VERÔNICA APARECIDA FAUSTINO

VIAS DE RESPOSTA CRUZADA DE PLANTAS DE SOJA SUBMETIDAS AO DÉFICIT HÍDRICO E HERBÍVORA POR *Anticarsia gemmatalis*

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Bioquímica Aplicada para obtenção do título de *Doctor Scientiae*

Orientadora: Maria Goreti de Almeida Oliveira

Coorientadores: Camilo Elber Vital
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RESUMO

FAUSTINO, Verônica Aparecida, D.Sc., Universidade Federal de Viçosa, janeiro de 2021. **Vias de resposta cruzada de plantas de soja submetidas ao déficit hídrico e herbívora por *Anticarsia gemmatalis*.** Orientador: Maria Goreti de Almeida Oliveira. Coorientadores: Camilo Elber Vital, Felipe Lopes da Silva, Gláucia Cordeiro e Humberto Josué de Oliveira Ramos.

Atualmente é consensual que as mudanças climáticas estão promovendo aumentos nas temperaturas globais, variabilidade na precipitação e surtos de pragas de insetos mais frequentes. Em adição, existem indicativos que a susceptibilidade das plantas ao ataque de insetos pragas pode ser aumentada em condições de estresses abióticos, tais como seca, e que estas respostas são variadas em função dos genótipos. Assim, a compreensão dos mecanismos moleculares de tolerância aos estresses bióticos e abióticos é crítica para o melhoramento genético das plantas cultivadas e sustentabilidade da produtividade. Estudos tem indicado que algumas respostas fisiológicas e cascatas regulatórias são compartilhadas quando desencadeadas por diferentes sinais de estresse. Desta forma, examinamos as cascatas regulatórias e as vias metabólicas de genótipos de soja tolerantes à seca (EMBRAPA 48), resistentes a infestação de *A. gemmatalis* (IAC17), comparando com um genótipo sensível à seca/ataque de insetos (BR16), e todos foram submetidos a ambos os sinais de estresse (abióticos e bióticos). Plantas em condições de seca foram menos suscetíveis ao ataque de insetos, promovendo menor sobrevivência da lagarta. As reduções de sobrevivência não foram dependentes dos fenótipos de tolerância à seca ou resistência a insetos, embora mais pronunciadas para IAC17. Além disso, perfis de metabólitos, expressão gênica e ensaios enzimáticos nos levam a concluir que apenas o sinal de seca não foi suficiente para explicar totalmente as reduções de sobrevivência. As atividades de inibição da protease e a expressão gênica se correlacionaram com os níveis de ABA, indicando que a sinalização de JA foi potencializada pelo ABA para aumentar a produção dos metabólitos dissuasores. Portanto, o aumento dos níveis de ABA durante o tratamento da seca pode estar agindo sinergicamente para aumentar a resposta de JA, não afetando os primeiros estágios das vias LOX e JA. Assim, “hub (s)” molecular (is) regulatório (s) integrando com múltiplos sinais podem ser alvos para engenharia genética de plantas com múltiplas tolerâncias a estresses ambientais.

Palavras-chave: Déficit hídrico. Inseto-praga. Resistência de plantas. Perfis metabólicos. Expressão gênica.

ABSTRACT

FAUSTINO, Verônica Aparecida, D.Sc., Universidade Federal de Viçosa, January 2021**. Cross response ways of soybean plants submitted to water and herbivora deficit by *Anticarsia gemmatalis*.** Advisor: Maria Goreti de Almeida Oliveira. Co-advisors: Camilo Elber Vital, Felipe Lopes da Silva, Gláucia Cordeiro and Humberto Josué de Oliveira Ramos.

Currently is predicted that climate changes are promoting increases in global temperatures, variability in precipitation and more frequent insect pest outbreaks. Thus, understanding the molecular mechanisms for tolerance of both biotic and abiotic stresses in critical for development of improved genotypes and sustainability of the productivity. As, some physiological responses and regulatory cascades have been shared when triggered by different stress signals, we examine the regulatory cascades and metabolic pathways of drought-tolerant (EMBRAPA48), resistant to *A. gemmatalis* infestation (IAC17) genotypes, comparing with one sensitive to drought/insect attack (BR16) under both stress signals. Plants under drought were less susceptive to insect attack promoting lower caterpillar survival. Survival reductions were not dependent of the drought-tolerance or insect-resistance phenotypes, despite more pronounced for IAC17. Furthermore, metabolites profiles, gene expression and enzymatic assays lead us to concluded that only the drought signal not was enough to explain the survival reductions. Protease inhibition activities and gene expression correlated with ABA levels, indicating that JA-signaling were potentialized by ABA for enhance the production of the deterrent metabolites. Increased of ABA levels during drought treatment may be acting synergistically to increase JA response, did not affecting the first stages of LOX and JA pathways. Thus, regulatory molecular hub(s) integrating signals may be targets to genetic engineering of plants with multiple tolerances to environmental stresses. Therefore, in current studies, we examined whether signs of drought could affect caterpillar survival for other genotypes characterized as drought tolerant and resistant to insect attack. they can assist in the development of better management strategies and new varieties.

Keywords: Water deficit. Insect pest. Plant resistance. Metabolic profiles. Gene expression.

SUMÁRIO

1 INTRODUÇÃO GERAL

A soja (*Glycine max* (L.) Merrill) é uma planta da família Fabaceae amplamente cultivada em todo o mundo, fornecendo uma valiosa fonte de proteína e óleo para consumo humano, alimentação animal e biocombustíveis (KIDOKORO *et al*., 2015). No Brasil, a soja tem grande importância no âmbito econômico, nutricional, e se destaca pelos altos índices de exportação de produtos derivados deste vegetal, tais como: grão, farelo e óleo (CATTELAN e DALL'AGNOL, 2018). Na última safra (2019/2020) o Brasil passou a liderar o ranking de maior produtor de soja do mundo (EMBRAPA SOJA, 2021).

Na safra anterior (2018/2019) a expectativa era que a produção brasileira ultrapassasse a produção dos Estados Unidos. No entanto, isso não foi possível devido as oscilações nas condições climáticas ao longo do ciclo da cultura e algumas intempéries, registrados especialmente entre o fim de 2018 e o início de 2019, de modo a prejudicar o rendimento médio de algumas regiões produtoras (COMPANHIA NACIONAL DE ABASTECIMENTO, 2019). Segundo o último levantamento realizado pela Companhia Nacional de Abastecimento (Conab), nesta safra a produção nacional atingiu 115 milhões de toneladas, ficando 3,6% abaixo do volume colhido em 2017/18.

As alterações climáticas ainda continuam afetando o rendimento da cultura da soja no Brasil. No início da semeadura da safra (2020/2021) ocorreu diminuição do volume da chuva, além do atraso na precipitação no período ideal para os produtores iniciarem o plantio. Isso pode retirar a “janela” (o intervalo de tempo considerado a melhor época para o plantio de culturas, levando em conta o menor risco de perdas de safra) para plantar a safra de milho, no início do próximo ano, na mesma área, após a colheita da soja o que compromete a renda do produtor e resulta em menor disponibilidade de grãos (TOMAZELA, 2020).

Essa queda na produtividade devido as mudanças climáticas pode ocorrer devido a mecanismos fisiológicos que a planta precisa ativar para lidar com a menor abundância de água. Nessa condição ambiental a planta estimula o fechamento dos estômatos para reduzir a perda de água para a atmosfera. No entanto, essas estruturas são responsáveis pelas trocas gasosas durante o processo de fotossíntese, logo, o fechamento ocasiona a redução da fotossíntese e consequentemente menor produção de fotoassimilados. Com isso, há menor crescimento da parte aérea da planta e menor translocação de nutrientes e fotoassimilados para os grãos. Adicionalmente, pode ocorrer abortamento de flores e de frutos (HUBBARD *et al*., 2010; POPKO *et al*., 2010; SARWAT e TUTEJA, 2017; WASILEWSKA *et al*., 2008).

Em resposta às mudanças ambientais, as plantas desenvolveram um conjunto de mecanismos morfológicos, fisiológicos e bioquímicos, para lidar metabolicamente com períodos de déficit de água. A rede metabólica deve ser reconfigurada para manter o metabolismo essencial e se aclimatar, adotando um novo estado estacionário das condições de estresse prevalecentes (FANG e XIONG, 2015; REJEB *et al*., 2014). De modo que as primeiras respostas comumente induzidas em plantas submetidas à estresses são: mudanças no fluxo de íons, alterações nas concentrações de espécies reativas de oxigênio (EROs) e transdução de sinais. A transdução do sinal resultante desencadeia a reprogramação metabólica em direção à defesa (REJEB *et al*., 2014), que pode levar à produção de compostos químicos, fito-hormônios e metabólitos secundários que podem interferir no metabolismo de defesa da planta (DAR *et al.,* 2015).

Entre os mecanismos de resposta de defesa os fito-hormônios (moléculas de baixo peso e reguladores de crescimento de plantas) são compostos que agem em concentrações muito baixas e regulam vários processos celulares e respostas perante as condições ambientais (WANI *et al*., 2016). Os hormônios vegetais mais investigados incluem citocininas (CK), auxinas (Aux), giberelinas (GA), ácido abscísico (ABA), etileno (ET), brassinosteroides (BR), ácido jasmônico (AJ) e ácido salicílico (AS). Dentre esses, o AJ, AS e ET estão envolvidos na defesa da planta, enquanto Aux, CK, GA e BR estão associados ao desenvolvimento da planta. Já o ABA é o principal hormônio que regula as respostas das plantas aos estresses abióticos (KAZAN, 2015).

O ABA é o fito-hormônio que regula vários processos fisiológicos e estágios de desenvolvimento da planta, incluindo dormência e desenvolvimento da semente, morfogênese embrionária, bem como a síntese de proteínas de armazenamento e lipídios. Além disso, é o mais responsivo ao estresse de seca, sinalizando para o fechamento estomático e dos hidatódios (GÓMEZ-CADENAS *et al*., 1996; SAVCHENKO *et al*., 2014). Vos *et al.,* (2019) relataram em seus estudos que o ABA também pode participar das interações inseto-planta no qual atua como um regulador positivo da resistência dependente de AJ através de compartilhamento de fatores de transcrição.

O AJ e seus derivados são hormônios vegetais de sinalização que podem estar envolvidos em diferentes respostas de defesas: abióticas (seca, altas temperaturas, radiação ultravioleta e congelamento); biótica (contra a herbivoria e infecções de patógenos). Eles também regulam os processos morfológicos, fisiológicos e bioquímicos, como a divisão celular, o crescimento da planta e da raiz, desenvolvimento do estame, floração e senescência da folha.

Eles também estão envolvidos na formação de metabólitos secundários e a adaptação ao ritmo sazonal e circadiano (WASTERNACK *et al*., 2014).

Além dos problemas causados pelo déficit hídrico, a cultura da soja também está sujeita aos fatores bióticos simultaneamente, como por exemplo, infestação da lagarta-da-soja (*Anticarsia gemmatalis* Hübner,1818). Esta lagarta é considerada uma das principais pragas desfolhadora de plantas de soja (HOFFMANN-CAMPO *et al*., 2000). Durante a herbivoria, em resposta aos insetos pragas, as plantas ativam vias de sinalização mediadas pelos fito-hormônios: AJ, AS e ET (GILL *et al*., 2010; SHIVAJI *et al*., 2010). Esses fito-hormônios podem agir individualmente, sinergicamente ou antagonicamente, a depender do tipo de ataque, o que proporciona a planta um alto potencial de regulação e pronta adaptação aos estímulos ambientais (VERHAGE *et al*., 2010).

Plantas são organismos vivos sésseis e, por este motivo, estão expostas à múltiplos estresses abióticos e bióticos que frequentemente impõem obstáculos ao seu crescimento e desenvolvimento, resultando em significativas perdas no rendimento de espécies, principalmente nas commodities, como é o caso da soja (KULCHESKI *et al*., 2011). A interação de plantas com múltiplos estresses, bióticos e abióticos, induz respostas bioquímicas complexas, como por exemplo, o acúmulo de certos metabólitos que atuam na resposta à estresses simultâneos (REJEB *et al*., 2014). Em plantas de soja submetidas à estresse hídrico moderado observou-se a potencialização da produção de inibidores de protease em resposta à herbivoria por *A. gemmatalis*, quando comparadas às plantas normalmente irrigadas (FAUSTINO *et al*., 2021).

Vários estudos revelaram que a resistência aos herbívoros pode ser afetada por estresses abióticos que ocorrem simultaneamente, como a seca (GUTBRODT *et al*., 2011). No entanto, apenas recentemente houve um aumento do interesse em identificar os mecanismos moleculares determinantes desses efeitos interativos (LU *et al*., 2015; DAVILA OLIVAS *et al*., 2016; FOYER *et al*., 2016; NGUYEN *et al*., 2016). A sobrevivência de lagartas foi reduzida quando alimentadas em plantas de soja previamente submetidas a déficit hídrico e as reduções observadas correlacionaram com maiores níveis de IPs (FAUSTINO *et al*., 2021), indicando que sinais de seca também podem estar atuando para aumentar a resistência a insetos.

Diante disso, os genótipos utilizados em nosso estudo foram a BR16, EMBRAPA48 e IAC17. O genótipo BR16 sensível à seca é uma variedade de ciclo semiprecoce, originado a partir dos cruzamentos (Mãe) D69-B10-M58 (Pai) Davis (Oya et al., 2004; Santos et al., 2015). Os teores de óleo é 22,6%; o de proteína é 39,0% e sua produtividade média no Estado do

Paraná é de 3.037 kg/há. Apresenta-se flor branca e hilo marrom-claro (Embrapa Soja, 2018). Já EMBRAPA48 é um genótipo tolerante a seca (LIMA *et al*., 2019; MESQUITA *et al*., 2020; COUTINHO *et al*., 2021) com alta estabilidade de produção e boas características para alimentação humana (Embrapa Soja, 2018b). Os teores médios de óleo e de proteína dos grãos são, respectivamente, de 21,4% e 39,1%; sua produtividade média é de 3.428 kg/há (Higashi et al., 1999). A sua origem foi a partir dos cruzamentos (Mãe) Davis x Paraná (Pai) IAS 4 x BR-5 (Higashi et al., 1999; Oya et al., 2004; Santos et al., 2015). Apresenta-se tipo de crescimento determinado, flor branca e hilo marrom-claro, pertence ao grupo de maturidade relativa 6.8 (EMBRAPA SOJA, 2018b). E, por último a IAC 17 resistente por lepidópteras nos quais apresentam maiores níveis de resistência a *A. gemmatalis* e alguns estudos indicam que a resistência da soja está associada a metabólitos secundários (GÓMEZ et al., 2020; ZHOU et al., 2011), incluindo flavonóides (GÓMEZ et al., 2018 , 2020; PIUBELLI et al., 2005; ZAVALA et al., 2015) Além disso, está caraterizado como variedade de ciclo precoce e médio. Foi originado a partir dos cruzamentos D 72-9601-1 x ‘IAC 8’, com caraterísticas de crescimento determinado. Os índices de produtividade descritos para o cultivar “IAC 17” são de 2953 kg.ha^{-1}, (SILVA, et al., 2009).

