

STÊNIO ANDREY GUEDES DANTAS

**ESTRATÉGIA DE INDUÇÃO DE DÉFICIT HÍDRICO EM SOJA E
DESENVOLVIMENTO DE GWAS PARA GERMINAÇÃO E VIGOR DE
SEMENTES**

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

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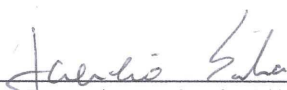
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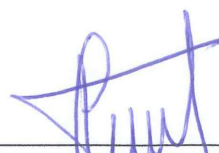
Eveline Teixeira Caixeta



Thais Roseli Corrêa



Laercio Junio da Silva
(Coorientador)



Fernando França da Cunha
(Coorientador)



Felipe Lopes da Silva
(Orientador)

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RESUMO

DANTAS, Stênio Andrey Guedes, D.Sc., Universidade Federal de Viçosa, março de 2018. **Estratégia de indução de déficit hídrico em soja e desenvolvimento de GWAS para germinação e vigor de sementes.** Orientador: Felipe Lopes da Silva. Coorientadores: Laercio Junio da Silva, Fernando França da Cunha, Tuneo Sedyama e Ivan Schuster.

A soja é a leguminosa de maior importância econômica do mundo, ocupando 6% de todas as terras aráveis do planeta. No Brasil, a soja é plantada em 55% de toda a área de cultivo. Devido sua grande área plantada, rotineiramente a soja está exposta a estresses, principalmente por déficit hídrico, que têm se tornado cada vez mais comum nos últimos anos. O objetivo do presente trabalho foi estabelecer uma metodologia viável e de baixo custo para avaliação de plantas de soja tolerantes ao estresse hídrico durante a fase de germinação e em plantas adultas em casa de vegetação. Posteriormente, com o auxílio de GWAS, explorar a associação entre SNPs e características de interesse relacionadas à germinação e dinâmica de reservas de sementes em diferentes condições de estresse, para seu potencial uso no melhoramento genético. Para isso foi realizado uma revisão sobre metodologias de aplicação de estresse em plantas em casa de vegetação, respondendo as perguntas: como, quando e quanto estresse aplicar nas plantas de soja? O qual indicou diferentes estratégias conforme o objetivo do estudo e disponibilidade de recursos financeiros. Em seguida foi definido como realizar o estresse hídrico em sementes, testando quatro diferentes níveis de estresse (0,0 Mpa, -0,2 Mpa, -0,4 Mpa e -0,6 Mpa), verificando que o potencial osmótico de -0,2 Mpa é o mais adequado para se selecionar genótipos tolerantes a seca durante a germinação, por expor a máxima variabilidade genética entre genótipos. Com essas informações foram realizadas análises de germinação e vigor de sementes em 97 genótipos de soja, genotipados com marcadores moleculares SNPs. A partir desses resultados se confirmou que as características relacionadas à germinação e vigor de sementes são governados vários por locus em diferentes regiões do genoma, independentemente do ambiente. Quanto à dinâmica de reservas de sementes para a germinação, foi verificado que somente a variável eficiência de conversão de reservas apresentou SNPs associados, e que essa característica também é de natureza quantitativa. Tanto para a germinação,

como para o vigor e dinâmica de reservas, há variabilidade genética a ser explorada, e essas características podem ser melhoradas. Conclui-se que existem metodologias viáveis e de baixo custo para seleção de genótipos tolerantes a seca utilizando plantas adultas e sementes, e, que há associação genômica entre SNPs e características relacionadas à germinação, vigor e dinâmica de reservas, com possibilidade de uso no melhoramento genético, para se produzir genótipos superiores.

ABSTRACT

DANTAS, Stênio Andrey Guedes, D.Sc., Universidade Federal de Viçosa, March of 2018. **Strategies to drought stress induction in soybean and development of GWAS for germination and seed vigor.** Adviser: Felipe Lopes da Silva. Co-advisers: Laercio Junio da Silva, Fernando França da Cunha, Tuneo Sedyama and Ivan Schuster.

Soybeans are the most economically important legume in the world, accounting for 6% of all arable land on the planet. In Brazil, soybeans are planted in 55% of the total area under cultivation. Because of its large area planted, soybeans are routinely exposed to stresses, especially water stresses, which have become increasingly common in recent years. The objective of the present work was to establish a feasible and low-cost methodology for the evaluation of plants tolerant to water stress during the germination phase and in adult plants under greenhouse conditions. And later with GWAS, we explore the association between SNPs and characteristics related to control of germination and seed reserve dynamics under different stress conditions for their potential use in genetic breeding. For that, a review was carried out on methodologies of stress application in greenhouse plants, answering the questions: how, when and how much stress apply in soybean plants? which indicated different strategies according to the objective of the study and availability of financial resources. It was also defined how to perform water stress in seeds during germination. Four different stress levels (0.0 Mpa, -0.2 Mpa, -0.4 Mpa and -0.6 Mpa) were used. The osmotic potential of - 0.2 Mpa was the most adequate to select drought-tolerant genotypes during germination, because it exposes the maximum genetic variability among genotypes. With this information, seed germination and vigor analyzes were performed in 97 soybean genotypes, using SNPs markers. These results confirmed that characteristics related to germination and seed vigor are governed by several loci, being a quantitative trait that appear in different regions of the genome, according to the environment, with or without stress. Concerning the dynamics of seed reserves for germination, it was verified that only the reserve conversion efficiency variable had associated SNPs and that this characteristic has also quantitative nature. Germination, vigor and reserve dynamics presented genetic variability to be explored, and these characteristics can be improved in breeding

programs. It was concluded that there are feasible and low-cost methodologies for selection of drought tolerant genotypes using adult plants and seeds. And that there are several loci associated with control of the germination, vigor and dynamics of reserves, but with the possibility of use in breeding, to produce better cultivars.

INTRODUÇÃO GERAL

A soja (*Glycine max* (L.) Merrill) é uma das culturas agrícolas mais importantes no mundo, ocupando aproximadamente 6% de todas as terras aráveis do planeta, e desde a década de 1970 a soja é a cultura que mais registra aumento percentual de área plantada no mundo inteiro, ano após ano (HARTMAN et al., 2011).

As evidências históricas e geográficas mostraram que a soja tem sua origem na China e foi domesticada pela primeira vez na região nordeste do país. A introdução da soja no Brasil ocorreu no Estado da Bahia em 1882, seguido de estudos em São Paulo, no Instituto Agrônomo de Campinas (IAC) e apenas em 1914 foi cultivada pela primeira vez no Rio Grande do Sul (SEDIYAMA et al., 1985).

Atualmente a soja é plantada em quase todos os estados brasileiros, no entanto, independentemente de onde a soja é cultivada, é clara a correlação entre estabilidade de produção de grãos e estabilidade na disponibilidade hídrica ao longo dos anos. Nos estados ou regiões com ambientes mais estáveis sob o ponto de vista de disponibilidade hídrica, percebe-se a existência de maiores investimentos em tecnologia e na melhoria do ambiente, formando um círculo virtuoso que beneficia toda a cadeia produtiva. Por outro lado, nas regiões com maior risco de déficit hídrico, os investimentos tendem a ser menores e as lavouras de soja ficam ainda mais sensíveis ou pré-dispostas aos efeitos prejudiciais da seca (ARIAS et al., 2011).

Uma das alternativas mais viáveis para minimizar as perdas de produtividade de soja, perante os cenários atuais e futuros de mudanças climáticas e consequentes secas é o melhoramento genético, por meio da descoberta de novos genótipos tolerantes. Várias metodologias têm sido propostas para se mensurar o estresse e agrupar genótipos e características de interesse (QTLs) relacionadas à tolerância ao estresse hídrico nos diferentes estádios fenológicos da planta. Durante a fase de germinação, tanto pelo método tradicional de avaliação de germinação e vigor (OYA et al., 2004 e TEXEIRA; DE LUCCA E BRACCINI; et al., 2008), como fazendo uso de marcadores moleculares para construção de mapas de ligação (WANG et al., 2012). O mesmo acontece nos estádios vegetativos e reprodutivos, onde são empregadas estratégias multifacetadas desde abordagens fisiológicas (RIES et al., 2012); (DEVI; GIRIDHAR, 2015) e

genômicas (MANAVALAN et al., 2009) ao uso de engenharia genética (BARBOSA et al., 2013).

Contudo, identificar esses genótipos tolerantes, por meio de metodologias de simulação de estresse confiáveis e com resultados reprodutíveis a nível de campo é o grande gargalo em um programa de melhoramento (MITRA, 2001). Não existe um consenso de quanto de estresse deve ser aplicado para esse fim, bem como, qual a melhor maneira de aplica-lo.

