpm. The supernatant was collected for analyses. 3 mL of 30% perchloric acid were added to the pellet, which rested overnight again for the extraction and quantification of starch.

The quantification of reducing sugars was conducted according to Miller methodology [72], and for total soluble sugars the methodology described by Yemm e Willis [73] was followed. The quantification of proteins followed the method proposed by Bradford [74] and the quantification of starch was conducted according to Yemm and Willis [73] methodology.

For the extraction of proline, 100 mg of dry plant material were macerated with 10 mL of sulfosalicylic acid at 3%. The solution rested in tubes and was mixed by agitation for 60 minutes at 255 rpm. After separating the material, the sample was filtered through filter paper and analyzed according to Bates et al. methodology [75].

4.7 Analyses of photosynthetic efficiency: Clorophyll a Fluorescence

For evaluation of photosynthetic efficiency, the measurement of clorophyll *a* fluorescence parameters was taken through a Mini-PAM II modulated fluorometer (Heinz Walz, Effeltrich, Germany Heinz). Rapid Light Curves (RLC) were obtained using the referred fluorometer. Photosynthetic active radiation (PAR) ranged from 0 to 1150 μmol fótons m^{-2} s^{-1} in 10 levels every 20 seconds in order to determine the electron transport rate (*ETR)* versus *PAR*. ETR was calculated as [(Fm'-Fs/Fm') x PAR x 0,5 x 0,84]. Using the software supplied with the equipment used (WinControl-3) it was possible, from these curves, to calculate the maximum electron rate (ETR_{max}) through the equation ETR = ETR_{max} x tanh(α x PAR/ETR_{max}) [76]. It was also possible to calculate *a* maximum light use efficiency, which corresponds to *ETR*/*PAR* curve inclination and the Ik Minimum saturating irradiance, which corresponds to ETR_{max}/α. 3 curves were made for each treatment (3 repetitions).

4.8 Trial design and Data analysys

Trial design was completely randomized (DIC) in 3x3 factorial design, through three treataments (WD, WD+TRE e WD+DER), three harvest seasons (1d, 12d and Rehydration) and four repetitions, totaling 36 experimental units. Changes in pots placement on the greenhouse benches were made periodically in order to minimize environmental interferences.

Means and ± standard error (SE) were calculated for each parameter. For statistical analysys of the results, variance analysys (ANAVA) and *Scott-Knott* means comparison test at 5% of probability ($p \leq 0.05$) were used, on Sisvar version 5.7 software (Universidade Federal de Lavras - UFLA, Lavras, Brasil).

5. Conclusion

The foliar application of the mixture of trehalose derivatives promoted a better response in the main characteristics analyzed compared with trehalose alone. Therefore, it can be concluded that the mixture of trehalose derivatives can contribute with studies to the formulation of bioestimulants, since it stimulates the antioxidant system and improves the photosynthetic efficiency in maize under water deficit.

Supplementary Materials. Video V1: Application of a mix of chitosan derivatives in maize plants under water deficit.

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Conflicts of Interest. The authors declare no conflict of interest.

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