Portanto, neste trabalho nós avaliamos se os sinais de seca poderiam afetar a sobrevivência da lagarta em genótipos caracterizados como tolerantes à seca e resistentes ao ataque de insetos. As cascatas metabólicas e de expressão gênica também foram avaliadas e correlacionadas com ensaios de sobrevivência, quando as folhas foram submetidas a ambos os sinais de seca e ataque de insetos. De maneira geral, os resultados indicam que o acúmulo de ABA sob o sinal de seca pode atuar modulando a ativação das cascatas regulatórias e melhorando a resistência ao ataque de insetos. Além disso, são discutidos os pontos chaves ao longo das cascatas regulatórias para integração dos sinais de seca e ataque de insetos, visando gerar alvos para a engenharia genética de plantas e para o desenvolvimento de plantas de soja com tolerância múltipla a estresses de seca e ataque de insetos.

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Soybean plants under simultaneous signals of drought and *Anticarsia gemmatalis* infestation trigger gene expression and metabolic pathways reducing the caterpillar survivals

Abstract

Currently is predicted that climate changes are promoting increases in global temperatures, variability in precipitation and more frequent insect pest outbreaks. Thus, understanding the molecular mechanisms for tolerance of both biotic and abiotic stresses in critical for development of improved genotypes and sustainability of the productivity. As, some physiological responses and regulatory cascades have been shared when triggered by different stress signals, we examine the regulatory cascades and metabolic pathways of drought-tolerant (EMBRAPA48), resistant to *A. gemmatalis* infestation (IAC17) genotypes, comparing with one sensitive to drought/insect attack (BR16) under both stress signals. Plants under drought were less susceptive to insect attack promoting lower caterpillar survival. Survival reductions were not dependent of the drought-tolerance or insect-resistance phenotypes, despite more pronounced for IAC17. Furthermore, metabolites profiles, gene expression and enzymatic assays lead us to concluded that only the drought signal not was enough to explain the survival reductions. Protease inhibition activities and gene expression correlated with ABA levels, indicating that JA-signaling were potentialized by ABA for enhance the production of the deterrent metabolites. Increased of ABA levels during drought treatment may be acting synergistically to increase JA response, did not affecting the first stages of LOX and JA pathways. Thus, regulatory molecular hub(s) integrating signals may be targets to genetic engineering of plants with multiple tolerances to environmental stresses.

Palavras-chave: Drought stress. Plant resistance. Metabolic profiles. Gene expression.

1 INTRODUCTION

Soybean (Glycine max (L.) Merrill) is a plant of the Fabaceae family widely cultivated in the world and its composition rich in proteins and oil allows applicability in animal and human food as well as biofuel (GOLDSMITH, 2008; BASAL *et al.*, 2020). Therefore, there is a growing demand to increase production, either by increasing the cultivated area or by increasing productivity. Earth climate is rapidly changing and predictions have indicate a continued increases in temperature and greater variability in precipitation culminating in more frequent infestation by insect pest (THE INTERGOVERNMENTAL PANEL ON CLIMATE CHANGE, 2014). However, plants have to constantly cope with a suite of biotic and abiotic stress factors. Their performance depends on the ability to quickly perceive changes in the environment and to express an adaptive response. Much effort has been made to understand the molecular mechanisms underlying plant adaptive responses because of their potential to improve agricultural production under adverse conditions (GRINNAN *et al.*, 2013).

In addition to the drought stress, soybean crops are affected by insect pests, such as the attack of *Anticarsia gemmatalis* that cause the defoliation of the plant, which reduces grain production causing great economic losses (BORTOLOTTO *et al.*, 2015; FUGI *et al.*, 2005). Drought and herbivory had the largest and the most consistent negative effects on plant performance, reducing the above- and below-ground biomass by 10-45 %, whereas increased temperature had little to no effect on plants (GRINNAN *et al.*, 2013). Thus, to maintain a sustainable productivity would be desirable that a cultivar show to be tolerant a multiple stress. Genotypes resistant to insect pests including IAC 17 and IAC 24 (FUGI *et al.*, 2005; BALDIN *et al.*, 2016; GÓMEZ *et al.*, 2020) and drought tolerant such as EMBRAPA48 have been developed in Brazil. The molecular mechanism of resistance or tolerance to environmental stresses of theses genotypes have been examined, especially for IAC17 (BALDIN *et al.*, 2016; GOMEZ *et al.*, 2018, 2020) and EMBRAPA48 (LIMA *et al.*, 2019; MESQUITA *et al.*, 2020; COUTINHO *et al.*, 2021). Adaptive plant responses to specific abiotic stresses or biotic agents are fine-tuned by a network of hormonal signaling cascades. Interesting, hormonal cross-talk modulates plant responses to abiotic stresses and defenses against insect herbivores when they occur simultaneously (NGUYEN *et al.*, 2016).

The physiological and molecular responses of the genotypes BR 16 (drought-sensitive) and EMBRAPA48 (drought-tolerant) under drought conditions have been studied by: Oya *et al*., (2004), Carvalho *et al*., (2015) and Lima *et al*., (2019); Mesquita *et al*., (2020); Coutinho *et al*., (2021). An integrative overview showed that tolerant plants maintain cell homeostasis and photosynthetic metabolism under stress conditions and involve remodeling of the cell wall and increase of the hydraulic conductance to the maintenance of cell turgor and metabolic processes (COUTINHO *et al*., 2021). In contrast, the drought signal promotes extensive gene expression reprograming in the drought sensitive BR16 which showed up-regulated several genes coding for protease inhibitors (PIs). The cultivars IAC 17 present higher levels of resistance to *A. gemmatalis* and some studies indicate that soybean resistance is associated to secondary metabolites (GÓMEZ *et al*., 2020; ZHOU *et al*., 2011), including flavonoids (GÓMEZ *et al*., 2018, 2020; PIUBELLI et al., 2005; ZAVALA *et al*., 2015;). This soybean genotype contains bioactive compounds that cause inhibitory effects on caterpillars, even when low amounts of the leaf etract are ingested. Metabolomic profiles revealed pathways involved in plant insect interactions, specifically for the synthesis of the largest amounts of methylquercetin and their glycoconjugates in the IAC 17 leaves (GÓMEZ *et al*., 2020). The cascades controlling the resistance did not involve jasmonic (JA) acid and abscisic acid (ABA), as suggested by the SA levels and LOX activity. Furthermore, these proprieties appear as constitutive characteristics, which are justified by higher levels of protease inhibition activity and flavonoid biosynthesis (GÓMEZ *et al*., 2020; PINHEIRO *et al*., 2021 under publishing).

Several ecological studies revealed that resistance to herbivores can be affected by simultaneously occurring abiotic stresses, such as drought (GUTBRODT *et al*., 2011). However, only recently there has been an increased interest to identify the molecular mechanisms underlying these interactive effects (DAVILA OLIVAS *et al*., 2016; FOYER *et al*., 2016; LU *et al*., 2015; NGUYEN *et al*., 2016). Caterpillar survival fed in plants previously submitted to water deficit was reduced which correlated with higher levels of PIs (FAUSTINO *et al.,* 2021), thus indicating that drought signals could also be acting to improve insect resistance. Therefore, in present studies we examining if drought signals could affect the caterpillar survival for other genotypes characterized as drought- tolerant and resistant to insect attack. The metabolic and gene expression cascades were also evaluated and correlated with survival assays when the leaves were submitted to both signals of drought and insect attack. Overall, the results indicate that ABA accumulation under drought signal may act modulating the activation of the regulatory cascades improving the resistance to insect attack. Furthermore, the key points for integration of the both drought and insect attack signals are discussed, aiming generate of targets to genetic engineering for development of soybean plants showing multiple tolerance to drought and insect attack.

2 MATERIAL AND METHODS

2.1 Plant growth, soybean genotypes and *Anticarsia gemmatalis* rearing

The soybean plants were cultivated in a greenhouse and then kept in cages, without addition of agrochemicals for applying the drought and insect attack stresses. The cultivar BR16 was designated as a semi-early cycle sensitive variety to drought, obtained from crossing D69-B10-M58 x Davis (OYA *et al.,* 2004; SANTOS *et al.,* 2015). The EMBRAPA48 drought-resistant cultivar (LIMA *et al.,* 2018; MESQUITA *et al.,* 2020; OYA *et al.,* 2004;) is a late-cycle variety, (OYA *et al.,* 2004; SANTOS *et al.,* 2015) with high production stability and good characteristics for human consumption, genealogy Davis x Paraná (getter) IAS 4 x BR-5 (HIGASHI *et al.,* 1999; OYA *et al.,* 2004; SANTOS *et al.,* 2015). Seeds of the BR16 and EMBRAPA48 genotypes were obtained from Empresa Brasileira de Pesquisa Agropecuária (EMBRAPA SOJA, Londrina, Paraná). The cultivar IAC17 is an early cycle variety (LOURENÇÃO *et al.,* 2010; SOUZA *et al.,* 2013), of genealogy D 72-9601-1 x 'IAC 8', considered a resistant genotype to lepidopteras (FUGI *et al.,* 2005; GÓMEZ *et al.,* 2018, 2020; MATOS *et al.,* 2002; PAIXÃO *et al.,* 2016;). The seeds of IAC17 were obtained from the Instituto Agronômico de Campinas (IAC). The germinated seeds were carefully transplanted to vessels containing 3L of soil. The plants were irrigated daily and kept in a greenhouse at 30 ± 5 °C and 60 ± 20% of relative humidity (RH).

Anticarsia gemmatalis larvae were obtained from a laboratory colony maintained at the Laboratory of Insects of the Department of Biochemistry and Molecular Biology at the Universidade Federal de Viçosa (UFV). These colonies are reared in plastic containers and fed *ad libitum* with artificial diet at 25 ± 2 ºC, 70 ± 10% relative humidity and under 14-h light:10-h dark photoperiod (GREENE, KEPPLA e DICKERSON, 1976).

2.2 Survival assays under drought stress

Two plants were grown in pots containing soil and commercial Plantmax® substrate in 1:1 ratio and kept in a greenhouse under normal water conditions until reaching the development stage V4 (fully expanded third trifoliate). The control plants were watered daily with approximately 30 mL water per plant. The plants were exposed to a slow drying soil treatment, which consisted of a reduction in irrigation to 40% of the daily normal (VALENTE *et al.,* 2009; COUTINHO *et al.,* 2019). The hydric regimes were assigned as irrigated (I) and under drought (WD). The leaf water potential (ψw) was measured in the third emerging trifoliate at dawn by using a Scholander pump (SCHOLANDER *et al.,* 1965) during the stress period.

For survival assays each, ten soybean plants (two plants by pot) from each genotype BR16 and Embrapa 48, IAC17 at V4 or V5 developmental stage were submitted to drought (WD) and irrigated (I) treatments, as described above. When the plants reached at Ψam = -1.0 were infested with 20 first instar *A. gemmatalis* caterpillars (2 caterpillars per plant) and kept in gage until the trifoliated leaf was completely consumed. After then, the caterpillars were transferred to new leaves and this procedure was repeated until pupal stage.

Larvae survival feeding each plant genotype was monitored daily during 20 days and estimated by the Kaplan-Meier method [23]. Survival curves were compared by the Log-Rank test [23].

2.3 Drought and caterpillar infestation stress assays

Two plants from each genotype were grown in 5 pots containing with soil mix and commercial Plantmax® substrate in 1:1 ratio and kept in a greenhouse under normal water conditions (I, Irrigated) and drought conditions as describe above. The leaf water potential (ψw) was monitored each day until the plant reach Ψam = -1.0. Afterward, two leaves of each plant from BR16, Embrapa 48 and IAC17 genotypes were infested (I, infested) or not (not infested) by two caterpillars (20 caterpillars by treatment). The caterpillars were kept in gages for 96 hours and the elicited leaves under insect attack were removed. Four leaves from each vessel were combined to generated pools. The biochemical evaluations were performed for 5 independent biological replicates (4 leaves by replicates). After the infestation period, the leaves were collected and stored at -80 °C for further analysis. The treatments were designated as: **1) INI** – Irrigated and not infested; **2) II** – Irrigated and infested; **3) WDNI** – under drought and not infested; **4) WDI** – under drought and infested exposed to infestation for 96 h in the three cultivars (BR16, EMBRAPA48 and IAC17).

2.4 Phytohormone Analysis by LC/MS

Metabolites were extracted from soybean leaves according to the methodology described by Coutinho *et al.,* (2019) and Lima *et al.,* (2019). Amounts of 100 mg of the powdered fresh tissues were weighed and 400 µL of extraction solvents were added (methanol: isopropyl alcohol: acetic acid 20: 79: 1). The samples were mixed in vortex 4 times for 20 sec (on ice), sonicated for 5 min, placed on ice for 30 min and then centrifuged at 13000 g for 10 min at 4°C. After centrifugation, 350 µl of supernatant was removed and transferred to a new tube. These extraction procedures were repeated was repeated with the pellet and then the supernatants were pooled.

Approximately 400 uL of the extracts were placed in vials and 5 uL were injected into the LC/MS system from NuBioMol (Center for Biomolecules Analysis-UFV, Brazil) using a chromatography column (Agilent Eclipse Plus, RRHD, 1.8 um, 2.1x50 mm) with a flow of 0.3 mL/min, coupled online to a mass spectrometer QqQ triple quadrupole (Agilent).

The generated mass spectra were processed using the Skyline software to obtain the extracted ion chromatograms (XIC) of each transition and the area values, as indicative of the abundance of each hormone (VITAL *et al.,* 2019). A standard curve of each hormone, in a concentration range of 0.1 to 300 ng/mL, was used to convert the area values from XIC in ng/g of plant tissue.

2.5 Determination of activity for lipoxygenases and protease inhibitors

The vegetable powders of each treatment were homogenized with 50 mM sodium phosphate buffer at pH 6.5 or Tris-HCl buffer at pH 8.2 according to the biochemical test performed in a ratio of 1 g of leaf to 3 mL of buffer and centrifuged at 17200g for 60 min at 4 °C. The supernatant obtained after centrifugation was stored at -20 ºC to assess LOX activity, proteinase inhibitory activity and total protein (OHTA *et al.,* 1986).

The LOX activity was determined using linoleic acid as substrate, by increasing the absorbance at 234 nm. The reaction mixture consisted of 1000 µL of 50 mM sodium phosphate buffer, pH 6.5, 10 µL of plant extract and 20 µL of 10 mM sodium linoleate. The reaction velocity was determined with the molar extinction coefficient of 25000 M^{-1} cm^{-1} for the formed product (AXELRED *et al.,* 1981).

Protease inhibitors were determined from the amount of trypsin inhibited, using L-BApNA as substrate and purified trypsin. The absorbance of the solutions was determined at 410 nm for 2.5 min of reaction. The analyses were performed in triplicate in the five replicates per treatment. The results were converted to mg of trypsin inhibited per gram of protein, according to the equation: mg of inhibited trypsin g^{-1} protein = A x B/(C x 1000 x P) where: A= absorbance at 410 nm of the control; B= sample dilution; P= protein concentration of the plant extracts (g mL^{-1}); C= trypsin factor, i.e. the product of the action of 1 μg of active trypsin on the L-BApNA substrate will give an absorbance reading of 0.019 at 410 nm (KAKADE *et al.,* 1974).