Uma das estratégias propostas é a seleção de genótipos tolerantes por meio de teste de germinação. Para estudos de seleção de genótipos de soja tolerantes a déficit hídrico, envolvendo testes de germinação e vigor, diversos autores observaram a eficácia desses testes em condições de baixo potencial osmótico simulado com polietileno glicol. Esse teste tem sido aplicado em estudos de diversidade genética, para diferenciação e agrupamento de genótipos de soja mais tolerante ao estresse hídrico (TEXEIRA; BRACCINI; et al., 2008 e TEXEIRA; DE LUCCA E BRACCINI; et al., 2008). Essa metodologia tem sido útil também para relacionar o comportamento dos genótipos durante a germinação com seu comportamento em campo e, subsequentemente com produtividade (KOSTURKOVA et al., 2008).

A vantagem da seleção de genótipos tolerantes a estresse hídrico por meio de teste de germinação é a facilidade de uso. Esse método além de ser de fácil execução, necessita de pouco espaço, pouco tempo, e poucos recursos, quando comparado a ensaios de campo, tornando o custo de avaliação de linhagens bem mais barato, e com resultados disponíveis em um curto espaço de tempo.

O estabelecimento inicial das plantas de soja no campo após o plantio é uma das fases mais importantes do desenvolvimento da lavoura, sendo esse desenvolvimento inicial diretamente ligado à produtividade, e, diretamente relacionado ao vigor da semente (Henning et al., 2010). Vigor é um conjunto de propriedades que contribuem para indicar a qualidade da semente, relatando assim o seu potencial para germinação, emergência de plântulas e capacidade de armazenagem sob condições ambientais diferentes daquela considerada padrão (Sun et al., 2007). A qualidade fisiológica das sementes, representada pela germinação e vigor, pode afetar o desempenho da lavoura, uma vez que, plantas provenientes das sementes de alto vigor apresentam maior área

foliar e produção de matéria seca. Além do alto vigor das sementes proporcionar maiores taxas de crescimento a partir dos 21 dias após a emergência (DAE) (Kolchinski et al., 2006).

No entanto, há discordâncias de que as diferenças quanto à resposta ao déficit hídrico na fase de germinação das cultivares é inteiramente consistente com a resposta ao déficit hídrico com base em dados de produtividade (MEDERSKI; JEFFERS, 1973 e OYA et al., 2004). Isso ocorre talvez porque ainda não exista um consenso em como simular o déficit hídrico, tanto nas sementes em fase de germinação e crescimento inicial, como em plantas adultas, para então correlacionar essas informações.

Além de não haver uma metodologia validada para a aplicação no melhoramento visando tolerância ao estresse hídrico por falta de água, essa é uma característica de controle genético quantitativo, com efeito aditivo e de forte interação entre genótipo e ambiente, além de apresentar forte interação com genes que estão diretamente envolvidos com a produtividade (ASHRAF, 2010).

Por se tratar de uma característica quantitativa, a associação genômica ampla (GWAS) pode ser usada para a identificação de locus (marcadores SNPs - single nucleotide polymorphisms) responsáveis pela variação fenotípica. A genômica aparece ultimamente com um grande potencial para o estudo e entendimento de características complexas em que a interação genótipo x ambiente exerce grande efeito, tal como a tolerância ao estresse hídrico (KUJUR et al., 2013). O uso deste tipo de metodologia explora a ligação mais próxima que existe entre marcadores moleculares e regiões cromossômicas de interesse para fazer a associação de caracteres quantitativos e regiões genômicas da planta, e, tem aumentado drasticamente nos últimos anos (HANSEY et al., 2011). A genotipagem de SNPs é uma valiosa ferramenta para a seleção assistida por marcadores moleculares em plantas (DAS et al., 2017).

Desse modo, o objetivo do presente trabalho foi estabelecer uma metodologia viável e de baixo custo para avaliação de plantas de soja tolerantes a estresse durante a fase de germinação e em plantas adultas em casa de vegetação. E, posteriormente, com o auxílio de GWAS explorar o controle genético da germinação, vigor e dinâmica de reservas de sementes em diferentes condições de estresse, para o entendimento e seu possível uso em programas de melhoramento genético.

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ARTIGO CIENTÍFICO 1 - SIMULATION OF WATER STRESS IN SOYBEAN CULTIVATED IN POTS AIMED AT THE SELECTION OF TOLERANT GENOTYPES: A REVIEW

ABSTRACT

Soybean is the main oleaginous plant cultivated commercially around the world and is affected by considerable loss of productivity due to water deficit. This issue has prompted a scientific race to achieve genotypes more tolerant to water deficit. However, there is no consensus on the best way to simulate the water-deficit stress and how much stress is necessary for selection of the best genotype. The objective of this study is to review methodologies for stress simulation in a protected environment. The methodologies described are the suspension of irrigation, irrigation suspension scale notes for wilt, gravimetric method and method from the curve of water retention in the soil. These methodologies are easy to perform and inexpensive, requiring only scale to perform them. Each method has its characteristics, advantages and disadvantages, which the researcher needs to take into consideration and adapt to local conditions.

Keywords: *Glycine max* (L.) Merrill, breeding for drought, methods of stress simulation.

INTRODUCTION

The soybean (*Glycine max* (L.) Merrill) is one of the most important commodities in Brazil, occupying 57% of the current growing area in the country and accounting for 44% of the withdrawn agricultural crops. According to reports of the Government Department of Strategic Management of Embrapa and the Government Department of Agriculture, cultivation of soybean will increase in terms of planted area over the next decade, with predictions of 31.44% expansion, occupying an anticipated area of 44,400,000 hectares in 2024 (Kist et al. 2014).

However, every year there have been recorded losses in productivity related to drought. In the harvest year 2011/2012, producing regions reported yields that were reduced by more than 9,000,000 tons (CONAB 2013). In the harvest year 2015/2016, even with an increased area, soybean income dropped by 0.8% compared in the previous year, due to climate change, mainly drought (CONAB 2016).

One of the most viable alternatives for minimizing loss in soybean productivity, before the current and future scenarios of climate change and consequent drought, is genetic breeding through the identification of new tolerant genotypes. However, identification of these tolerant genotypes using reliable stress simulation methods which produce reproducible results in the field is a major bottleneck in the breeding program (Mitra 2001). There is no consensus on the amount of stress that must be applied for this purpose, or on the best way to apply this stress.

The first step in selecting tolerant genotypes to any kind of stress, including the water deficit, is to answer the following questions: When to simulate stress, taking into account when the plant is most sensitive to this stress? How to simulate the stress? How much stress to apply? The answers to these questions guide the planning of research and indicate the need for any improvements, if necessary.

Thus, the objective of this study is to review methodologies for stress simulation in plants cultivated in pots for the selection of soybean genotypes that are tolerant to water stress.

WHEN TO SIMULATE WATER STRESS?

The phenological growth stage which is most suitable for the simulation of water stress will depend on the purpose of the study. In general, stress can be applied at any stage of plant development, such as germination, seedling stage, young plants in the vegetative stage and adult plants in the reproductive stages. Table 1 lists some studies with different objectives and the respective developmental stages of soybean when water stress was imposed.

Table 1. Phenological stages of stress simulation according to the study objective.

Study objective	Phenological stage	Reference
Transcriptomic analysis	V2	Tripathi et al., 2016
	V2	Marcolino-Gomes et al., 2016
	V5	Prince et al., 2015
	R2	Shin et al., 2015
Gene expression	V1	Quach et al., 2014
	V2	Kidokoro et al., 2015
	V6	Le et al., 2012a
	V6, R2	Le et al., 2012b
Activity of antioxidant enzymes	VE	Kausar et al., 2012
	V6, R5	Devi e Giridhan et al., 2015
	R1	Zoz et al., 2013
Physiological parameters	V5	Stolf-Moreira et al., 2010
	V6	Mutava et al., 2016
	V6, R5	Devi e Giridhan et al., 2015
Productivity	R5, R6	Pardo et al., 2015
	R5	Brevedan e Egli 2003

“V” represent the vegetative stage and "n" represents the number of nodes on the main stem with fully developed leaves beginning with the unifoliolate leaves. “R” represent the reproductive stages, being R1 Beginning bloom, R2 Full bloom, R3 Beginning pod, R4 Full pod, R5 Beginning seed, R6 Full seed (Fehr et al., 1971)

When the goal of the study is to evaluate yield and productivity, simulation of water stress during the reproductive stages, especially during grain filling (R5), is the most recommended. The phenological stage of R5 is the period when the plant is most

likely to decrease in productivity due to water stress (Desclaux et al. 2000; Brevedan and Egli 2003; Pardo et al. 2015). This phase comprises the early formation of the seed and is characterized by the presence of seeds of at least 3 mm in diameter in the last four pods, (Sedyiama et al. 2015).