2.6 Proteolytic activity from caterpillar intestine

To obtain the enzymatic extract for the analysis of proteolytic activities, three caterpillars were collected for each repetition of their respective treatments (five biological replicates). *A. gemmatalis* larvae were dissected to collect their midguts. The midguts were removed, frozen in liquid nitrogen, and crushed using a mortar to obtain the powder. The powder was resuspended with a 10^{-3} M HCl solution and centrifuged in 2 mL plastic microtubes at 10000g for 30 min at 4 °C. The supernatant was stored at -20 ºC for further analysis regarding total-proteases and trypsin-*like* (OLIVEIRA *et al.,* 2005).

Total-protease activity was determined using 2% (w/v) azocasein as substrate in 0.1 M Tris-HCl buffer, pH 8.2 containing 20 mM $CaCl_2$ at 37 °C. The reaction mixture contained 50 μL of substrate and 60 μL of enzyme extract. The reaction was stopped by adding 240 μL of 10% (w/v) trichloroacetic acid. Next, the samples were homogenized by vortexing, maintained on ice for 15 min, and centrifuged at 10000 g for 5 min at 25 ºC to remove the precipitated protein. An aliquot of 240 μL of supernatant was poured into tubes containing 280 μL of 1 M NaOH, and then absorbance was obtained at 440 nm wavelength (TOMARELLI, 1949).

Trypsin-*like* amidase activity was determined using the substrate L-BApNA. The reaction mixture contained 0.5 mL of substrate, 0.5 mL of the buffer and 10 μL of the enzyme extract. The initial rates were determined by the formation of *p*-nitroanilida product at 440 nm wavelength as a function of time (2.5 min). The calculations were performed using the molar extinction coefficient of 8.800 (M^{-1} x cm^{-1}) for the product. To calculate the specific activities of total-proteases and trypsin-*like* amidase the absorbances obtained were divided by the total protein concentration (OLIVEIRA *et al.,* 2005).

2.7 RNA extraction, cDNA synthesis and expression analysis by qRT-PCR

The leaves were pulverized with liquid nitrogen. Total RNA was extracted from leaf tissues using Trizol reagent (Invitrogen) according to the manufacturer's instructions. RNA quality was examined in agarose gel that was stained with 0.1 µg/mL ethidium bromide (EtBr) and quantified using a Thermo Scientific NanoDrop 2000c. A total of 4 µg of RNA was used for cDNA synthesis with the FireScript RT cDNA Synthesis kit (Solis BioDyne) following the manufacturer's instructions. Gene expression was evaluated using an ABI 7500 fast thermal cycler (Applied Biosystems, Foster City, CA, USA) and Fast Master SYBR Green Master Mix (Thermo Fisher Scientific). The amplification reactions were performed with the cycling conditions:10 min at 95 ºC and 40 cycles of 94 ºC for 15 s and 60 ºC for 1 min, followed by a melting curve. RT-qPCR-specific primers were designed using the Primer-BLAST software (https://www.ncbi.nlm.nih.gov/tools/primer-blast/), with a melt temperature (T) of 59 to 61 °C, a length of 18 to 23 bp, an amplifier product size of 120 to 150 bp, and a 40 to 60% GC content. Three technical replicates were performed for each one of the three biological replicates. Gene expression was quantified using the ΔCT method and the absolute expression levels were calculated as $2^{-\Delta CT}$.

2.8 Metabolite profiling by LC/MS

Leaf extracts were prepared by grounding 150 mg of leaves in liquid nitrogen and adding 500 µl of extraction solution (75% methanol and 0.1% formic acid) to each sample according to Rogachev and Aharoni, 2007 with some modifications (GÓMEZ *et al.,* 2018). Before injections in the LC/MS system, the 2.0 mL tubes containing 500 ul of methanolic extracts were agitated for 20 seconds and centrifuged by 14,000 g for 10 min at room temperature. After centrifugation, the supernatant was filtered using a PVDF membrane of pore size 0.22 µm and 80 µl of the solution was then transferred into a vial. The analysis was carried out by injecting an aliquot of 10 µl in a Nano Liquid Chromatography – Mass Spectrometry (nanoLC/MS) using the nanoACQUITY UPLC system (Waters, Milford, MA, USA), containing a trap column and a capillary column ProteCol GHQ303 C18 3.0 µm – 300 µm × 150 mm, operating at a flow rate of 5.0 $\mu l.min^{-1}$.

The eluted metabolites were injected automatically into the micrOTOF QII mass spectrometer (Bruker Daltonics, Bremen, Germany), working in online mode with a microESI ionization needle. Before starting the LC/MS runs, the samples were desalting automatically, on-line coupled to an analytical column, using a trap C18 column for 10 min at 10 µl/mL of a mobile phase composed of 5% (B) acetonitrile and 0.02% of acetic acid (v/v). The mobile phase solutions used in this gradient program were as follows: (A) water and 0.02% of acetic acid (v/v) and (B) acetonitrile and 0.02% of acetic acid (v/v). The following gradient program was used: a linear rising ramp starting at 5%, increasing to 50% (B) for 35 min, 50% (B) for 5 min; a linear rising ramp starting at 50%, increasing to 95% of (B) for 5 min; 95% (B) for 51 min, a linear gradient descent starting at 90%, decreasing to 5% (B) for 5 min, followed by a steady condition at 5% (B) for 3 min.

The ion source operated at 200 ºC, using nebulizer gas at 0.8 Bar, dry gas at 4.0 Bar and capillary voltage at 4,500 Volts. The ion scanning for MS1 spectra in positive mode was carried out for masses ranging between 100 and 1000 m/z and between 50 and 1000 m/z for the MS2 spectra. The MS2 spectra were acquired using a data depending MS2 acquisition for the most intense peak, which presented intensities above 2000 au (arbitrary units), for chromatographic a peak ranging 1.0 min was presented. The MS2 mode was operated by AutoMS2 using argon gas for CID fragmentation and the collision stepping mode for automatically ramping of the fragmentation energy.

The data were acquired over 57 min in each LC-MS/MS analysis, using the Hystar software program, version 3.2 (Bruker Daltonics, Bremen, Germany) and the spectra were processed through the DataAnalysis software program, version 4.0 (Bruker Daltonics, Bremen, Germany), using the default settings for metabolomics. The mass spectrometer was operated in Auto-MSn mode, which collected MS2 spectra for the most intense ions of each whole scan spectrum.

A detailed description of all the steps of the profile LC/MS analyzes is shown by Gouveia *et al.,* (2019). The raw mass spectrometry data were converted into the mzXML format using the Proteowizard convert tool, through the peak-picking algorithm (TAUTENHAHN *et al.,* 2012). The list peaks were also generated in the generic format (*mgf*) by the Data Analysis software program, and the putative metabolites were identified using NIST library, containing the MS2 spectra for standard compounds downloaded from MassBank of North America, and converted for the NIST format (GOUVEIA *et al.,* 2019).

The raw data converted into mzXML were also used to compare the LC/MS metabolite profiles from leaf extracts by XCMS platform (https://xcmsonline.scripps.edu). The alignments were performed using the default parameters for the UPLC/Qtof system, with 20 ppm accuracy for MS1 and a metabolite database for *Arabidopsis thaliana*. The Multi group method was used to contrast each genotype (IAC17, BR16, EMBRAPA48) in the following treatments: II x INI x WDI x WDNI. The Multi-Omic module was used for pathway inference against the metabolite database from *Arabidopsis thaliana*. A metabolite table containing the intensity of all ions detected in the runs was exported from the XCMS platform and used as an input for statistical analysis which was carried out by the MetaboAnalyst web-based platform (http://www.metaboanalyst.ca/). The quality filters based on standard deviation methods were used to automatically remove low-quality data. The intensity values were then normalized by the median and the data were converted using the Pareto Scaling method. The data were analyzed Partial Least Squares - Discriminant Analysis (PLS-DA) to group the treatments and classify the ions (VIP score) by order of importance in the samples. One-way analysis of variance (ANOVA) and *Heatmps* were used to visualize the compounds with differential abundance.

2.9 Effect of leaf extract added to the diet of *A. gemmatalis*

For confirmed the presence of deterrent compounds in the soybean submitted drought and insect infestation stress, leaf extracts were added in the diets and the larvae development were evaluated. The Extracting solution (75% methanol and 0.1% formic acid) was added to the plant material and the containers were kept on ice for 30 minutes. Then the material was centrifuged at 10,000 g, 4 ° C, for 20 min and the recovered supernatant was lyophilized. The control was prepared using only the extraction solution that was subjected to the same procedures as the vegetable sample. The lyophilized material was solubilized in deionized water using a probe-type ultrasonic homogenizer (UltraSonic Processor, Model GE 50) for 5 cycles of 5–10 s in ice bath and centrifuged at 10,000 g, 4 ° C, for 10 min (GÓMEZ *et al.,* 2020).

The diets were prepared for the caterpillars as described by Hoffmann-Campo *et al.,* (2000). The fusion of 55g of diet was performed using the water bath and the leaf extract was added to the diet at a concentration of 1% (0.2mL of extract + 1.4mL of water) and 7.5% (1.6mL of extract). For the control treatment, the sample prepared from the extracting solution was added.

For the evaluation of the survival test, a caterpillar (5th day after hatching) was placed per cell (totaling 20 caterpillars per treatment) with a sufficient diet for its growth and development. These diets were changed daily. The treatments consist of diets plus extracts from the INI, II, WDNI, WDI treatments that were evaluated in the IAC17, BR16, and EMBRAPA48 genotypes, in concentrations of 1% and 7.5%.

3 RESULTS

3.1 Survival analysis

To evaluated the effect of drought stress treatment on the soybean resistance to caterpillar attack, three soybean genotypes were submitted or not to water deficit previously to the exposition to insect infestation (**Fig. 1**). The water supply was carried out to maintaining the leaf water potential under -1.0 MPa during caterpillar infestation, while the control plants were maintained irrigated. The genotypes showing contrasting response to water deficit. As expected, the EMBRAPA48 genotype reached the potential of -1.0 MPa two days after the BR16 (LIMA *et al.,* 2019) which is a drought sensitive genotype. Furthermore, the IAC17 was the genotype more sensitive to drought stress (-1.0 MPa at 13° day).

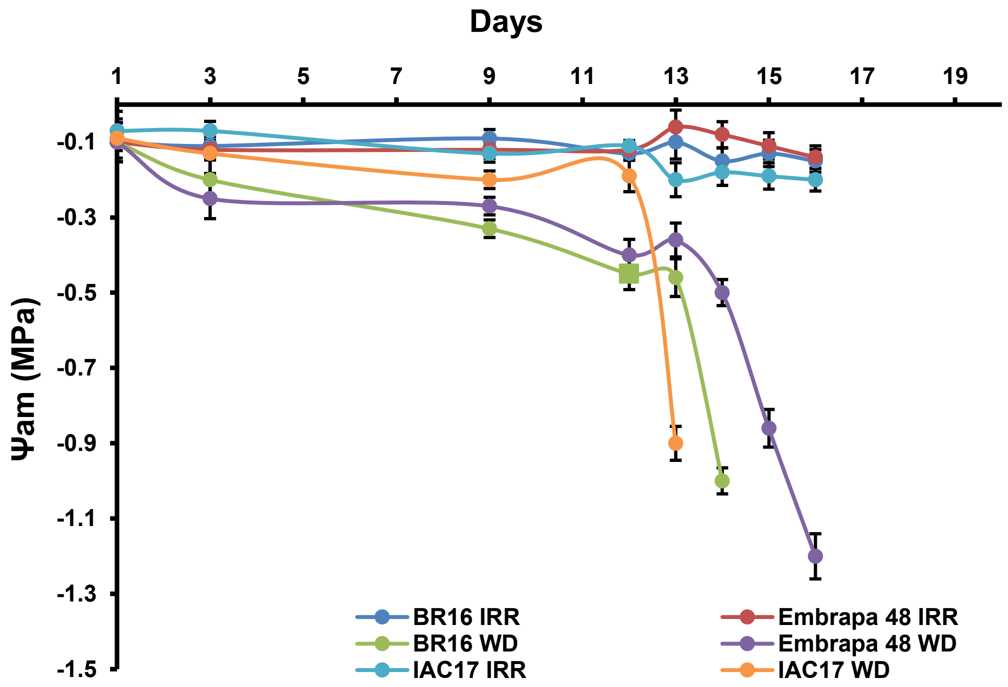

Figure 1. Temporal profile of leaf water potential (Ψam) in the morning from soybean genotypes, BR16, EMBRAPA48 and IAC17 under drought (WD) and irrigated (IRR) conditions. The bars represent the standard error of the mean (n = 5, where n represents the number of plants).

After caterpillar infestation, the survival curves were obtained using the Kaplan-Meier estimators, which indicated significant differences in the percentage of survival between larvae fed with irrigated (IRR) and under drought (WD) soybean plants (log-rank $X^2 = 14.20$, $p = 0.01$), as indicated in **Figure 2**. A gradual decline in the survival rate has observed over time.

However, this was more evident in the treatments in which the caterpillars were fed with under drought soybean plants (WD). This trend was verified for the three genotypes, however in different magnitudes. As expected, lower survivals were observed for the IAC17, a genotype resistance to caterpillar infestation (GÓMEZ *et al.,* 2018*,* 2020). Insects fed on plants elicited by drought (under drought from both the BR16 and EMBRAPA48 genotypes showed a reduction in the survival of 42% and 40%, respectively, evaluated when all the larvae that survived became pupae. However, the mortality rate of larvae fed with under drought soybean leaves was more pronounced in the IAC17 genotype, which dropped by 72% until the end of the experiment (**Fig. 2**). Furthermore, the effect of the drought treatment, related to the irrigated plants on the caterpillar survival, was more pronounced for the EMBRAPA48. Thus, the survival assays indicated that plants stressed by drought reduced the caterpillar survival and its magnitudes were genotype-dependent.

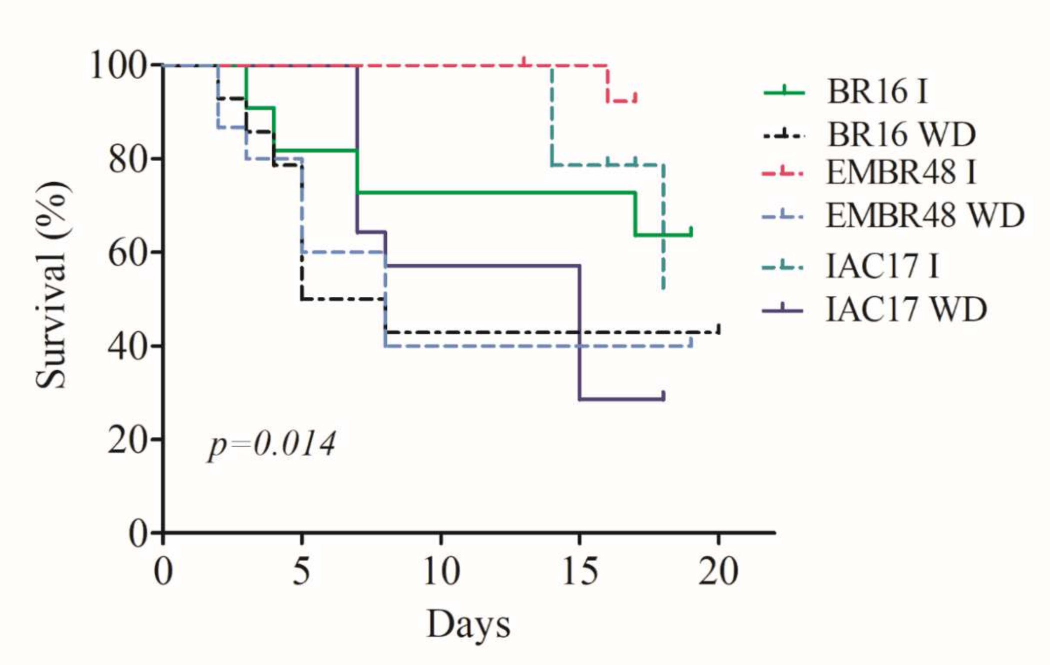

Figure 2: Survival of the Anticarsia gemmatalis larvae fed on soybean leaves of the BR16, EMBRAPA48 and IAC17 genotypes submitted to irrigated (**IRR**) and drought (**WD**) conditions.

3.2 Phytohormonal profiling

Plants need to defend themselves against environmental stresses with different molecular mechanisms triggered by signal perception which induce phytohormone biosynthesis. They act synergistically to activate signaling cascades that regulate downstream transcriptional responses. Therefore, we evaluated the absolute concentrations of phytohormones by LC/MS QqQ in soybean leaves under drought and caterpillar attack.

Jasmonic acid (JA) is the most responsive phytohormone in plant-insect interactions (YANG *et al.,* 2019). As expected, higher abundances were observed in leaves under caterpillar infestation for JA and Me-JA. JA and Me-JA levels were not modified under drought treatment (**Fig. 3** and **4**), it was possible to observe a downward trend, especially when combined under drought and infestation stresses, being more pronounced in the BR16 genotype. Methyl jasmonate which have also been related as responsive to insect infestation, also showed higher levels in the leaves of EMBRAPA48 and IAC17 genotypes (**Fig. 4**).

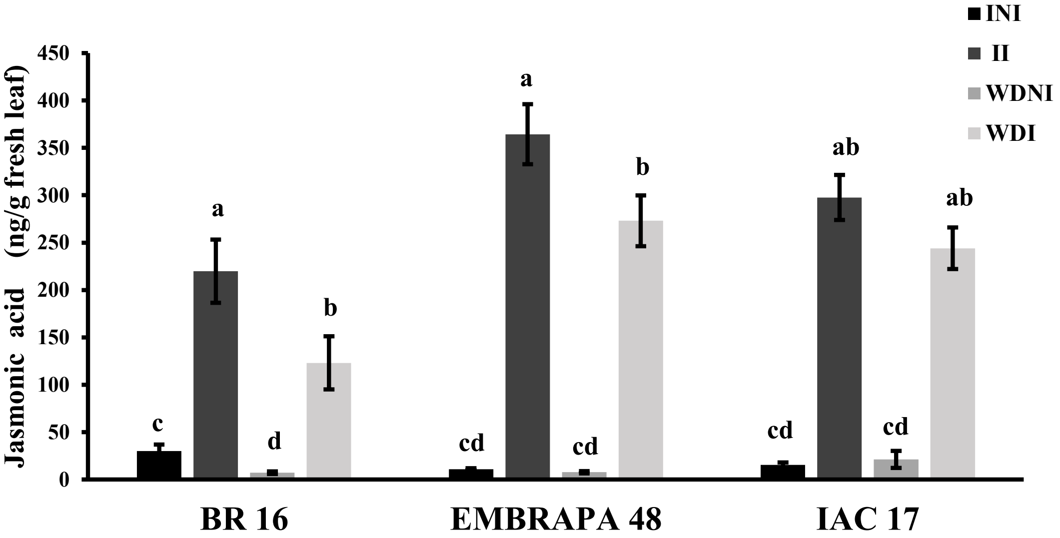

Figure 3. Levels of the phytohormone Jasmonic acid (JA) from soybean leaves of the genotypes IAC 17, EMBRAPA48 and BR16, under stresses of drought (**WD, under drought**) and of infestation by *A. gemmatalis* (**I, infested**) or in absence of caterpillar infestation (**NI**, non-infested) or under irrigation (**I, Irrigated**). Treatments: **INI** (Irrigated and Non-Infested), **II** (Irrigated and Infested), **WDNI** (Under drought and non-infested) and **WDI** (Under drought and Infested). Lower case letters represent the groups created by ANOVA and Duncan test. Categories that share the same letter did not differ in abundance. The bars represent the standard error of the mean (n = 5).

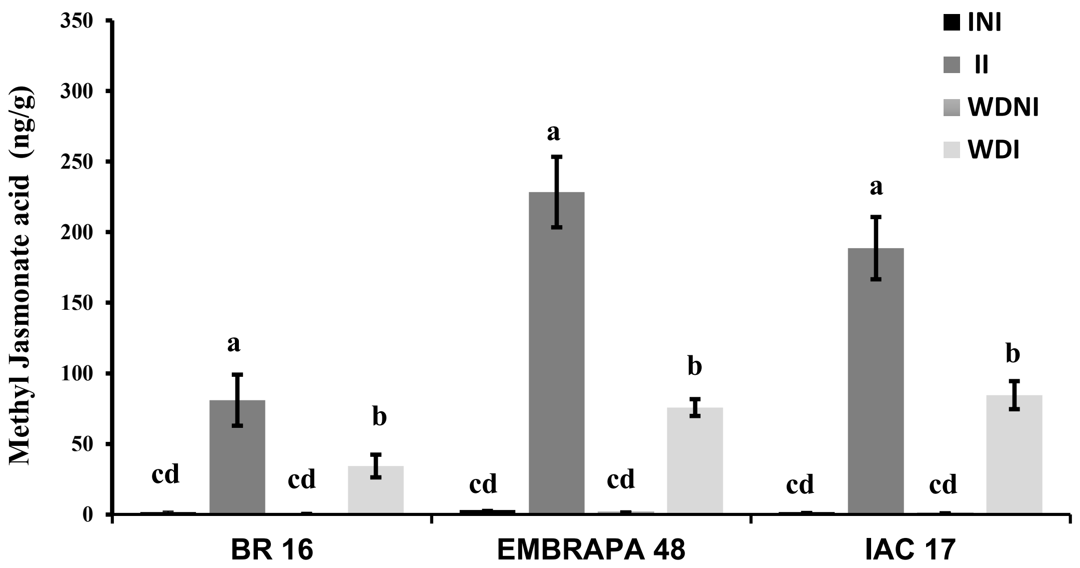

Figure 4: Levels of the phytohormone Methyl-Jasmonate (Me-JA) from soybean leaves of the soybeans genotypes IAC 17, EMBRAPA48 and BR16, under stresses of drought (**WD, under drought**) and of infestation by *A. gemmatalis* (**I, infested**) or in absence of caterpillar infestation (**NI**, non-infested) or under irrigation (**I, Irrigated**). Treatments: **INI** (Irrigated and Non-Infested), **II** (Irrigated and Infested), **WDNI** (Under drought and non-infested) and **WDI** (Under drought and Infested). Lower case letters represent the groups created by ANOVA and Duncan test. Categories that share the same letter did not differ in abundance. The bars represent the standard error of the mean (n = 5)

Despite the role of abscisic acid (ABA) in insect-plant interactions being less understood, it also plays an important role in the abiotic stress tolerance by acting as a positive regulator of JA-dependent resistance (VOS *et al.,* 2013, 2019). The levels of ABA were increased in the leaves under drought treatment (**Fig. 5**), however for EMBRAPA48 and BR16 genotypes the infestation and irrigated treatment also increased the ABA levels. A positive interaction between abiotic and biotic stresses was observed when both treatments were applied, especially for EMBRAPA48 genotype (**Fig. 5**). Interestingly, for the resistant IAC17 genotype the changes in the ABA levels were less pronounced in contrast with the increasing detected for EMBRAPA48. Thus, the higher reduction in the caterpillar survival (**Fig. 2**), observed for EMBRAPA48 and BR16 correlated to the levels ABA induced under drought conditions. Furthermore, this effect was increased when the leaves were submitted to both stresses. This hypothesis is in accordance with the synergistic effect of ABA with JA to induced the cascades controlling the plant response to insect attack (TIWARI *et al.,* 2017).

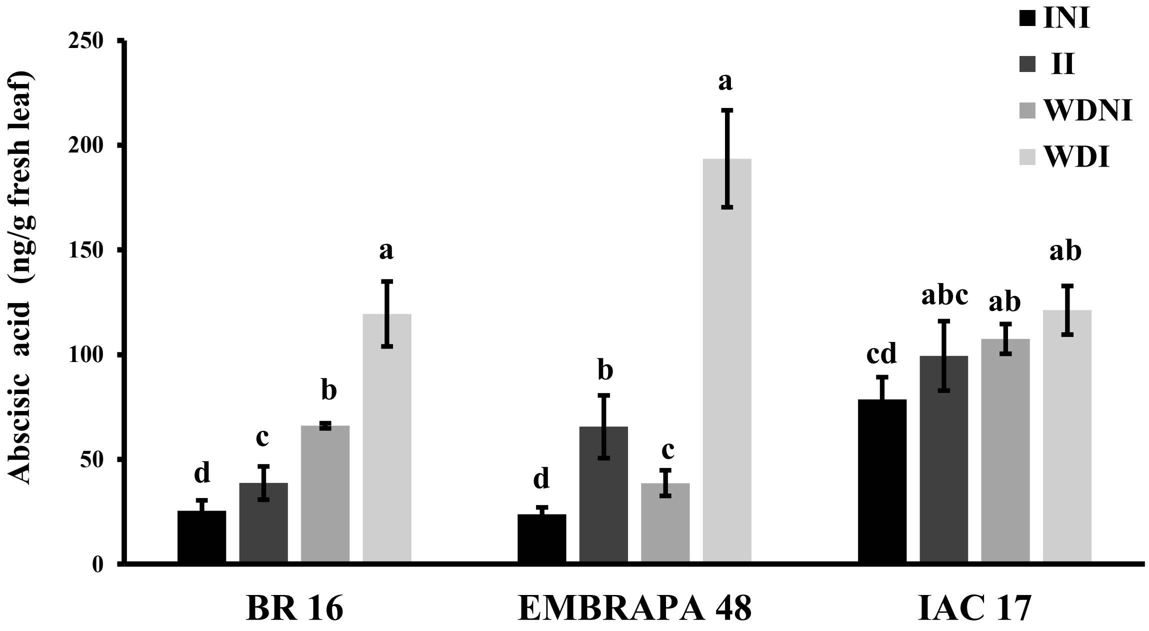

Figure 5. Levels of phytohormone Abscisic acid by LCMS QqQ of soybean leaves of the soybean genotypes IAC 17, EMBRAPA48 and BR16, under stresses of drought (**WD, under drought**) and of infestation by *A. gemmatalis* (**I, infested**) or in absence of caterpillar infestation (**NI**, non-infested) or under irrigation (**I, Irrigated**). Treatments: **INI** (Irrigated and Non-Infested), **II** (Irrigated and Infested), **WDNI** (Under drought and non-infested) and **WDI** (Under drought and Infested). Lower case letters represent the groups created by ANOVA and Duncan test. Categories that share the same letter did not differ in abundance. The bars represent the standard error of the mean (n = 5).

The role of salicylic acid (SA) in plant responses to pathogens has been well documented, but its direct and indirect effects on plant responses to insects are not very well understood. SA levels did not show changes in response to caterpillar infestations, however it was evident a reduction under drought conditions (**Fig. 6**). We could highlight the highest levels detected for the IAC17, however it appears was not related to the effect of the drought treatment in the caterpillar survival acting in the resistant genotype. Although exceptions occur, SA and JA usually have antagonistic effects (PIETERSE *et al.,* 2012, HOYSTED *et al.,* 2017), and this can modulate the expression of plant resistance, however it was not the case for the resistant genotype IAC17 (**Fig. 6**).

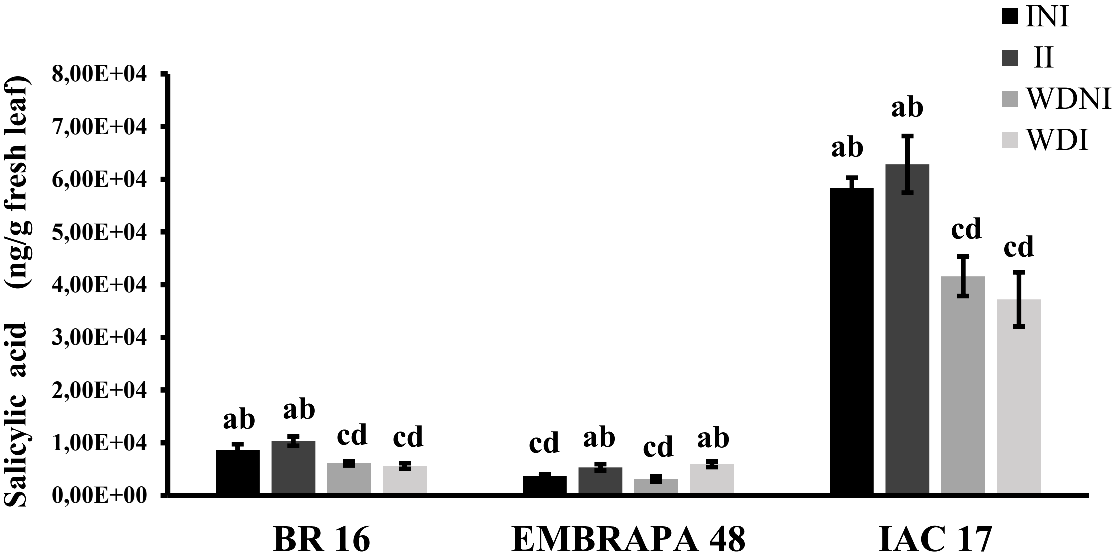

Figure 6. Levels of the Salicylic acid (SA) by LCMS QqQ of soybean leaves of the soybean genotypes IAC 17, EMBRAPA48 and BR16, under stresses of drought (**WD, under drought**) and of infestation by *A. gemmatalis* (**I, infested**) or in absence of cartepillar infestation (**NI**, non-infested) or under irrigation (**I, Irrigated**). Treatments: **INI** (Irrigated and Non-Infested), **II** (Irrigated and Infested), **WDNI** (Under drought and non-infested) and **WDI** (Under drought and Infested Lower case letters represent the groups created by ANOVA and Duncan test. Categories that share the same letter did not differ in abundance. The bars represent the standard error of the mean (n = 5).

3.3 Effects of drought stress and insect herbivory on the LOX and PIs activities

As Jasmonic acid (JA) is an important signaling molecule in the plant defense which is synthetized through lipoxygenase (LOX) pathway, we also evaluated their activities in response to drought (**Fig. 7**). The lipoxygenase activity increased after caterpillar treatment for the IAC17 and BR16 genotypes. However higher levels were detected under drought conditions, except for the EMBRAPA48 which were detected higher levels in the absence of the caterpillar infestation. Furthermore, the LOX activity under drought did not follow the behavior observed for JA levels (**Fig. 3**) which were reduced under drought conditions. Thus, another regulatory mechanism could be acting to regulated the LOX activity under drought signaling.