The imposition of stress at certain stages of development significantly affects the result of the study. Lack of water in the soil during the initial period of flowering until the early formation of seeds (R1-R5) reduces the number of seeds, while the lack of soil moisture after this period (R5-R7) reduces seed size (Snyder et al. 1982; Andriani et al. 1991; De Souza et al. 1997; Brevedan and Egli 2003).

HOW TO SIMULATE THE STRESS?

Suspension of water supply

One of the simplest ways to simulate and to apply stress in plants is by the total suspension of water supplied to the plant for a specific period. The main advantage of this methodology is its ease of application. Syed et al. (2015) and Mutava et al. (2015), with the aim of studying gene expression and understanding mechanisms of stress tolerance, respectively, used pots with a capacity of 26.5 L and applied stress by means of the total suspension of water supply for a period of 21 days, after which irrigation was re-introduced. Syed et al. (2015) classified this type of stress as severe and described medium stress as the absence of irrigation over a period of 11 days.

Weber et al. (2014) used the method of suspending irrigation for six and twelve days during the R3 stage to study soybean plants genetically modified with a drought tolerance gene; the plants were grown in polyethylene tubes with a height and diameter of 35 cm and 100 cm, respectively. The same authors, in a preliminary experiment, also evaluated genotype tolerance to water stress by the suspension of water supply for 10 days in plants 21 days after sowing and monitored the groundwater and the efficiency of the genotype.

Interruption of the water supply for a specified period was also used by Hossain et al. (2014) in young plants at the R2 stage over a 10-day period. In this study, physiological parameters of plants under stress and irrigated plants were evaluated. A similar procedure was performed by Thu et al. (2014) in an investigation of potential

drought-tolerant genotypes, where plants from 12 days old had irrigation suspended and were evaluated until 27 days after sowing

The method of suspending the water supply for a specific period presents the disadvantage of applying non-standardized levels of stress. Different types of soil have different water contents, and coarse textured soils have a lower water retention compared to those with a fine texture. Another factor that should be considered is the size of the pots, since different volumes of soil have differing water content. The vegetative stage of the plant, as well as the micro and macro climatic conditions, also influences the evapotranspiration rate.

Therefore, the stress in a given period of time will be of varying intensity depending on the soil type, pot size, and conditions of climate or plant phenological phase.

Suspension of water supply with wilting scale

The wilting scale is an alternative to the quantification of stress applied by the suspension of water supply involving direct measurement of the level of wilt in the leaves. In this method, in addition to the plants that will be evaluated, a standard genotype with a scientifically-known low tolerance to drought, such as BR-16 (Stolf-Moreira et al. 2011; Souza et al. 2013) and Pana (Pathan et al. 2014), is planted. All plants are treated with the same water regime until the specified period for imposition of stress. At the time of stress enforcement, water supply is suspended for all genotypes, including the standard genotype. When the standard genotype of susceptibility presents a moderate to severe wilt, other genotypes are scored using the phenotyping scale of 1 to 5: 1 = no wilt; 2 = wilt affects a few leaves from the top; 3 = wilt affects half of the leaves; 4 = severe wilt, with about 75% of the leaves show symptoms of wilting; and, 5 = severely wilted and dead plants. After the period of stress, adequate supply of water is resumed until plants reach the stage of physiological maturity (Pathan et al. 2014).

This method of evaluation, from wilting scale, was also used in the cultivation of soybeans by Abdel-Haleem et al. (2012) for genetic mapping of QTLs that explains the tolerance for young plants, by Ries et al. (2012) in studies of physiological parameters responsible for drought tolerance, Prince et al. (2015) in transcriptomic studies of contrasting strains for tolerance to wilt and by Hwang et al. (2016) which found the

existence of stable QTLs for the characteristic, what indicates the possibility of using of these markers to assist drought selection.

Weighing method and pot water holding capacity

In addition to the suspension of irrigation method, there are more laborious methods that seek to standardize and quantify the stress level. These methods start from one point in common, that is the water-holding capacity of field or pot. The water holding capacity (WHC) represents the ideal level of water available to the plant and is commonly expressed as percentage humidity or as soil water tension. Thereby, the WHC serves as a standard and a starting point for calculating the stress applied. For example, the stress will be a water content that is 50% of the WHC or a soil tension 10 times less than the WHC tension.

This method has the main advantage of the possibility of quantifying and controlling the stress applied in different environments, types and sizes of pots. However, it is important to estimate the WHC using a reliable method of a good standard, and thus quantify the stress level more precisely.

There are basically two ways to estimate the WHC in pots, one involving the drainage time (gravimetric method) and the other through the water retention curve in the soil. The methods are as follows:

Determination of WHC in pots using drainage time

One definition of WHC is water content of soil with no vegetation and no evaporation, held against the action of gravity, after the soil is fully saturated and left to drain freely for a certain period of time (Veihmeyer and Hendrickson 1931). That is, it is the maximum water retained in the soil after drainage, after which the loss of water due to gravitational force ranges from minimal to negligible.

Based on this concept, the establishment of the soil at WHC is defined by the time that elapses until the drainage of water is considered to be insignificant. In several studies involving soybean (Brevedan and Egli 2003; Pardo et al. 2015), common bean (Coletto et al. 2014), millet (Kholová et al. 2010), *Arabidopsis thaliana* (Bacso et al. 2008), corn (Chapuis et al. 2012) and sunflower (Pereyra-Irujo et al. 2007) an average period of 12 hours after saturation or during an entire night has been reported as the

time required for the soil to reach WHC, and this value has been used as the basis for the definition of the applied stress level.

For this method, all plants receive an adequate water supply up to the phenological stage which represents the time for simulation of stress. At that moment, the pots are saturated with water in the late afternoon. The next morning, after draining for a whole night, the pots are weighed and this is determined as the WHC. Subsequently, the pots are weighed once, twice or three times a day, in order to apply the treatments.

For example, the weight of the pot in the morning after being saturated and left to drain for a whole night is 10 000 g; this represents WHC. At the end of the first day of treatment, the same pot weighs 9000 g; the soon to be added 1000 g water raises the current value to the initial weight (WHC) without applying stress to this plant. In the case of stress treatment, also with an initial weight of 10 000 g (WHC) and, at the end of the first day of stress, 9000 g, if the treatment is 50% of the WHC, the plant will receive half of what would be necessary, i.e. 500 g water. This will be repeated during the whole period of stress.

This method, despite being postulated and widely used has a serious problem, namely the overestimation of WHC values which are based only on the drainage time. According to Casaroli and Lier (2008), the time taken for drainage to reach a negligible value is a subjective criterion and varies between authors and situations; values ranging 12 to 120 hours have been reported. The inaccurate reporting of WHC values in studies of water stress in plants can lead to unreliable results, especially if the goal of the experiment is to study light or moderate stress.

This problem can be demonstrated by an experiment conducted during the period of May to August 2015 in protected and controlled environment, with a temperature ranging from 20 ± 30 °C and $60 \pm 70\%$ relative humidity, with the crop soybean, cultivars MG/BR 46 Conquista and Anta 82, and, two types of soil (sandy and loam). The objective of this study was to investigate whether short periods of moderate stress (6, 9 and 12 days at 40% WHC) during the phenological stage R5 would be sufficient to reduce the productivity of the species. The method of stress simulation

used was the gravimetric method, with saturation and drainage for 12 hours for determination of WHC.

There was no effect of any period of stress with either soil type. This lack of effect was because the 12-hour drainage period with both soils was insufficient time for the soil to reach WHC (Table 2). Due to the WHC being over-estimated, the corresponding amount of water to be applied under stress was also over-estimated, causing an excessive application of water and consequently an ineffective stress. In addition, the imposition of water deficit is not constant and increases as a function of time. This is due to the water content in the soil reducing due to the accumulation of water deficit applied in the previous days.

Table 2. Soil moisture in WHC determined by different methods.