Cascades regulated by JA include the increase of the abundance of protease inhibitors (GÓMEZ *et al.,* 2020) under insect attack as detected for all genotypes evaluated. As verified by Gomez *et al.,* (2020), the protease inhibitory activities were higher in the resistant genotype IAC17 when compared to other genotypes (**Fig. 8**), mainly in the presence of the caterpillar infestation. The protease inhibition was 4-fold higher in the leaves of resistant genotype IAC17 in the presence of elicitation by caterpillar. Interestingly, the protease inhibitors levels were increased in response to drought in the BR16 genotype. Otherwise, it was reduced for the IAC17 under drought and return to background levels when the water deficit stress and infestation were combined. However, for EMBRAPA48 the drought treatment reduced the protease inhibitors levels and it was restore to background when combined the both stresses (**Figure 8**). These results are following the survival curves from the BR16 and IAC17 genotypes and suggest that the reduction in the caterpillar survival could be related to the protease inhibitors production at least for the BR16 and IAC17. Consequently, the protease inhibitors may contribute partially to the differences of resistance to *A. gemmatalis* showed by the genotypes.

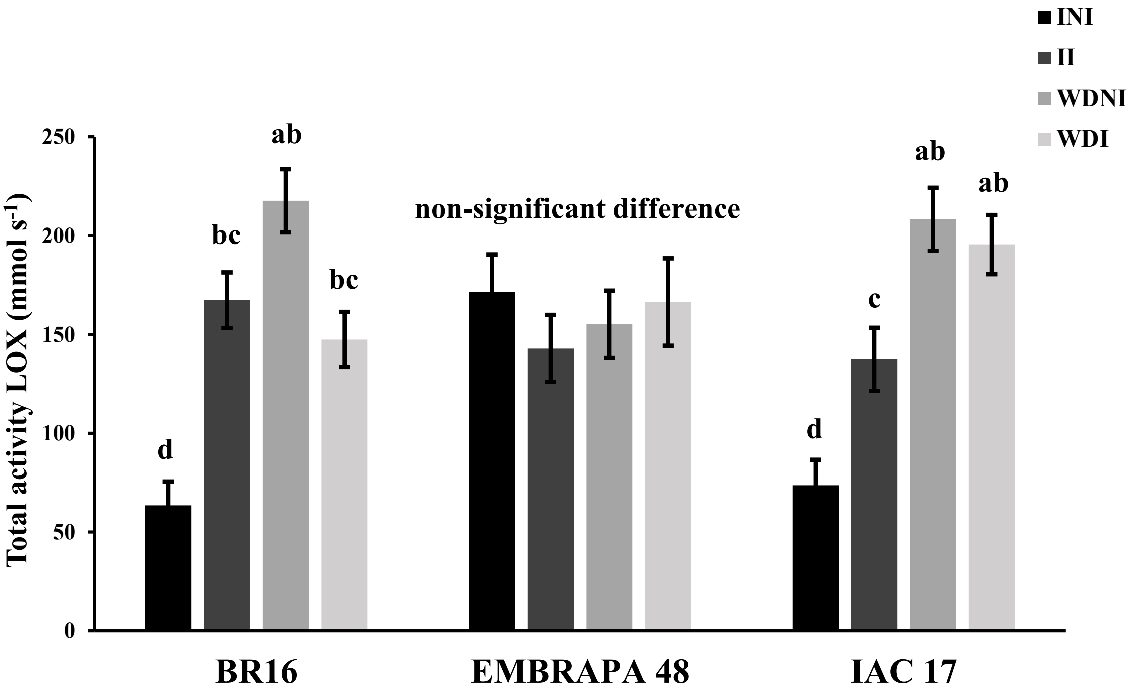

Figure 7. Lipoxygenase activity in the leaves of the soybeans genotypes IAC 17, EMBRAPA48 and BR16, under stresses of drought (**WD, Under drought**) and of infestation by *A. gemmatalis* (**I, infested**) or in absence of caterpillar infestation (**NI**, non-infested) or under irrigation (**I, Irrigated**). Treatments: **INI** (Irrigated and Non-Infested), **II** (Irrigated and Infested), **WDNI** (Under Drought and non-infested) and **WDI** (Under Drought and Infested). Lower case letters represent the groups created by ANOVA and Duncan test. Categories that share the same letter did not differ in abundance. The bars represent the standard error of the mean (n = 5).

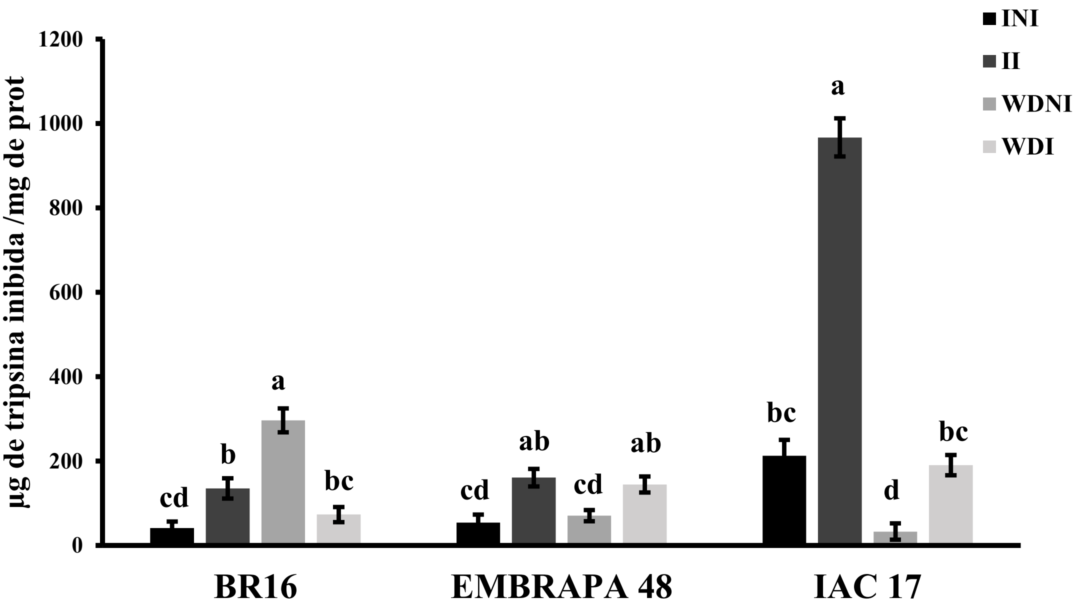

Figure 8. Activity of protease inhibitors in the leaves of the soybeans genotypes IAC 17, EMBRAPA48 and BR16, under stresses of drought (**WD, under drought**) and of infestation by *A. gemmatalis* (**I, infested**) or in absence of caterpillar infestation (**NI**, non-infested) or under irrigation (**I, Irrigated**). Treatments: **INI** (Irrigated and Non-Infested), **II** (Irrigated and Infested), **WDNI** (Under drought and non-infested) and **WDI** (Under drought and Infested). Lower case letters represent the groups created by ANOVA and Duncan test. Categories that share the same letter did not differ in abundance. The bars represent the standard error of the mean (n = 5)

3.4 Enzymatic activity in the intestine of *Anticarsia gemmatalis*

Plant resistance to caterpillar infestation by antibiosis has been related to the reduction in intestine digestibility by plant defense proteins that limit the rate of enzymatic conversion of ingested leaf. The major class of defense protein is protein inhibitors (PI). Thus, the proteolytic activities of the intestines of *A. gemmatalis* were also evaluated to verify if the survival reduction by drought treatment correlated with the inhibitory effect on the digestive capacity of the caterpillars (**Fig. 9**). As expected, total trypsin activity of the intestines was significantly reduced for caterpillars fed with leaves from resistant to insect attack IAC17 (**Fig. 9A**). However, the amidasic trypsin activities were lower in the intestines of caterpillars fed with leaves of all genotypes under drought stress (**Fig. 9B**). The highest reductions were verified for BR16 (49.8%) and IAC17 (44.6%) genotype (**Figure 9B**). These results are in accordance with the levels of the protease inhibition activities detected in the soybean leaves (**Fig. 8**) and it is an indicative that reduction in caterpillar survival under drought stress can involve protease inhibitors for IAC17 and BR16. Otherwise, for EMBRAPA48 the effect in the survival is not related to induction of the protease inhibitor biosynthesis signaled by water deficit (**Fig. 8**).

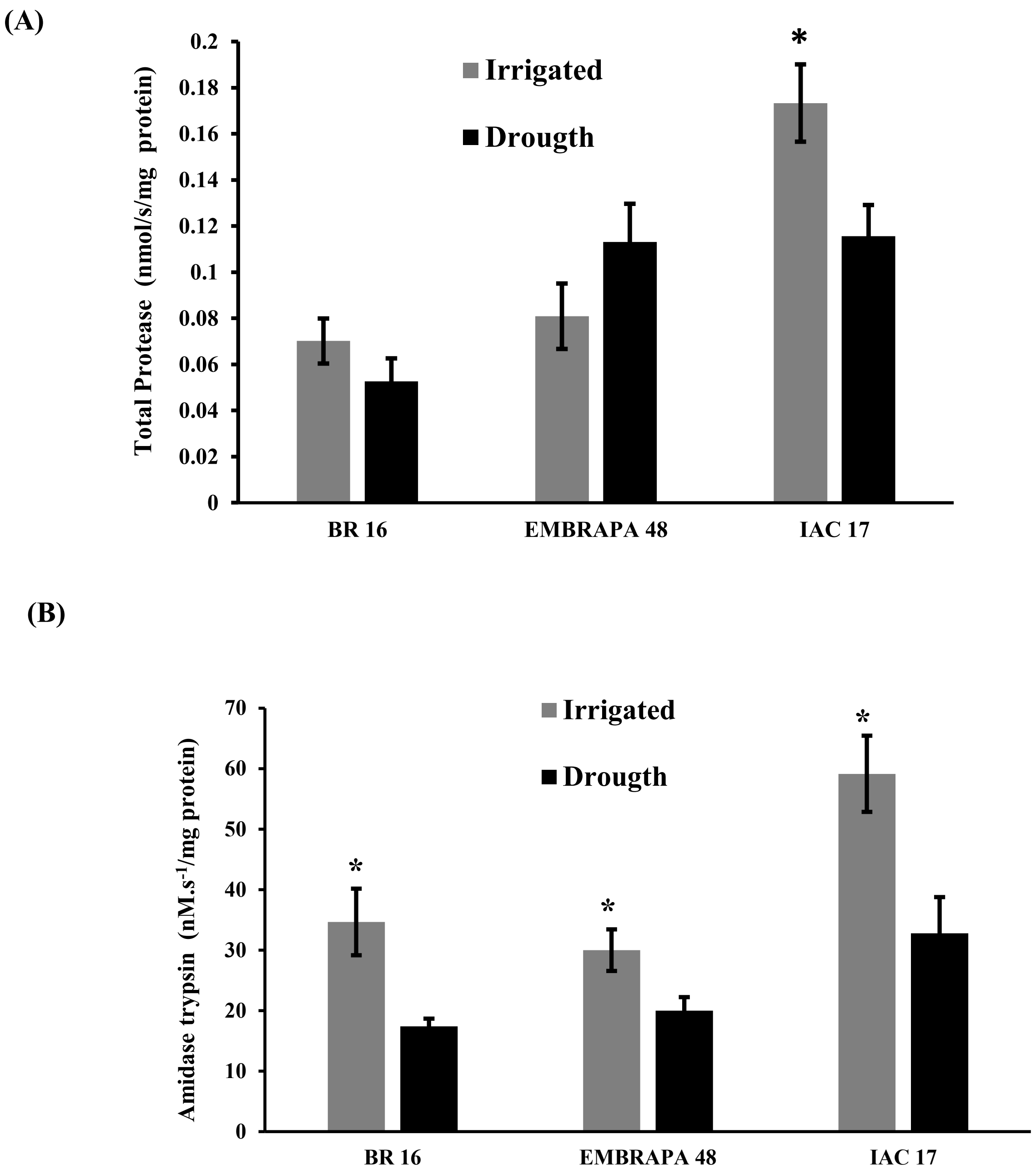

Figure 9: Enzymatic activity of protease total (**A**) and trypsin like (**B**) and from *A. gemmatalis* intestines fed with irrigated soybean leaves and water deficit. Pair-wise comparations were performed to evaluated the effect of the drought in the protease activities and asteristic indicate significative differences by Student's test ($P < 0.05$). The bars represent the standard error of the mean (n = 5).

3.5 Gene expression analyzes by qRT-PCR

The expression of Lipoxygenase and serine protease inhibitor (Bowman-Birk e SKTI) genes that are comment involved in the caterpillar's response was evaluated, thus their expressions were evaluated in response to drought treatment in plants under or not to herbivory

(**Fig. 10**). As expected, the expression levels for LOX, BBI and SKTI genes were upregulated for genotypes IAC17 and BR16 under caterpillar infestation (contrast INI x II). Exceptions were observed for the downregulation of the PIs in the EMBRAPA48, despite of the LOX gene expression has been induced. Thus, for EMBRAPA48 the LOX and jasmonic acid pathways did not induced PIs expression, as also indicated by LOX activity (**Fig. 7**), JA levels (**Fig. 3**) and PI activity (**Fig. 8**).

However, these expression patterns were altered when the plants were submitted to drought (WD) treatment before submission of caterpillar infestations. For all genotypes, the drought treatment induced the LOX expression, despite of this increase in their transcription not induced BBI expression for IAC 17 and EMBRAPA48 (**Fig. 10**). However, for SKTI gene the LOX induction correlated with the increase in the LOX expression for these genotypes. Thus, the drought signaling can or not culminate in the gene regulation by the LOX and JA cascades, dependent of the genetic backgrounds of soybean genotype evaluated.

PI expression levels by qRT-PCR correlated partially with the results of the protease inhibitor activities which upregulations were observed for SKTI in IAC17 and EMBRAPA48 genotypes. However, the increase in inhibitory activity was observed only for BR16 in response to drought treatment. Furthermore, these increases in inhibitory activity observed for BR16 may be attributed to BBI expression or by upregulation of the other PI genes in soybean plants in response to drought, as observed by Coutinho *et al.,* (2021) in the transcriptomic analysis by RNAseq. In this study, a large number of PI genes were detected as upregulated only for BR16 and not for EMBRAPA48 (COUTINHO *et al.,* 2021).

Even though the expression of the BBI gene has been repressed under drought for IAC17 and EMBRAPA48, when the plants under water deficit were submitted to caterpillar infestation the expression levels were observed a higher than treatments with only infestation or drought (**Fig. 10**). Thus, when both signals were applied simultaneously the expression was potentialized, acting synergistically. This observation is in accordance with the JA levels (**Fig. 3**) that were maintained high in plants under both stresses. Interspersing, the ABA levels were always higher for the treatments whose plants under both stresses, despite of this increase has been minor pronounced in the resistant genotype IAC17.Thus, the ABA phytohormone may be involved in the synergism processes for gene expression inducer and in the cascades culminating in the reduction of the caterpillar survivor in plants under drought stress.

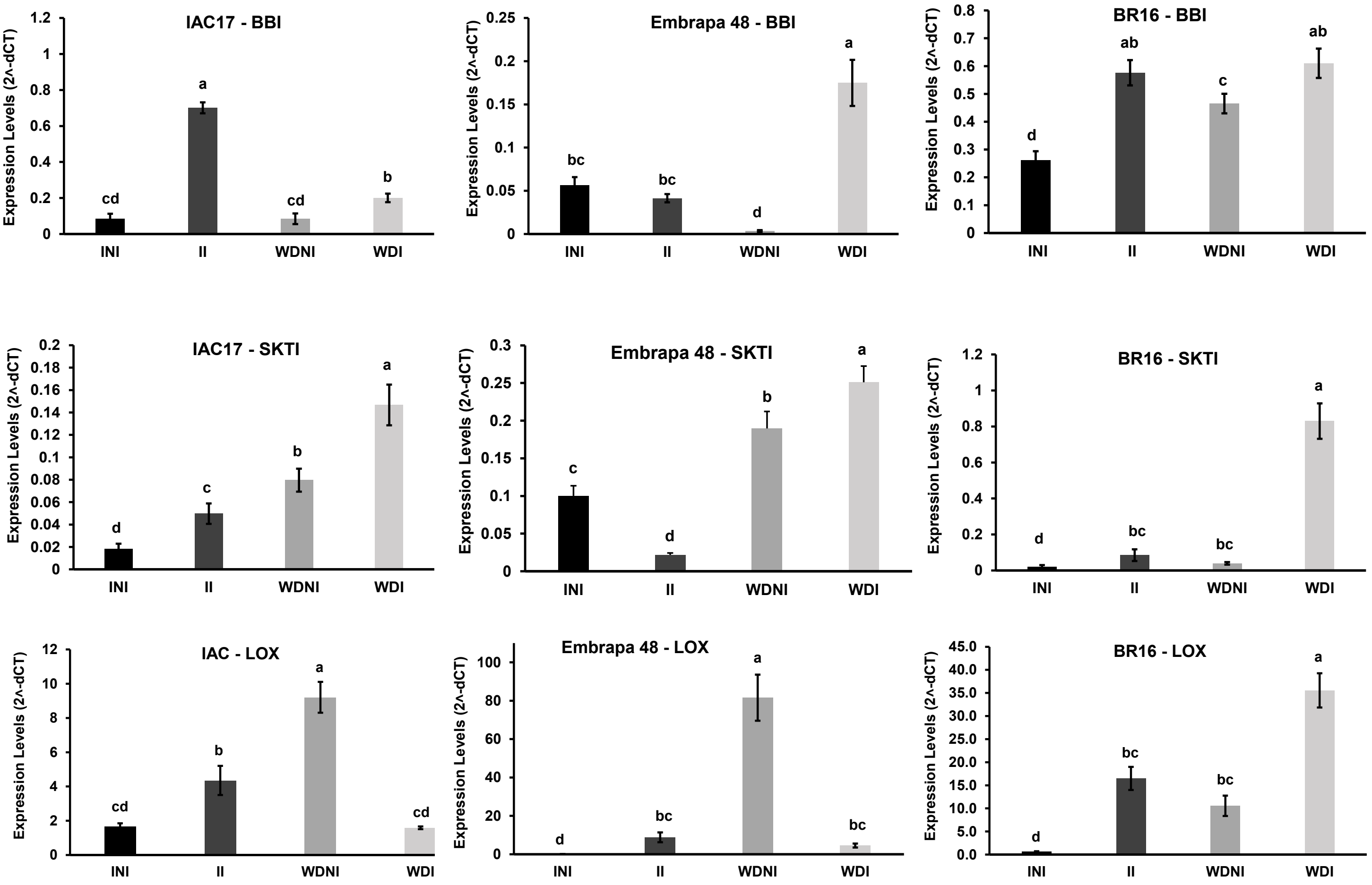

Figure 10: Gene expression analysis of the Lipoxygenase (**LOX**) gene and of the genes encoding for protease inhibitors (PIs) Bowman-Birk (**BBI**) and Kunitz trypsin inhibitor (**SKTI**) genes by qRT-PCR of the soybeans genotypes IAC 17, EMBRAPA48 and BR16, under stresses of drought (**WD, under drought**) and of infestation by *A. gemmatalis* (**I, infested**) or in absence of caterpillar infestation (**NI**, non-infested) or under irrigation (**I, Irrigated**). Treatments: **INI** (Irrigated and Non-Infested), **II** (Irrigated and Infested), **WDNI** (Under drought and non-infested) and **WDI** (Under drought and Infested). Lower case letters represent the groups created by ANOVA and Duncan test. Categories that share the same letter did not differ in abundance. Categories that share the same letter did not differ in abundance. The expression levels were obtained using the $2^{-\Delta CT}$ method and analyzed by (Student's test ($P < 0.05$). Bars: mean ± SE; n = 3.

3.6 Metabolite profiling

Many secondary metabolites do not affect the normal growth and development of plant, but they reduce the palatability of the tissues, acting as repellents or reducing the insect survival (antixenosis or antibiosis). Several compounds and metabolic pathways have been related to insect resistance, mainly flavonoids in the soybean genotypes IAC17 showing resistance to *A. gemmatalis* (GÓMEZ *et al.,* 2018; 2020; PIUBELLI *et al.,* 2005). Thus, a LC/MS-based non target method was applied to compare dysregulated compounds specifically present in the leaves that explain the differences in the caterpillar's survival as observed in Figure 1.

Converted LC/MS raw data were used to compare the metabolite profiles between the contrasting genotypes and assessment of the fluctuation of the metabolites from soybean leaves under both drought and insect attack stresses.

The PCA and PLS-DA methods were used to analyse the samples according to the most significant variables and for differential grouping of the contrasting genotypes (**Fig. 11**). As expected, the profiles showed a distinct general behavior of the soybean genotypes IAC17, BR16 and EMBRAPA48 due to their different genetic backgrounds. However, their responses to the presence of the caterpillars as a pre-treatment were also distinct (**Fig. 11**).

PLS-DA method was used to analyse the samples according to the most significant variables and determine how the differential accumulation of all metabolites correlated with treatments. As expected, the PLS-DA plot of the LC/MS profiles (**Fig. 11**) indicated distinct patterns of response to both stresses between genotypes in absence of the inoculations because the genotypes used are contrasting related to tolerance to drought and resistance to caterpillar attack (COUTINHO *et al.,* 2019; GÓMEZ *et al.,* 2020; LIMA *et al.,* 2019). BR16 genotype has been considered to be both drought sensitive and susceptible to *A. gemmatalis* infestation, thus the replicates of the four treatments shown distant grouping (**Fig. 11A**). Lower magnitude of response was observed for infestation (NI x II, 9.2% of the total variance) related to response to drought (INI x WDNI, 32.1% of the total variance), as also expected. Interesting, the group separation was also high when comparing the variability of the metabolite abundances in plants being infested by caterpillar elicited or not by the drought treatment and responding to both stress signals (II x WDI, 32.1% of the total variance).

In contrast, for the genotypes resistant to caterpillar infestation IAC 17 and drought tolerant EMBRAPA48 the grouping separation was less evident (**Fig. 11 B** and **11C**). As expected also, a lower magnitude of the variability abundance was observed for drought of the EMBRAPA48 (INI x WDNI, 28.3% of the total variance) compared with BR16 (**Fig. 11A**), as also observed by Lima *et al.,* (2019) and Coutinho *et al.,* (2021). Surprisingly, the PSL-DA results for EMBRAPA48 did not show group separation for a response to infestation, but only for plants infested by caterpillar elicited or not by the drought treatment and, as observed for BR16, responding to both stress signals (II x WDI, 28.3 % of the total variance). For IAC 17, the grouping was more evident for the response to infestation (**Fig. 11C**).

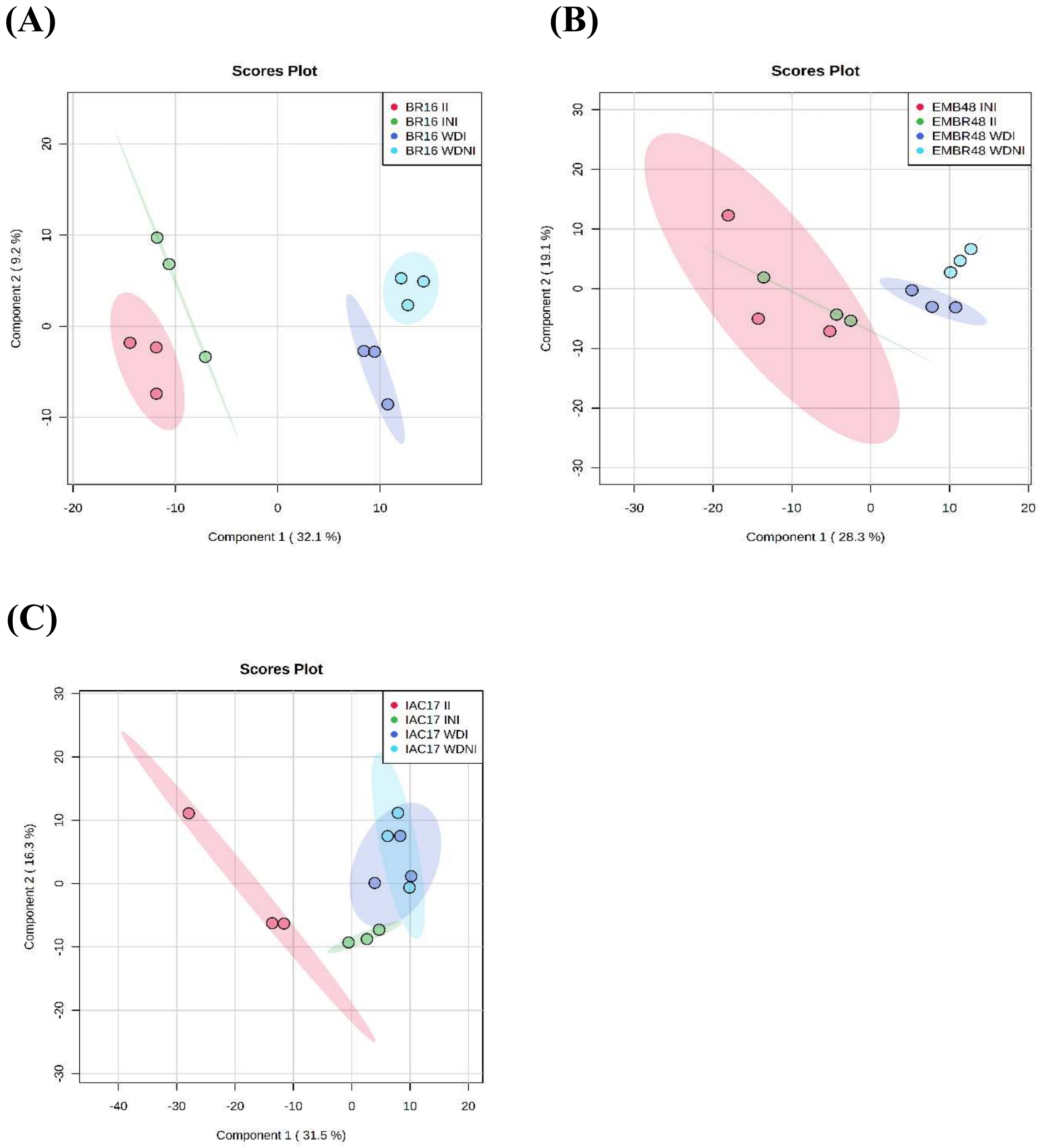

Figure 11: Analysis of 2D Scores Plot by Partial Least Squares Discriminant Analysis (PLS-DA) of the metabolite profiles by LC/MS from soybean leaves. In **(A)** BR16, **(B)** EMBRAPA48 and **(C)** IAC17, under stresses of drought (**WD**, with drought) and of infestation by *A. gemmatalis* (**I**, infested) or in absence of caterpillar infestation (**NI**, non-infested) or under irrigation (**I**, Irrigated). Treatments: **INI** (Irrigated and Non-Infested), **II** (Irrigated and Infested), **WDNI** (With Drought and non-infested) and **WDI** (With Drought and Infested). Points represent replicates analyzed.

After alignment and retention time corrections, the extracted ion chromatograms (XICs) were used for comparisons of the m/z and intensities between treatments. The variance analysis by ANOVA and a heatmap plot were also used to verify the dysregulated metabolites from leaf extracts with higher relative abundances under infestation and compare with those also increase and responsive to drought (WDNI) that justify the reduction of the caterpillar survival (**Fig. 12**). First of all, we searched the ions responsive only to caterpillar infestation that were also increased in leaves of the drought-stressed plants compared with the irrigated plants, to verify if this stress signal altered the abundance metabolites naturally responsive to insect attack. For implemented this approach, the ions dysregulated for the contrasts INI x II and INI x WDNI were evaluated for each genotype. In general, the detected ions up dysregulated in response to infestation were not responsive to drought for all genotypes. In contrast, many for these compounds were down dysregulated, as for example the clusters M373T46 to M351T31 for the BR16 (**Fig. 12A**), M184T61 to M501T61 for EMBRAPA48 (**Fig. 12B**) and, M625T24 to M146T15 and M623T66 to 357T11 for IAC17 (**Fig. 12C**). Therefore, none putative metabolites, acting in the reduction of the caterpillar survivor with low molecular mass and non-polar, which were present in the methanolic extracts and detected by LC/MS, were not up-dysregulated by just by drought signal.

However, some ions were observed up-dysregulated forming groups only for plants under both stress signals, for example the clusters M177T33 to M213T51 from BR16 profiles (**Fig. 12A**), M255T32 to M245T8 from EMBRAPA48 profiles (**Fig. 12B**) and M131T56 to M276T54 from IAC17 profiles (**Fig 12C**). Thus, as observed in gene expression results, the signaling may be acting in synergism to modulated the leaf metabolism to increase the abundance of metabolites reducing the caterpillar survivor in plants under drought stress.