Soil	Moisture of WHC after drainage of 12 h	Moisture of WHC at 10 kPa ¹
1	40.54%	27%
2	28.88%	16%

¹ Water hold capacity of soil determined by the water retention curve in soil at a tension of 10 kPa

Determination of pot capacity by the water retention curve in soil

For water to be retained in the soil after saturation, it is necessary that forces act to it. These forces are called the soil matric potential, quantified by energy tension (kPa, MPa, mca, Bar or ATM). The matric potential required to keep the soil moisture tension varies depending on the soil type, density and organic matter content. However, conventionally accepted moisture tensions of -33 kPa for clay soils and -10 kPa for sandy soils. This methodology is a more precise alternative to the gravimetric method since it involves soil moisture tension and not drainage time.

Therefore, this methodology requires initial physical analysis of the soil to be performed to determine the soil type. Then, a water-retention curve of the same soil, in strains of -6, -10, -30,-60,-100,-400 and-1500 kPa, is constructed. With the water content of the soil for each strain, is set the regression equation of type potential, as the example in Figure 1, which was used a clayey soil texture.

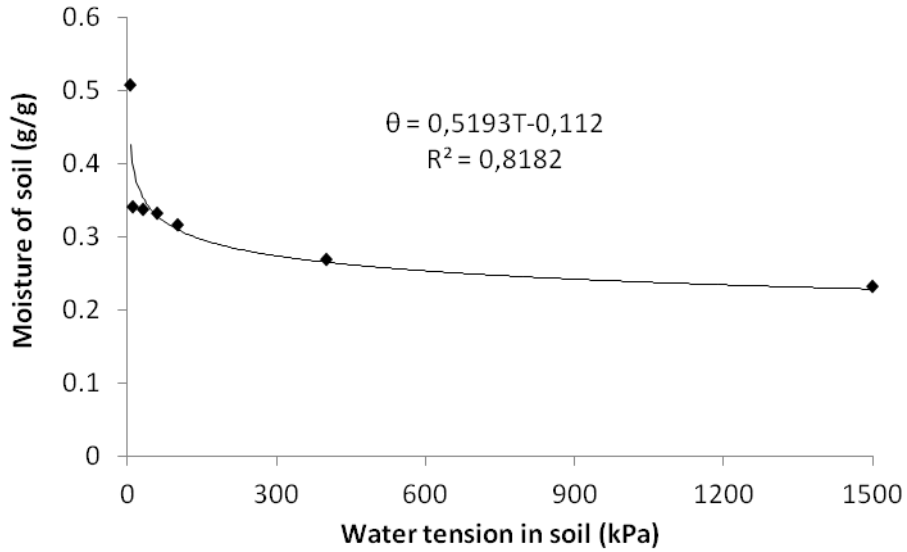


Figure 1. Water retention curve in soil, where θ represents the percentage water content, and T represents the tension of water in the soil.

Based on this information, the water content of the soil at WHC and at the desired stress level is calculated. For example, given a clay soil where the WHC tension is equivalent to 33 kPa, the situation of stress applied was a tension of 900 kPa.

$$Moisture_{WHC} = 0.5193 \times 33^{-0,112} \therefore Moisture_{WHC} = 0.3510 \text{ or } 35.10 \%$$

$$Moisture_{stress} = 0.5193 \times 900^{-0,112} \therefore Moisture_{stress} = 0.2424 \text{ or } 24.24 \%$$

To use this method it is necessary to standardize the volume of soil in all pots. For this, all the pots and soil are weighed at the time of filling. At this point, it is important that the water content of the soil is standardized. Therefore, it is recommended that soil is spread in layers, not in mounds, at least two days before the filling of the pots. At the time of filling the pots, a soil sample is collected for determination of water content.

The initial weight of the pot (W_i) is made up of the mass of the empty pot (W_p) plus the mass of moist soil (W_{ms}), and represented by the equation: $W_i = W_p + W_{ms}$. W_{ms} can be calculated using the equation $W_{ms} = W_i - W_p$. Then, the mass of dry soil (W_{ds}) would be a W_{ms} less the moisture present in that soil, represented by the equation $W_{ds} = W_{ms}/(1 + M_{db})$, where M_{db} is the soil moisture in dry base. Values

obtained in this study were as follows: Mdb 33.36%; Wi 9150 g; Wp 299.8 g; Wms 8850.2 g and Wds 6636.3 g.

Using information on Wds and the moisture of the treatments with and without stress, the actual amount of water to be applied with each treatment has been calculated. It is known that $Mdb = W_{water}/Wds$. Therefore, for conditions where Mdb is 35.10% (no stress) and 24.24% (in stress conditions), the mass (content) of water is determined as follows:

$$\text{Non stress} : 0.3510 = W_{water}/6636.3 \therefore W_{water} = 0.3510 \times 6636.3 \therefore W_{water} = 2329.3 \text{ g}$$

$$\text{Stress} : 0.2424 = W_{water}/6636.3 \therefore W_{water} = 0.2424 \times 6636.3 \therefore W_{water} = 1608.6 \text{ g}$$

Once the experiment is set up, the total weight of the system will be composed of mass of pot, plant, dry soil and water, represented by the equation $W_{total} = W_{pot} + W_{plant} + W_{dry\ soil} + W_{water}$. The mass of the plant can be determined by measurement of fresh plant matter in its phenological stage which will be evaluated. For this, additional plants are grown in the plot in parallel and are weighed when they reach the desired phenological stage. It is important that the root mass should be considered in the system too, so the roots are removed, cleaned and weighed together with the rest of the plant. In this study the mass of the plant was of 170.6 g.

Based on this information, a spreadsheet can be prepared where the weight of the system with the respective treatments will be recorded monitored as the weight to be reached every day through irrigation management. For that, during the period of imposition of stress, representative samples from the experiment, or all the pots, are weighed at 10:00 hours and 16:00 hours. Based on the difference in mass, an amount of water is applied for each irrigation treatment to balance the mass of the different systems

As for the stress level applied in soil water tension, there is no consensus on what is the most appropriate level. Pardo et al. (2015) used a tension of 650 kPa to select and validate a method of selection of soybean genotypes tolerant to water deficit. Pereyra-Irujo et al. (2012) in a study testing a new platform model for soybean phenotyping considered the tensions of 440 kPa to be moderate stress and 940 kPa as

severe stress. Desclaux et al. (1996, 2000), studying the effect of water stress on soybean plant phenology, applied stress of up to 750 kPa to find out how water stress affects the characteristics of soybean plants.

The efficiency of this method of stress simulation was evaluated in tests carried out in the Plant Science Department of the Universidade Federal de Viçosa during the last two years. Fifty soybean genotypes were used in each assay, using a tension level of 33 kPa for stress-free plants and 900 kPa for stressed plants. The stress period was applied when the plants reached the phenological stage of R5 and was continued for 15 days, following the method strictly, and with daily weighing in the morning and afternoon.

According to results presented in Table 3, the methodology generated data of good quality with a normal distribution. Most important, the mean values with the stress environment were significantly different to those with the non-stress environment. The high coefficient of variation in the test carried out in the second half of 2016 can be attributed to the clay content present in the soil (Table 4), demonstrating that this methodology is more efficient and reliable when using soils of the argillaceous-sandy textural class.

Table 3. Average values, coefficient of variation (CV%), heritability (h²), normality according to Shapiro Wilk methodology and t-test of soybean components (number of pods, number of seeds and seed mass in grams) grown in environments without stress and with water stress, simulated by the pot capacity method using information from the soil water retention curve.

Year/ experiment	Treatment	Average ^a	CV (%) ^a	h ^{2a}	Nomality ^a P value	t-test ^a p-value
2016.1	Number of pods					
	Control	106.66	16.93	0.45	0.47 ⁺	0.02 ^{**}
	Stress	91.54	15.80	0.63	0.92 ⁺	
	Number of seeds					
	Control	238.60	14.09	0.74	0.94 ⁺	0.00 ^{**}
	Stress	196.17	14.15	0.64	0.55 ⁺	
	Seeds weight (g)					
	Control	43.34	9.08	0.02	0.00	0.00 ^{**}
	Stress	30.79	9.23	0.23	0.11 ⁺	
2016.2	Number of pods					
	Control	29.71	40.91	0.61	0.00	0.00 ^{**}
	Stress	17.38	44.27	0.42	0.39 ⁺	
	Number of seeds					
	Control	56.81	37.60	0.36	0.10 ⁺	0.00 ^{**}
	Stress	32.85	44.98	0.39	0.09 ⁺	
	Seeds weight (g)					
	Control	6.15	50.80	0.49	0.01	0.00 ^{**}
	Stress	3.44	64.23	0.57	0.00	
2017.1	Number of pods					
	Control	113.34	30.59	0.15	0.00	0.00 ^{**}
	Stress	85.55	17.91	0.48	0.36 ⁺	
	Number of seeds					
	Control	236.51	16.46	0.61	0.46 ⁺	0.00 ^{**}
	Stress	176.47	19.84	0.38	0.00	
	Seeds weight (g)					
	Control	29.07	13.49	0.13	0.00	0.00 ^{**}
	Stress	22.35	14.15	0.30	0.34 ⁺	

^aAnalyses performed using R Studio statistical software version 3.4.1 and using the integrated base package (<https://www.R-project.org/>)

⁺ Significant by the Shapiro-Wilk normality test

^{*}, ^{**} Significant at 5% and 1% by the t-test

Table 4. Summary of the physical analysis of the soils used.