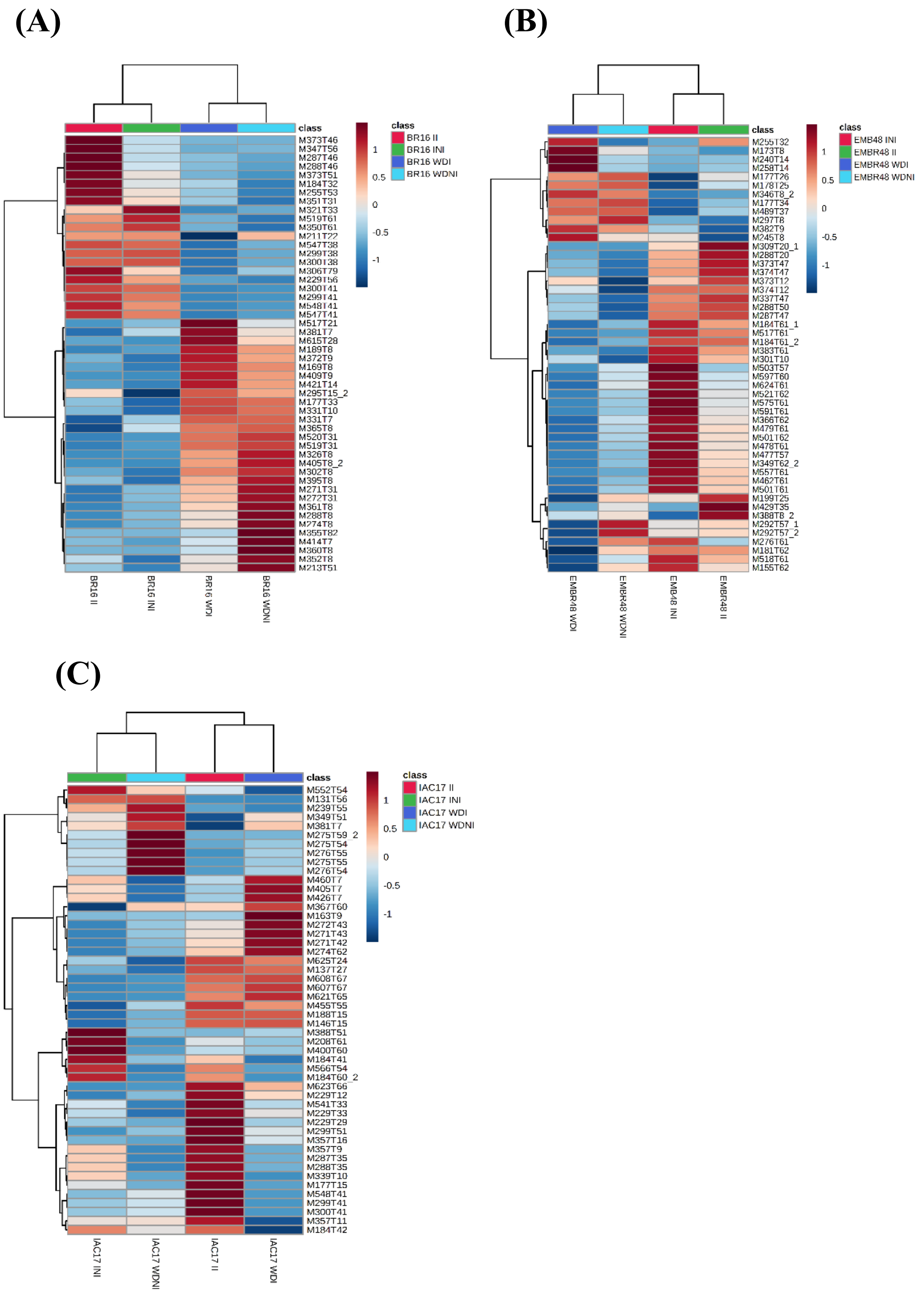

Figure 12: Clustering analysis by HeatMap method of the characterized metabolites in the leaves of the soybean genotypes (A) BR16, (B) EMBRAPA48 and (C) IAC17, under stresses of drought (**WD**, under drought) and of infestation by A. gemmatalis (**I**, infested) or in absence of caterpillar infestation (**NI**, non-infested) or under irrigation (**I**, Irrigated). Treatments: **INI** (Irrigated and Non-Infested), **II** (Irrigated and Infested), **WDNI** (Under drought and non-infested), and **WDI** (Under drought and Infested). This shows the differences in the abundance of the metabolites analyzed by LC-MS in response to treatments. Blue color represents a decrease, and red color an increase. The ions from LC/MS were exported from XCMS package as features where “**M**” designed the nominal mass and “**T**” the retention time.

Using the MS2 spectra for searcher against NIST spectral library, it was possible identify some dysregulated compounds showing higher leaves under both drought and infestation stresses, as indicated in the **Figure 12**. Some compounds belonging the class of the flavonoids and coumarins, identify as 6-O-malonylgenistin, 7-formy 6-nitrocoumarin, 3Methoxyapigenin (Chrysoeriol) and 6-O acetylgenistein (**Fig. 13**)

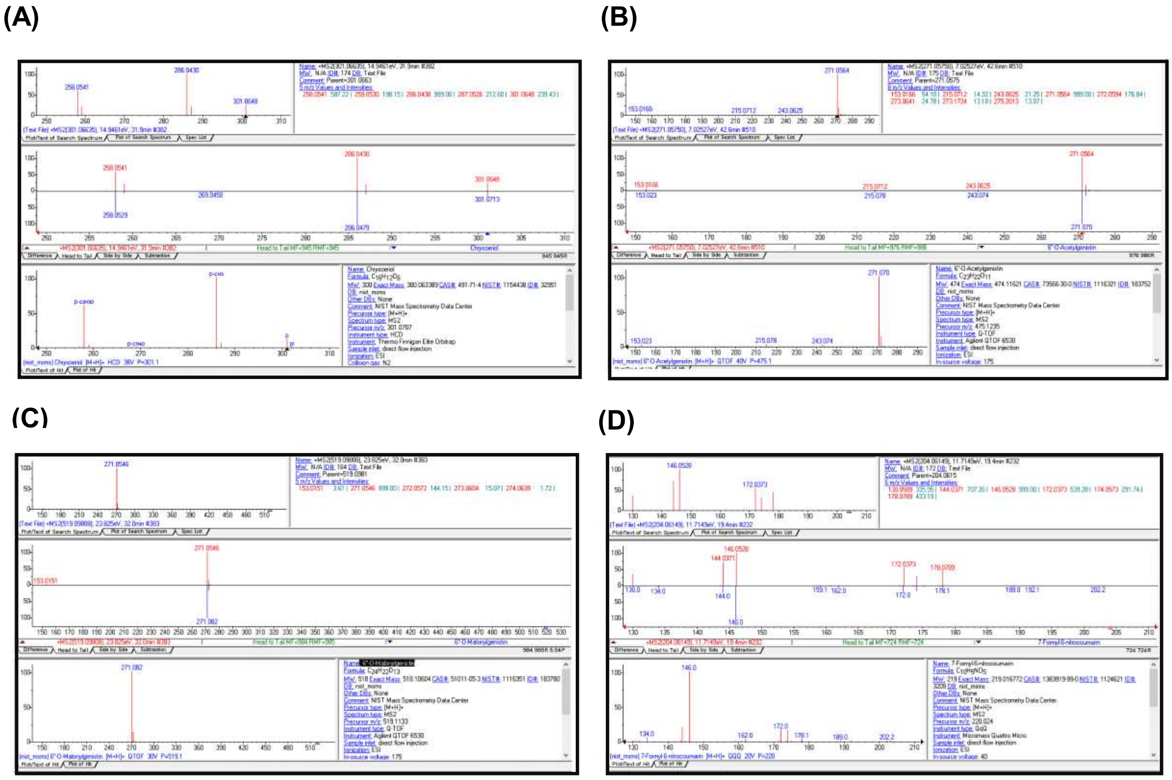

Figure 13: Dysregulated compounds identify by search against NIST spectral library. In **(A)** 3'-Methoxyapigenin (Chrysoeriol); in **(B)** 6-O acetylgenistein; in **(C)** 6-O-malonylgenistin and **(D)** 7-formy 6-nitrocoumarin.

3.7 Survival analysis of caterpillars fed diets supplemented with leaf extracts

Analyses of plant resistance and susceptibility to insect attack were performed by evaluating the larva-adult development and survival after the caterpillars were fed the leaves (**Fig. 1**). In these assays, the plants were submitted to water deficit and followed the infestation by the caterpillar were allowed, thus at this moment the previous trigger signal by drought are combined also with the signal of insect attack. Thus, using this assay make it impossible to observed the effect of the drought signal to triggering the biosynthesis of deterrent compounds in the soybean leaves.

Thus, the survival assays were also carried out for caterpillars fed diets containing extracts from soybean leaves in response to drought treatment in plants under or not to herbivory. In addition, the leaf extracts were added in the diets in two concentrations and evaluated the caterpillar survival and pupal weight (**Fig. 14**). In the conditions and concentration used in the testing survival, the pupal weight and survival of the caterpillars were slightly affected. Some reductions on survival were observed for BR16, however it was not significative (**Fig. 14**). On the other hand, the survivals were significantly reduced by the presence of the leave extracts from resistant genotype soybean IAC17 in the diet. Interestingly, reductions of the survival were incremented when were added extracts from plant leaves elicited by both stresses (WDI). These results could be explained by low concentrations of the deterrent metabolites from methanolic extracts. Probably, the amount of leaf material, to the BR16 and EMBRAPA48 genotypes used for extraction preparations, have been insufficient. The reductions in the survival observed for IAC17 can be explained by the fact of compounds acting as deterrents have been detected in higher levels in the leaves even in absence of the herbivory elicitation. Thus, for IAC17 resistance to *A. gemmatalis* appear to be a characteristic constitutive (GÓMEZ *et al.,* 2018 2020).

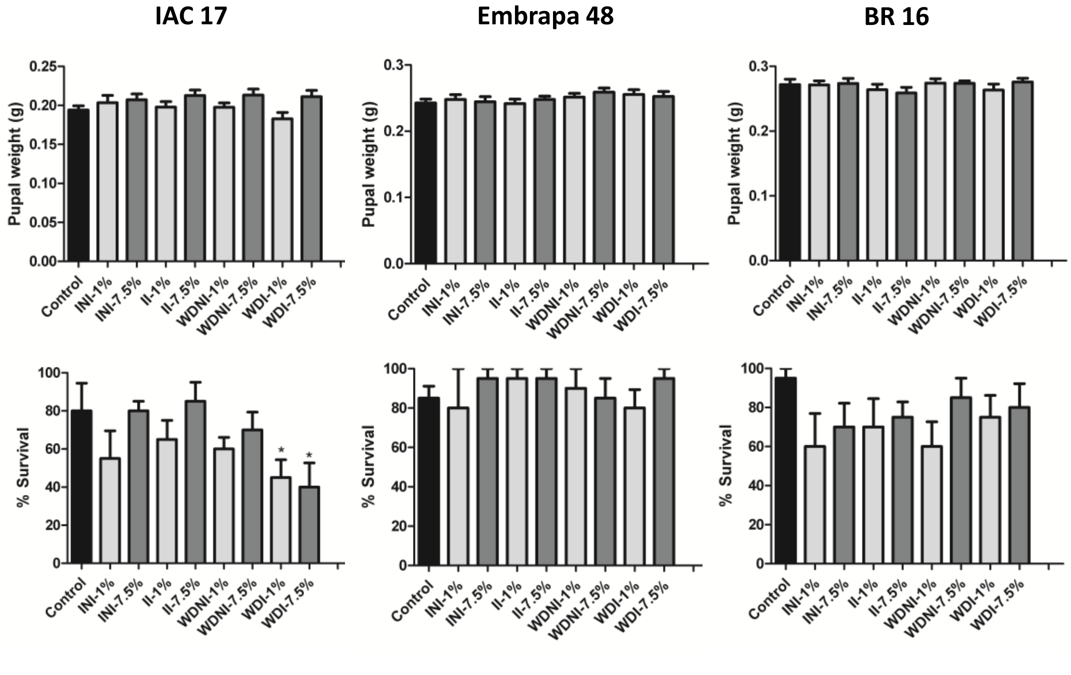

Figure 14. Effect of the diet supplementation with metabolite extracts from soybean leaves of the genotypes IAC17, Embrapa 48 and BR16 under drought and insect attack stresses in the (A) pupal weight and (B) survival of the *A gemmatalis* caterpillars. Treatments: **INI** (Irrigated and Non-Infested), **II** (Irrigated and Infested), **WDNI** (Under drought and non-infested), and **WDI** (Under drought and Infested). The extracts were prepared at 1.0 and 7.5%. Statistical tests were performed according to ANOVA followed by Duncan's test. * Indicates significant difference between treatments and control (p <0.05).

Despite the less effect in the caterpillar survival and pulpal weight, the pre-pupal stage durations were significantly affected by extracts from IAC17 and EMBRAPA48 when added in the diets (**Fig. 15**).

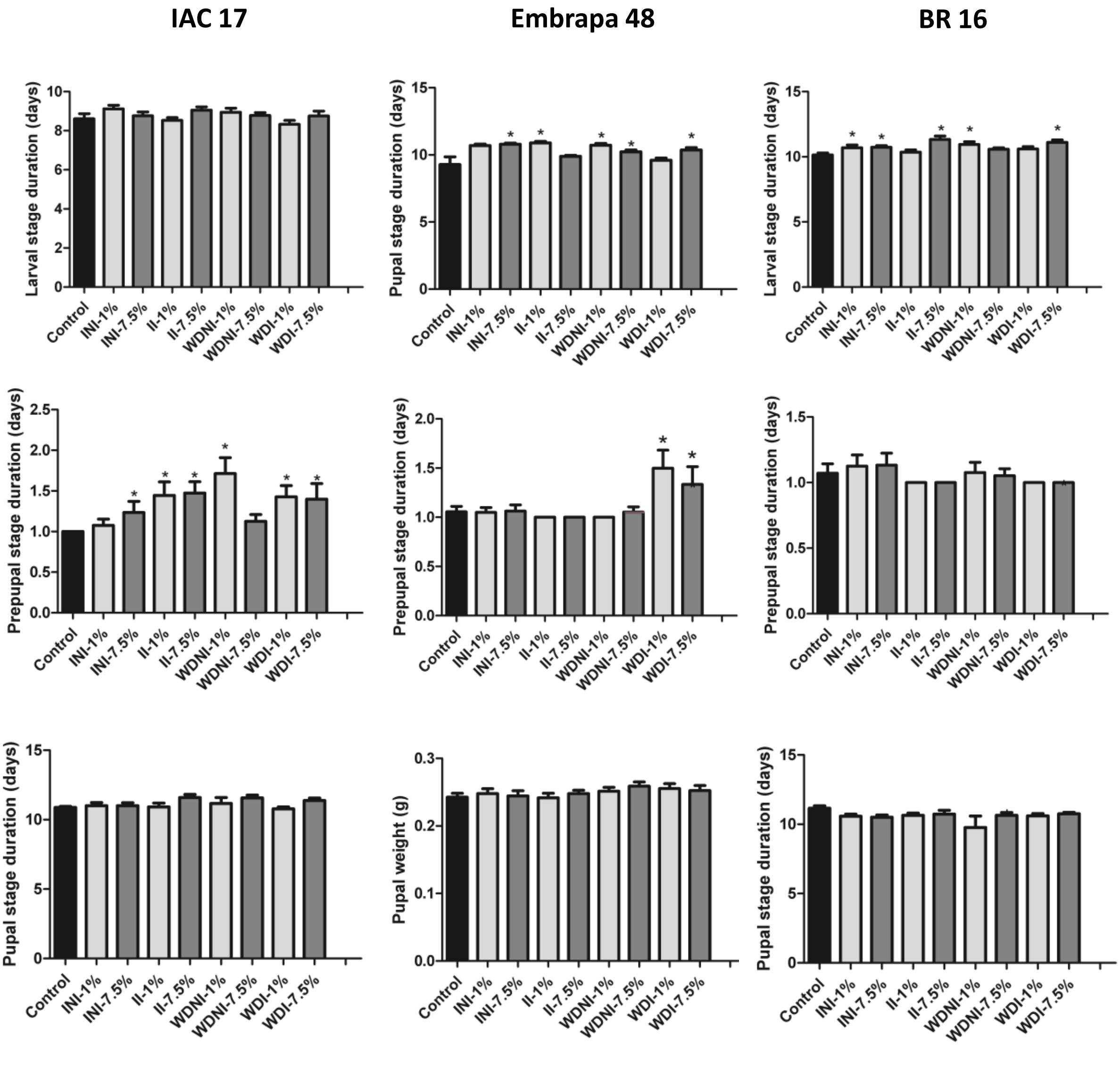

Figure 15: Effect of the diet supplementation with metabolite extracts from soybean leaves of the genotypes IAC17, Embrapa 48 and BR16 under drought and insect attack stresses in the (A) larval stage duration (B) prepupal stage duration (C) pupal stage duration of *A gemmatalis* caterpillars fed with artificial diet plus soy leaf extract. These extracts come from treatments INI, II, WDNI, WDI that were evaluated in genotypes IAC17, BR16 and EMBRAPA48. The extracts were prepared at 1 and 7.5%. Statistical tests were performed according to ANOVA followed by Duncan's test. * Indicates significant difference between treatments and control (p <0.05).