Year	Sand	Silt	Clay	Textural class
Experiment	-----%-----			
2016.1	30	20	49	Sand clay
2016.2	56	13	31	Clay
2017.1	43	7	50	Sand clay

The high clay content in the soil causes a shorter time interval for the plant to achieve a high stress level; this is due to the clay retaining water and, therefore, not making it available to the plant (Figure 2). As a result, the safety interval between irrigation shifts is reduced, causing the plants to reach stress faster than normal and with much more severity, thus causing the high CV observed in the 2016.2 experiment. To solve this problem, it would be necessary to weigh more frequently throughout the day, instead of only twice. However, this can make the service unfeasible, depending on the number of pots being studied. Thus, for the application of this methodology, the use of soils of argillaceous-sandy texture is recommended.

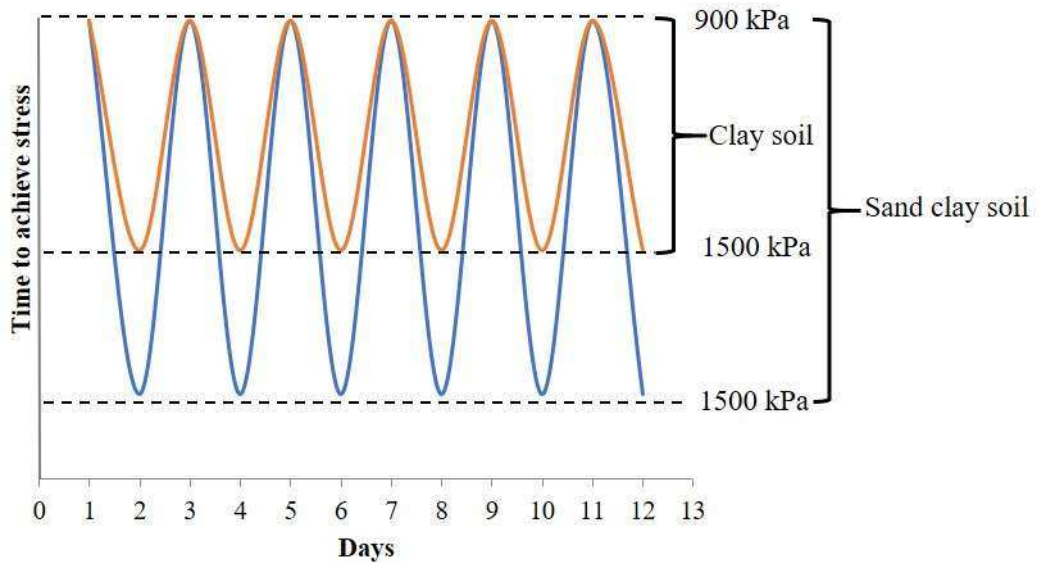


Figure 2. Time required for different soils to reach high stress level (-1500 kPa) according to their clay content within the same irrigation shift.

FINAL CONSIDERATIONS

Each methodology has characteristics that vary according to the complexity and ease of execution, from simple irrigation suspension to the voltage monitoring of water in the soil by means of daily weighing. Selection of a methodology will be determined by the number of genotypes to assess, the objective of the research and the availability of manpower. All the methods presented are cheap and easy to use, requiring only balance for weighing. This study introduced only a few methodologies, depending on the researcher use them singly or unify them in order to extract the better results, as well as, make the adjustments to their particular situations.

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ARTIGO CIENTÍFICO 2 - STRATEGY FOR SELECTION OF SOYBEAN GENOTYPES TOLERANT TO DROUGHT DURING GERMINATION

DANTAS, Stênio Andrey Guedes; SILVA, Francisco Charles do Santos; SILVA, Laércio Junio; SILVA, Felipe Lopes. Strategy for selection of soybean genotypes tolerant to drought during germination. **GMR**, v 16, n. 2, p. 1-8. 10 maio 2017.

ABSTRACT

Water deficit is the main reason for instability in the context of soybean culture. The development of strategies for the selection of more tolerant genotypes is necessary. These strategies include the use of polyethylene glycol 6000 solutions (PEG-6000) for conducting the germination test under conditions of water restriction. Thus, the objective of this study was to determine the osmotic potential and the main characteristics that promote the discrimination of soybean genotypes with regard to water stress tolerance during germination and the vigor test. Thirteen soybean cultivars were used. The seeds were allowed to germinate on sheets of germitest paper moistened in solution with PEG-6000, simulating different levels of water availability, which is expressed as osmotic potential (0.0, -0.2, -0.4, and -0.6 MPa). We assessed germination, length, and dry mass for seedlings and seeds, as well as reserve dynamics. Germination and variables related to the dynamics of reserves have great influence on the expression of variability in environments under stress. Among the different osmotic potentials, the -0.2 MPa was the most efficient for the expression of genetic variability among the cultivars. Conducting the germination test with PEG-6000 solution to -0.2 MPa was efficient for selecting soybean cultivars tolerant to water stress. This was accomplished by evaluating the percentage of germination, along with variables related to the dynamics of reserves.

Key words: *Glycine max* (L.) Merrill; Genetic breeding; Stress level; Polyethylene glycol (PEG-6000); Drought tolerance

INTRODUCTION

While the effects of water stress following the establishment of cultures have been widely studied and elucidated, the survival of plants under drought during the establishment of culture has rarely been considered. The initial establishment of seedlings is obviously the first step to succeeding in the production of diverse cultures, primarily in water-deficit conditions, in which potential differences between the survival rates of plants under these conditions may result in better training of booth, ensuring greater stability (Marrou et al., 2015).

The establishment of the culture of soybean is directly related to the vigor of seed (Henning et al., 2010). Vigor is a set of properties that contribute to indicate the quality of the seed; these properties related to its potential for germination, seedling emergence, and storage capacity under different environmental conditions are considered standard (Sun et al., 2007).

The initial vigor of plants can be measured through the evaluation of hypocotyl, root, and seedling characteristics, as the hypocotyl length (HL) and root length (RL) and dry mass (Vanzolini et al., 2007), as well as through the analysis of seed reserves (Henning et al., 2010). It is important to note that these features when present genetic variability can be considered in breeding programs (Pereira et al., 2015) and a strategy on the selection of superior genotypes.

For studies of selection of soybean genotypes tolerant to water deficit involving germination and vigor tests, several authors have observed the effectiveness of these tests in conditions of low osmotic potential simulated with polyethylene glycol (PEG), applying from the use in studies of genetic diversity, differentiation and grouping of soybean genotypes most tolerant to water stress (Teixeira et al., 2008a,b); as well as to the relationship of genotype response during this test with its performance on the field, and subsequently with productivity (Kosturkova et al., 2008).

However, there is no consensus that the differences regarding the response to water deficit in the germination stage of cultivars are entirely consistent with the response to water deficit by evaluating data of productivity (Mederski and Jeffers, 1973; Oya et al., 2004). This is perhaps because there is not yet a standardized methodology

for the simulation of water deficit and the level of stress that the seed must be submitted during the germination test for discrimination of tolerant or susceptible genotypes, and the identification of important features to be assessed that would lead to such discrimination.

The objective of this study was to determine the osmotic potential and the main characteristics to be evaluated that promote discrimination of soybean genotypes as tolerance to water stress during germination and vigor.

MATERIAL AND METHODS

The test was conducted in the Oilseed Breeding Laboratory, Plant Science Department of University Federal of Viçosa (UFV), Minas Gerais, Brazil. Thirteen soybean cultivars were used: UFV 16, UFVS 2001, UFVS 2002, UFVS 2003, UFVS 2004, UFVS 2008, UFVS 2009, UFVS Quartzo, UFVS Turquesa, UFVS Berilo, UFVS Opala, TMG 1179 RR, and MG/BR Conquista. The seeds used in this study were produced under field conditions in 2014/2015 in experimental field of UFV, and, immediately after harvesting, drying at room temperature, and cleaning, the seeds were standardized as to size in mm sieve, and then was carried out the test, thus ensuring that all tests were conducted with freshly harvested seeds, as recommended by Soltani et al. (2006). The seeds were subjected to the following tests and measurements.