4 DISCUSSION

The increase in soybean productivity has been impaired due to abiotic and biotic environmental stresses. Among the abiotic stresses, highlight the water deficit, a climatic phenomenon that causes damage to plant growth and development (ISHIBASHI *et al.,* 2011; RADHAKRISHNAN e LEE, 2013; TAIZ, ZEIGER e MAFFEI, 2013). Insect pests are an important biotic stress and the soybean caterpillar *A. gemmatalis* Hübner (Lepidoptera: Noctuidae) is one of the main defoliating pests of the crop, which causes losses in the photosynthetic rates of plants (Blanco *et al.,* 2016), affecting the productivity. However, the plants development strategies to avoid or tolerate these environmental stresses involving complex multigenic reprograming affecting diverse physiological process (BECHTOLD e FIELD, 2018). Soybean (*Glycine max* (L.) Merrill) contributes substantially to the Brazilian economy, with Brazil being the largest soybean producer in the world which has led to the development of genotypes showing improved resistant or tolerant by genetic breeding (BALDIN *et al.,* 2016; COUTINHO *et al.,* 2021; FUGI *et al.,* 2005; LIMA *et al.,* 2019; MESQUITA *et al.,* 2020).

Furthermore, the plants could be submitted simultaneously a several stresses. Thus, to maintain a sustainable productivity would be desirable that a cultivar show to be tolerant a multiple stress. In fact, some physiological responses and regulatory cascades have been shared when trigger by different stress signals (NGUYEN *et al.,* 2016; VOS *et al.,* 2019;) which means that a genotype characterized as tolerant to drought could be also show tolerance to insect attack. For example, drought tolerant soybeans shown higher levels some flavonoid precursors of phytoalexins (RODRIGUES *et al.,* 2021 under publishing), while other genotypes produce large amounts of protease inhibitors (COUTINHO *et al.,* 2021). Furthermore, different profiles flavonoid and protease inhibitors biosynthesis have been verified in soybean genotypes tolerant to *A. gemmatalis* (GÓMEZ *et al.,* 2018; 2020). Evaluating caterpillars infesting soybean plants under drought stress, Faustino *et al.,* (2021) observed a reduction of the caterpillar survival fed in plants previously submitted to water deficit, which correlated with higher levels of PIs. Thus, the regulatory cascades and metabolic pathways can be trigger by both stress of drought and insect attack. Despite the characterization of the molecular determinants for the phenotype of resistance or tolerance to be challenging, we have focusing in the molecular characterizations of the drought tolerance in the cultivar EMBRAPA48 (COUTINHO *et al.,* 2021; LIMA *et al.,* 2019; MESQUITA *et al.,* 2020.) and of resistance to insect attack in the genotype IAC17

(GÓMEZ *et al.,* 2018, 2020). Aiming to examine the regulatory cascades and metabolic pathways shared during biotic and abiotic stresses, we submitted these genotypes to both signals of drought and insect attack and compared theirs metabolic and genetic reprograming with the susceptive genotypes BR16.

Drought treatment reduced significantly the survivals of the caterpillars fed on the leaves of all evaluated genotypes, including the genotype BR16 used as reference of susceptibility to insect attack (PIUBELLI *et al.,* 2005). In the assays of water deficit imposition, the IAC17 plants were the more sensitive to drought reaching early lower potentials, however showed lowers survival indexes. On another hand, the drought-tolerant EMBRAPA48 promotes lower survival under water deficit than the drouth-sensitive BR16. Despite of these observations, under conditions of normal water supply the survival levels were not significantly distinct in the drought-tolerant EMBRAPA48 compared with sensitive-drought ones. Thus, for genotype EMBRAPA48 the deterrent compounds were not produced constitutively due to genetic background of drought-tolerance. Furthermore, the insect resistance has been shown as a constitutive character for the IAC17 (GÓMEZ *et al.,* 2018, 2020). Interesting, this drought-tolerant genotype presented less pronounced gene and metabolic reprograming in response to drought signal than BR16 (COUTINHO *et al.,* 2021), but the drought signal appears trigger the producing of molecular agents against caterpillars. In contrast, the BR16 showed upregulation of several genes encoding for PIs under drought treatment (COUTINHO *et al.,* 2021).

Phytohormonal cascades have been critical for plant response to drought and herbivory (NGUYEN *et al.,* 2016). Insect signal perception culminate in the induction of the phytohormone biosynthesis, including jasmonic acid (JA), abscisic acid (ABA) and ethylene (ET). They act synergistically to activate signaling cascades that regulate downstream transcriptional responses (NGUYEN *et al.,* 2016). For drought, the response cascades could be ABA-dependent or not, thus phytohormonal profiles can be indicative of the regulatory processes affecting the caterpillar survival under drought treatments. Under drought stress, the JA levels were not altered and for some conditions decreased, thus the cascades controlling biosynthesis of metabolites responsible by the survival reductions appear to be JA-independent. When the plants were previously submitted to drought and then were subsequently infested, the JA and JA-Met leaves were lowers compared with plants elicited only by infestation, despite of the IAC17 and BR16 showed increased levels of LOX activities under drought. In fact, the metabolites related to the resistance phenotype in IAC17 have been also shown be JA and ABA-independent **(**GÓMEZ *et al.,* 2018*,* 2020) and appears be related to SA and ET levels in the absence of infestation (GÓMEZ *et al.,* 2020).

Otherwise, the ABA levels were increased specially when both stresses signal were applied in the soybean leaves. Increases in the PIs activities and gene expressions partially correlated with ABA levels. Higher PIs activities under drought were detected only for BR16. However, the SKTI gene expressions were induced for all genotypes in accordance with the ABA levels. especially in the plants under both stresses. For the other evaluated PI gene, encoding for protease inhibitor BBI, it was observed an upregulation only in the EMBRAPA48 when the plants were elicited by both stress signals. Thus, signalling cascades may be acting in an ABA-dependent synergism for active the genes of resistance to insect attack in soybean plants, only when elicited simultaneous by both stresses.

The increased LOX activity in the leaves of irrigated soybean plants after *A. gemmatalis* herbivory is a common biochemical response against herbivory (THAKUR e UDAYASHANKAR, 2019). Signals of the herbivory trigger release of polyunsaturated fatty acids, generating substrate for LOX enzyme which mediate the production of the hormone jasmonic acid (JA) (BRUXELLES *et al.,* 2001; ROACH *et al.,* 2015). Drought stress (moderate and severe) applied alone also increased the LOX activity in the leaves of soybean plants (FAUSTINO *et al.,* 2021), however ours results indicated that drought signal not acting directly in the cascades involving LOX to increase JA levels. Instead, cascades downstream regulated by JA signaling could be being potentialized by ABA to enhance the production of the metabolites that reduce the caterpillar survival.

In *Arabidopsis thaliana*, the JA response pathway consists of two antagonistic branches that are regulated by MYC- and ERF-type transcription factors (VERHAGE *et al.,* 2011). Production of ABA induced in response to leaf-chewing *Pieris rapae* caterpillars was required for both the activation of the MYC-branch and the suppression of the ERF-branch during herbivory (VOS *et al.,* 2019). Thus, in this study was concluded that ABA is essential for maximizes defenses against caterpillars in *Arabdopsis*. Studies using exogenous applications of ABA in the absence of herbivory did not altered the expression of the marker genes for the branches *VSP2* and *PDF1.2*, indicating that ABA alone is not sufficient for influencing the expression levels of these marker genes, but requires additional activation of the JA pathway (VOS *et al.,* 2019). Thus, these findings justify the restoring of the JA levels observed when in the soybean leaves under drought and *A. gemmatalis* infestation.

However, others hub genes and regulatory pathways could be associated with the convergency and synergism between drought and insect attack observed for soybean and *A. gemmatalis* interaction. GmNAC genes have been upregulated during the onset of leaf senescence and in response to several treatments simulating drought, ER-stress and biotic stress

(MELO *et al.,* 2018). Specifically, GmNAC085 was downregulated during natural senescence and up-regulated only in the intermediary-to-late stages of PEG treatment (MELO *et al.,* 2018). ANAC019, ANAC055, and ANAC072 are close-related genes which are induced by multiple stresses and display the same expression pattern under ABA treatment (JENSEN *et al.,* 2010) and during age-induced senescence (BREEZE *et al.,* 2011). Microarray analysis in ANAC019 and ANAC055 mutants identified that these genes are differentially involved in flavonoids, JA and SA signaling. In ANAC019 mutants, genes involved in flavonoid biosynthesis and SA signaling were down-regulated, including DFR (dihydroflavonal reductase) and F3H (flavone 3-hydroxilase) (QI *et al.,* 2011; HICKMAN *et al.,* 2013; LIANG *et al.,* 2014), whereas JA biosynthesis genes are up-regulated, including LOX2 (lipoxygenase 2) and PR4 (HICKMAN *et al.,* 2013). Interesting, some of these up regulated genes also were responsive to *A. gemmatalis* infestation in soybean plants IAC17 (PINHEIRO *et al.,* 2021 under publishing).

Some isoforms for MPL proteins (Bet-v1) were differently expressed under *A. gemmatalis* infection (PINHEIRO *et al.,* 2021 under publishing), which are also involved in the phytohormonal cascades controlling the secondary metabolites triggered downstream signal transductions (WANG *et al.,* 2016). Interesting, physiological and biochemical analyzes performed by Wang *et al.,* (2016) indicated that MLP43 was involved in stress responses by ABA and drought in Arabidopsis through modulation of the primary metabolic profile and gene expression. Experiments with induction of MLP in Arabidopsis also showed that the total flavonoid content in plants was double in treatment compared to control, suggesting that MLP overexpression is involved in the changes in the plant of the flavonoid and secondary metabolites concentrations (CHEN e DAI, 2010). Furthermore, it has been suggested that expression of MLP-like protein may increase the Coumestrol (CMS) isoflavone accumulation in response to stress conditions (HA *et al.,* 2019). In agreement, the levels of specific flavonoids were detected in highest concentration in the in the resistant IAC 17 genotype after treatment with the caterpillar (GÓMEZ *et al.,* 2018), thus MPL-like protein could be under control of ABA-dependent cascades in the regulation of production de secondary metabolites against *A. gemmatalis* infestation. Metabolic profiles by LC/MS also indicated that drought and insect signaling attack may be acting in synergism in plants under both stresses signals to inducing the biosynthesis of specific compounds that may be involved in reduction of the caterpillar survival. Interesting, some these identified compounds belonging the flavonoids and coumarins classes identified as 6-O-malonylgenistin, 7-formy 6-nitrocoumarin, 3'-Methoxyapigenin (Chrysoeriol) and 6-O acetylgenistein were showed higher abundance in soybeans leaves under both stress signals. Presence of deterrent compounds in the soybean leaves were also examined by supplementation of the caterpillar diets with methanolic extracts containing metabolites of low molecular mass. Reductions of the survival and alteration on pulpal stage duration were incremented when added extracts from plant leaves elicited by both stresses were.

5 CONCLUSIONS

Soybean plants under drought stresses were less susceptive to *A. gemmatalis* attack as indicated by reductions of the caterpillar survivals. Overall, the results suggested that these survival reductions were not dependent of the of the drought-tolerance or resistance to insect attack phenotypes, despite only a drought-tolerant and a resistant genotype have been evaluated. Furthermore, the metabolites profiling, gene expression and enzymatic assays lead us to concluded that only the drought signal not is enough to promote the increase of resistance to insect attack.

Under drought stress, the JA levels were not altered and, for some conditions decreased, thus the cascades controlling biosynthesis of metabolites responsible by the survival reductions appear to be JA-independent. However, Increases observed for PIs activities and gene expression partially correlated with ABA levels. Thus, cascades downstream regulated by JA signaling were potentialized by ABA to enhance the production of the metabolites that reduce the caterpillar survival. Inductions of the PIs gene expressions have been used as reference to evaluated the downstream induction of the JA-dependent cascades, thus the increased of ABA levels during drought treatment may be acting synergistically to increase JA response. Our results indicate also that the first stages of insect-soybean interaction were less affected by drought signal as indicated by JA levels, despite of the LOX activities have been increased and its gene expression.

ABA accumulation under drought signal may act modulating the activation of the MYC-branch as has been observed in others studies or by possibly by activating the others transcription factors, such as NAC. Thus, the characterization of the regulatory molecular hub (s) for integration of the both drought and insect attack signals may generate targets to genetic engineering, aims the development of soybean plants showing at genetic background multiple tolerance to drought and insect attack.

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CONCLUSÃO GERAL

As plantas de soja sob estresse hídrico foram menos suscetíveis ao ataque de *A. gemmatalis*, conforme indicado pela redução da sobrevivência da lagarta. No geral, os resultados sugeriram que essas reduções de sobrevivência não foram dependentes da tolerância à seca ou resistência aos fenótipos de ataque de insetos, apesar de apenas um genótipo tolerante à seca e um genótipo resistente a inseto ataque terem sido avaliados. Além disso, os perfis de metabólitos, expressão gênica e ensaios enzimáticos nos levam a concluir que apenas o sinal de seca não é suficiente para promover o aumento da resistência ao ataque de insetos.

Sob estresse hídrico, os níveis de AJ não foram alterados e, para algumas condições, diminuíram, portanto, as cascatas que controlam a biossíntese de metabólitos responsáveis pelas reduções de sobrevivência parecem ser AJ-independentes. No entanto, os aumentos observados para atividades de IPs e expressão gênica também podem estar parcialmente correlacionados com os níveis de ABA. Assim, cascatas a jusante reguladas pela sinalização de AJ foram potencializadas pelo ABA para aumentar a produção dos metabólitos que reduzem a sobrevivência da lagarta. Como as induções das expressões gênicas dos IPs têm sido usadas como referência para avaliar a indução a jusante das cascatas dependentes de AJ, concluímos, assim, que o aumento dos níveis de ABA durante o tratamento da seca pode estar agindo sinergicamente para aumentar as respostas AJ-dependentes. Nossos resultados indicam também que os primeiros estágios da interação inseto-soja foram menos afetados pelo sinal de seca conforme indicado pelos níveis de AJ, apesar das atividades de LOX e sua expressão gênica terem sido aumentadas.

O acúmulo de ABA sob o sinal de seca pode atuar modulando a ativação da cascata dependente de MYC, como foi observado em outros estudos, ou possivelmente pela ativação de outros fatores de transcrição, como o NAC. Assim, a caracterização do (s) hub (s) molecular (is) regulatório (s) para integração dos sinais de seca e ataque de insetos podem gerar alvos para a engenharia genética, visando o desenvolvimento de plantas de soja que apresentem características genéticas de tolerância múltipla à seca e ao ataque de insetos.