Determination of the moisture and dry mass of seeds

To determine seed water content, three replications of 50 seeds were weighted (W1) and taken to air circulation oven at 105°C for 24 h, and then reweighed (W2) on analytical scales. The seed dry mass (SDM) was calculated as $((W1 - W2) / W2)$. For the purpose of this study, the SDM was considered as the total reserves of the seed available to be mobilized to seedlings.

Germination

The germination test was conducted with four replications of 50 seeds, distributed on three sheets of germitest paper moistened with solutions in different stress levels, expressed as osmotic potential: 0.0, -0.2, 0.4, and 0.6 MPa. Each stress level has been obtained by adding PEG-6000 to distilled water at a temperature of 25°C, in accordance with the recommendation of Vilela (1991). The roles were moistened

with their respective solutions at a rate of three times their dry weight. After the distribution of the seeds on two sheets of germitest paper, these were covered with a sheet of germitest paper and made the rolls, which were brought to germination at the regulated temperature of 25°C, with a 12-h light/12-h dark as stated in rules for seed analysis (Brasil, 2009).

The counts of the number of normal seedlings were conducted at five (first count of germination - FC) and 7 days (germination - GR) after sowing. The results are reported as percentage.

Seedling growth and reserve dynamics

Seedling growth test was performed with four repetitions of 10 seeds each, distributed longitudinally on the upper third of the germitest paper with their micropil parts directed to the base of the paper. Seven days after sowing, the rootlets and the hypocotyls of normal seedlings were measured with a ruler. The results are reported in cm.

Then, the radicle, the hypocotyl, and the cotyledons were separated and placed to dry at $80^{\circ} \pm 1^{\circ}\text{C}$ for 24 h. After this period, the plant material was weighed on analytical scales, separately, for evaluation of radicle dry mass (RDM), hypocotyl dry mass (HDM), and cotyledon dry mass (CDM).

Due to the need for standardization with the SDM data, which is an average of 50 seeds put out to dry, the values obtained individually from dry mass for each plant tissue, described earlier, were added and multiplied by 50, which yielded an approximate value of total dry mass estimated for 50 plants (PDM) at 100% germination (Soltani et al., 2002, 2006; Pereira et al., 2015).

From the remaining CDM and the SDM it was possible to obtain the value of the reduction of seed reserves (RSR) for estimator: $\text{RSR} = \text{SDM} - \text{CDM}$. Following the same reasoning and based on the values of PDM, the efficiency of conversion of reserves (ECR) was determined, which corresponded to the SDM conversion in seedling dry mass, using the estimator: $\text{ECR} = \text{PDM} / \text{RSR}$. And finally, it was determined the rate of RSR (RRSR), obtained by the estimator: $\text{RRSR} = \text{RSR} / \text{SDM}$,

which allows to identify the cultivar that mobilized the greater SDM for the establishment of seedlings (Soltani et al., 2006).

Statistical analyses

Statistical analysis of the data was divided into five stages: the first stage was a multivariate analysis of the Mahalanobis distance among four stress environments, considering all the genotypes as a single source of variation, including cluster analysis based on the link between averages (UPGMA); in the second stage, analysis of variance was performed to study the interaction genotype x stress levels; the third stage was to determine the osmotic potential great for discrimination of varieties as the stress level; the fourth stage was to identify the features that contribute most to the cultivar discrimination osmotic potential great; and the fifth was a simple correlation analysis between the characteristics in the potential of -0.2 MPa.

For the third step, the data were organized so that stress levels were considered as variables. The analysis of relative contribution to genetic diversity was conducted using the methodology of Singh (1981) within each variable and all simultaneously.

All analyses were performed using the Genes program (Cruz, 2013).

RESULTS AND DISCUSSION

The levels of simulated stress achieved with PEG-6000 showed divergence (0.0 MPa), when compared in terms of Mahalanobis distance. Between the stress conditions, the environment conditioning by potential -0.2 MPa proved to be distinct from the other; however, no significant difference were observed between the potential -0.4 and -0.6 MPa, indicating that these stress levels cause the same effects in similar magnitudes on the seeds (Figure 1).

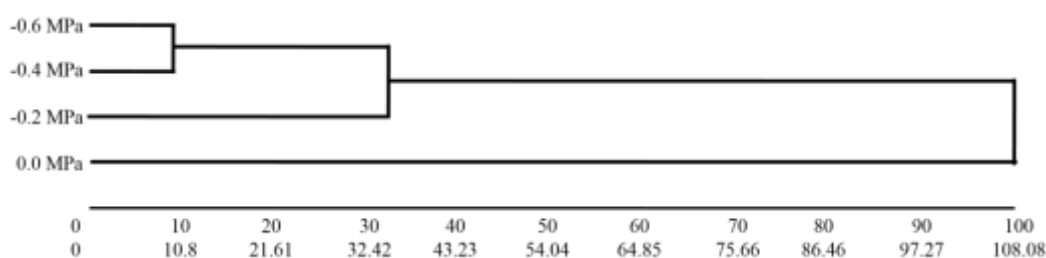


Figure 1. Cluster analysis based on connection between groups (UPGMA) for four potential osmotic conditions on 13 genotypes and 10 variables.

There was statistical significance for interaction genotype x stress levels for the F-test at 5% probability for all traits evaluated, demonstrating that the cultivars respond differently at each stress level. Analyzing the unfolding of the interaction, it was found the expression of genetic variability among genotypes to be explored within each level of stress (results not shown), being the need for identification of osmotic potential great for discrimination of cultivars in tolerant and susceptible.

The stress level 0.0 MPa (control) had a high influence on the genetic variability for all analyzed variables influencing 37% of general diversity (Table 1). The high variability within the 0.0 MPa stress due to the natural genetic diversity exists between cultivars for characteristics related to seed vigor, a fact noted as much on culture of soybean (Santos et al., 2012; Pereira et al., 2015), as well as in other cultures, such as rice (Yu et al., 1999), wheat (Soltani et al., 2001), and corn (Sun et al., 2001).

Among the four osmotic potentials tested, 0.2 MPa allowed for the greatest expression of genetic variability among the genotypes studied (43%) (Table 1).

Table 1. Relative importance of levels of stress (osmotic potential) induced by PEG-6000 for the genetic diversity of 13 characteristics of soybean genotypes.

Level of stress (MPa)	FC	GR	RDM	HDM	CDM	RL	HL	RSR	ECR	RRSR	All variables
0.0	82%	19%	53%	45%	45%	28%	21%	49%	12%	54%	37%
-0.2	18%	15%	33%	20%	29%	47%	16%	32%	31%	29%	43%
-0.4	0%	24%	8%	10%	19%	18%	36%	18%	9%	16%	14%
-0.6	0%	42%	6%	25%	7%	7%	28%	1%	48%	1%	6%

FC - first count; GR - germination; RDM, HDM, and CDM - dry mass of root, hypocotyl, and cotyledons; RL and HL - root and hypocotyl length; RSR - reduction of seed reserves; ECR - efficiency of conversion of reserves; RRSR - reduction rate of seed reserves.

When considered in isolation each feature within the four levels of stress, one can realize that of the ten that were evaluated, the stress of -0.2 MPa was responsible for the largest variation in six (FC, RDM, CDM, RL, RSR, and RRSR), while the stresses -0.4 and -0.6 MPa contributed equally to two variables each (Table 1). In this way, the stress of -0.2 MPa stands out as being the most suitable as a potential stress level for

discrimination of genotypes tolerant and susceptible to drought imposed during the germination process.

Rossetto et al. (1977) reported that the percentage of germination of soybean seed lots is reduced when water is retained in the substrate at tensions of approximately -0.2 MPa, which is the most influential potential with respect to genetic diversity. Soltani et al. (2006) observed in a study with wheat that there was an interaction between genotype x level of osmotic potential applied during germination, and showed that significant differences among genotypes were clearer in stress levels smaller than -1.5 MPa, much larger than the potential evaluated in the present study; however, in different species.

The vigor of seed can be affected by many factors and can be expressed by germination, root, and shoot length, fresh and dry mass of seedlings, germination test in cold or high temperature condition, reserve dynamics and others, as shown in Table 1. The aforementioned characteristics are controlled by multiple genes, being of quantitative nature (Dickson, 1980), affected by environmental factors during seed maturation, harvest and storage, which makes genetic analysis of seed vigor too hard (Sun et al., 2007).

An alternative when trying to analyze so many variables and optimize the efficiency of selection in genetic studies is selecting features with greater influence, especially in situations requiring the evaluation of many genotypes. For this, we used an analysis of importance of characters in different stress levels (Figure 2).

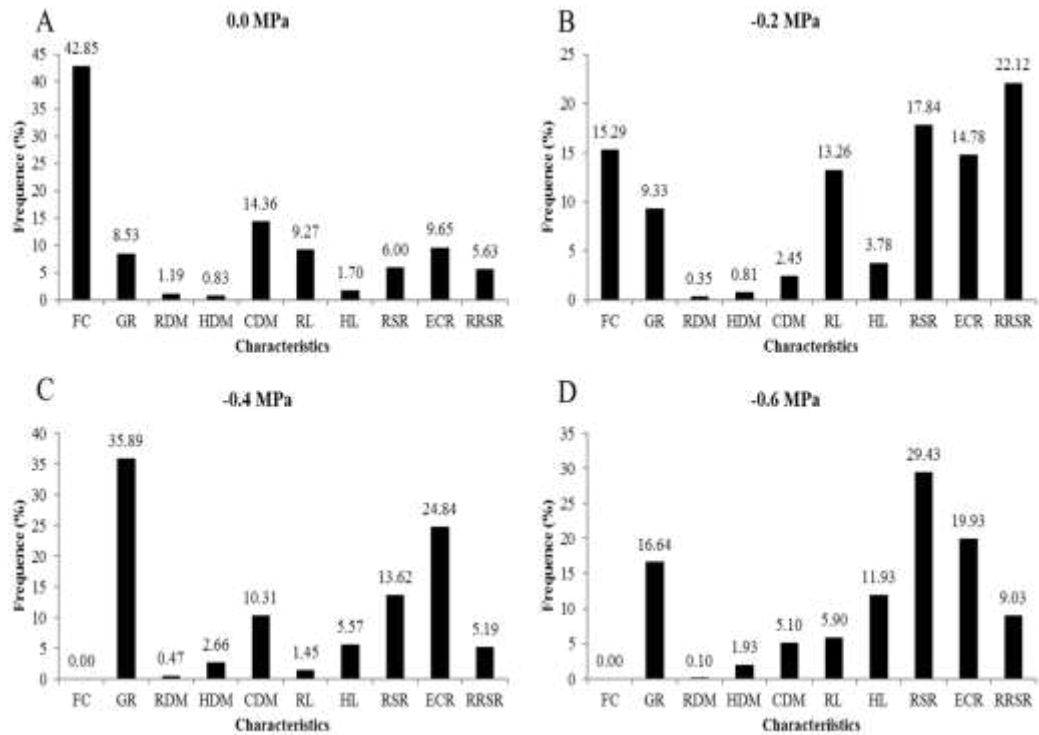


Figure 2. Relative importance of various characteristics to the genetic diversity of 13 soybean genotypes at four levels of stress (osmotic potentials) induced by PEG-6000 during germination. FC - first count; GR - germination; RDM, HDM, and CDM - dry mass of root, hypocotyl, and cotyledons; RL and HL - root and hypocotyl length; RSR - reduction of seed reserves; ECR - efficiency of conversion of reserves; RRSR - reduction rate of seed reserves.

As much as the level of stress increased, characteristics related to dynamic reserves also increased influencing the diversity. In all stress conditions (Figure 2B, C, and D), among the three characteristics that had the most influence on the genetic diversity, at least two in each stress level were related with dynamics of reserves and with RRS emphasis, which was always the second most important feature. This demonstrates the importance of using these variables to distinguish the most tolerant and the most sensitive genotype.

The first count and germination are highly important under all environmental conditions. However, as they increase, the stress levels at the first count lose influence on the germination and other remaining characteristics, which delay the germination of seeds. Moreover, not always the seed germination ensures the establishment of the plant, if it does not have sufficient reserves to appropriate initial seedling growth. For

this reason, the properties related to the dynamics of reserves emerge as potential features for selection of genotypes tolerant to stress conditions.

The RSR, or remaining dry mass in cotyledons, is indicative of how much the initial reserve present in cotyledon was relocated during the stage of seed germination, i.e., the higher the RSR more energy is available to the process of germination. Features of use of seed reserves, like RSR, play important roles in seed vigor, implementing its germination and heterotrophic growth (Cheng et al., 2013).

Seed germination is followed by the mobilization of cotyledon reserves, providing essential energy to feed seedling growth until the seedling becomes photoautotrophic. Because of the significance of this process during and after germination, the mobilization of reserves during germination has been widely studied (Pritchard et al., 2002).

There are disagreements about the amount of energy available to seeds and its effective use (ECR), that is, processing as dry matter (Pereira et al., 2015). These variables are greatly influenced by seed size and initial mass (Soltani et al., 2006). Therefore, a simple linear correlation analysis was performed between these assessed characteristics in soybean genotypes subjected to a stress level of -0.2 MPa (Table 2), which is the level of stress that provided the greatest expression of genetic variability.

Table 2. Estimated phenotypic correlation coefficients for various characteristics based on the averages of the evaluated cultivars under water stress level of -0.2 Mpa.

	FC	GR	RDM	HDM	CDM	RL	HL	RSR	ECR	RRSR	SDM
FC	-	0.20	0.61*	-0.09	0.15	0.42	-0.02	-0.08	-0.03	-0.10	0.09
GR	-	-	0.44	-0.05	0.40	0.42	0.29	-0.42	0.28	-0.44	0.06
RDM	-	-	-	0.48	-0.14	0.87**	0.38	0.27	-0.25	0.24	0.19
HDM	-	-	-	-	-0.44	0.49	0.55*	0.64*	-0.54	0.63*	0.31
CDM	-	-	-	-	-	-0.20	-0.10	0.74***	0.81*	-0.87**	0.31
RL	-	-	-	-	-	-	0.50	0.29	-0.42	0.30	0.15
HL	-	-	-	-	-	-	-	0.37	-0.37	0.32	0.38
RSR	-	-	-	-	-	-	-	-	-0.73**	0.98**	0.40
ECR	-	-	-	-	-	-	-	-	-	-0.81**	0.06
RRSR	-	-	-	-	-	-	-	-	-	-	0.20
SDM	-	-	-	-	-	-	-	-	-	-	-

FC - first count; GR - germination; RDM, HDM, and CDM - dry mass of root, hypocotyl, and cotyledons; RL and HL - root and hypocotyl length; RSR - reduction of

seed reserves; ECR - efficiency of conversion of reserves; RRSR - reduction rate of seed reserves; SDM - seed dry mass. *, **, *** Significant at 5%, 1% and 0.01% probability by the *t*-test.

According to the results in the potential of -0.2 MPa, no correlation were found between seed size and the characteristics related to the reservation dynamics, showing that the initial seed size does not influence at all the vigor of the seed.

In conclusion, we observed that germination test driving under water stress, induced by PEG-6000 and osmotic potential of -0.2 MPa is efficient for the exploitation of greater genetic variability between soybean genotypes; and that the percentage of germination and analysis of seed reserve dynamics are potential variables to be considered when selecting genotypes tolerant to drought during germination.

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ARTIGO CIENTÍFICO 3 - GENOME-WIDE ASSOCIATION STUDY OF GERMINATION AND VIGOUR IN SOYBEAN SEEDS UNDER DROUGHT CONDITIONS

ABSTRACT

Soybean is the most economically important legume planted in the world. The objective of this work was use the genomic association in study of characteristics related to the germination and vigor of soybean seeds. The seeds of 97 cultivars, genotyped with SNP markers, were then germinated under stress-free conditions and under water stress conditions induced by polyethylene glycol 6000. The results showed that genotypes behaved differently according to the level of stress, with significant statistical interactions between genotypes and environment. Significant values for genetic variability and heritability were found. A genomic association study showed SNPs significantly associated with control of vigor characteristics when germinated in the environment without stress. In stress environment, significant SNPs were found associated with dry mass of cotyledon and root length. Root dry mass was the only variable that had significant SNPs at both stress levels. It was concluded that the genetic control of germination and vigor of the soybean seeds differs depending on the environment, but there is a possibility of improvement of these characteristics, both through classic breeding and through the use of GWAS.

Key words: GWAS, Breeding to drought, *Glycine max* (L.) Merrill, Drought stress

INTRODUCTION

Soybean (*Glycine max* (L.) Merrill) is the fourth most planted agricultural species in the world, being the most economically important legume (SILVA et al., 2017). Soy is currently planted from the Southern Hemisphere to the North on all continents except Antarctica. Expansion of crop planting, along with climate change, have led soybeans to be increasingly exposed to abiotic stresses, which tend to reduce productivity. Water deficit is considered one of these main limiting factors (SENTELHAS et al., 2015).

Lack of water during germination of soybean seeds compromises the initial growth and establishment of the plants in the field, affecting their uniformity and density in the affected area, which directly affect their productivity (GULLUOGLU et al., 2017). Breeding by the selection of genotypes that are water stress tolerant during the initial stages of development is a potential solution to this problem, and has also been studied in other species such as alfalfa (MA et al., 2017), brassicas (TAN et al., 2017) and corn (XIANG et al., 2017), using different approaches.

The adaptation of the plants to water deficit is complex, involving the interaction of several genes. This complex adaptation is also true for seedling vigor during the initial phase of plant development, both in stress and non-stress conditions, which is controlled by several genes (DICKSON, 1980) at the transcriptional, translational and metabolic level (RAJJOU et al., 2012). It is thus difficult to understand the genetic control of these traits for possible future use in breeding. In order to understand the interactions between plants and water stress, the use of genomic selection integrated with physiology and other sciences have been suggested (VADEZ et al., 2014). With the reduction in genotyping costs and constant improvements in statistical methods, genomic wide association selection (GWAS) and genome wide selection (GWS) studies are promising tools for the genetic breeding of complex traits in agricultural species (ZHANG et al., 2016).

GWAS is an approach of molecular breeding using the identification of association between molecular markers or loci and characteristics of interest in a given population of individuals, and is an efficient method for the identification of genomic regions associated with complex quantitative genetic control characteristics (TAN et al., 2017).

The objective of this study was to analyze the genetic control of characteristics related to the germination and vigor of soybean seeds in environments with and without water stress, exploring the possibility of using GWAS to support this analysis and to apply it in the selection of more tolerant genotypes.

MATERIALS AND METHODS

Obtaining seeds and phenotyping

Seeds of 97 soybean cultivars were planted in the 2015/2016 (Table S1) in the Experimental Station of Coimbra, Minas Gerais, Brazil. The plants received all the cultural traits according a field of grain production. Seed phenotyping was conducted at the Oilseed Breeding Laboratory, at the Department of Plant Science, Universidade Federal de Viçosa, Minas Gerais, Brazil.

A germination test was conducted with four replicates of 50 seeds, distributed over two sheets of germitest paper moistened with solutions at two different osmotic potentials: 0.0 and -0.2 MPa. The 0.0 MPa solution corresponded to the control condition, without stress; the -0.2 MPa solution provided the stress condition. This value was adopted according to previous experiments where it was verified that an osmotic potential of -0.2 is performs best in the selection of stress tolerant genotypes (DANTAS et al., 2017). To achieve the desired stress level, polyethylene glycol (PEG 6000) was added to distilled water at 25 °C (VILLELA et al., 1991). The papers were moistened with their respective solutions at a ratio of three times their dry weight. After the seeds were distributed, they were covered with a sheet of germitest paper and rolls were prepared, and placed in a germinator at 25 °C, with a photoperiod of 12 hours of light and 12 hours of dark («Regras para Análise de Sementes — Ministério da Agricultura, Pecuária e Abastecimento», [S.d.]).

Counts of the number of normal seedlings (germination - GR) were performed seven days after sowing. The results were expressed as a percentage.

A seedling growth test was performed with four replicates of ten seeds each, distributed in the upper third of the germitest paper with its micropiles directed to the base of the paper. Seven days after sowing, the radicles and hypocotyls were

photographed and measured using ImageJ software (ABRAMOFF et al., 2004). The results were expressed in cm / seedling.

The radicle, hypocotyl and cotyledons were then separated and placed to dry in a forced air circulation oven at 80 ± 1 °C for 24 hours. The plant tissues were then weighed separately to evaluate the dry masses of seedlings, radicles (MSR), hypocotyls (MSH) and cotyledons (MSC).

Statistical analysis of phenotypes

Phenotypic analyses were performed using Selegem-REML / BLUP software (RESENDE; RESENDE, 2016). Initially, the model 105 was adopted to calculate the maximum, minimum and standard deviation. The variance components were calculated considering the evaluation of genotypes in several replicates, with several observations per plot, in a single environment and a single harvest. Each stress level was considered as an environment, and each components of variance for each were estimated separately and then together. For this purpose, model 81 was used, which is represented by: $y = Xu + Zg + Wp + e$, where y is the data vector, u is the effect of the general mean, g is the vector of the genotypic effects (assumed to be random), p is the vector of plot effects, and e is the vector of errors or residues. Capital letters represent the incidence matrixes for these effects. Analysis of variance was performed in the statistical software R Studio version 3.4.1 and using the integrated base package («R: The R Project for Statistical Computing», [S.d.]).

The coincidence index was obtained using the ten best and worst genotypes ranked by the average of each evaluated characteristic, the index being a relation between the number of coincident genotypes (Cn) divided by the number of evaluated genotypes n , which in this case was ten, according to the formula: $Ci (\%) = (Cn/n) \times 100$.

Genotyping

Genotyping was performed at Deoxi Biotecnologia Ltda, Araçatuba / SP, using the Illumina iScan platform and the Infinium iSelect HD Custom Genotyping BeadChips 6k panel (Illumina, Inc., San Diego, CA, USA), customized for soybean with 24 samples by BeadChip. Genotyping was performed according to the protocol described by the manufacturer («MiSeq Support - Documentation & Literature», [S.d.]).

Genomic association and linkage disequilibrium

The analysis was performed using a compressed mixed linear model (ZHANG; ERSOZ; LAI; TODHUNTER; TIWARI; GORE; BRADBURY; YU; ARNETT; ORDOVAS, 2010) implemented in the GAPIT R package (LIPKA et al., 2012a). Significant SNPs with $MAF \geq 0.025$ and $FDR \leq 0.95$ were considered significant.

The linkage disequilibrium between SNPs located in the same region of the genome forming a locus was performed in TASSEL 5.0 (BRADBURY et al., 2007) with a distance of 50 bp, a total of 19025 comparisons.

Allele contribution analysis

To perform this analysis, the phenotypic mean for each allele of each significant SNP was sampled. The alleles were later grouped into locus and represented by the SNP with the highest P-value. The percentage difference was then calculated between the phenotypic value of the highest and lowest allele at each locus.

RESULTS

Phenotype statistics

Phenotypic values varied according to the characteristics evaluated and the environment. The germination presented a range of variation from near zero to almost one hundred percent in the environment without stress. Under stress conditions the range was 0 to 100%, with a germination average 24.21% lower than the control. This result shows that the stress reduced germination, but that there are genotypes that even under unfavorable conditions, such as water deficit, can achieve 100% germination. Some genotypes showed a coincidence of rank among the environments, where it was verified that among the ten genotypes with the highest and lowest traits values, there are a coincidence of genotypes between the best and the worst ranks (Table 1).

The germination was the trait with the highest coincidence index, in both the environments, with 40% and 50% of coincidence in the best and the worst environment, respectively. The genotypes that correspond to the better performance in germination, independently of the stress level, were the cultivar CD 202, Ocepar 3, CD 251 RR and MSOY 7901.

The traits related to root, length and dry mass, had low best coincidence index, compared to the coincidence index of germination. The root length showed 20% of coincidence, represented by cultivars SG 770RR and Fundacep 33. The dry mass of roots had just one genotype that appear among best in both stress levels, the cultivar CD 202. The hypocotyl length and dry mass showed similar results to the traits related to roots, when, to the hypocotyl length just the cultivar Ocepar 3 was listed among the ten bests. The hypocotyl dry mass had 20% of coincidence between the best genotypes, represented by cultivars Fundacep 96RR and NK 8350. For the cotyledons dry mass, no one genotype showed coincidence when was listed between the better in both environments.

The cultivar CD 202 appear among the best in the traits germination and root dry mass. While the cultivar Ocepar 3 is listed between the best in the traits germination and hypocotyl length. These cultivars are the unique that appear more than one time in de coincidence index, representing a potential tolerant cultivar to be explored and studied in breeding programs.

The characteristics related to the dry mass of the seedlings showed different responses to stress according to the organ evaluated. The dry mass of the root and the hypocotyl had values corresponding to zero under stress conditions, which is a reflection of the genotypes that did not germinate. Meanwhile the dry mass of cotyledons increased under stress conditions, because the seed soaks up the solution and increases in volume and mass, but does not carry out the translocation of reserves for the emission of the radicle and hypocotyl due to the stress. In general, the root dry mass had a low average difference of values observed between the two environments (5.02%), while the masses of the hypocotyls and cotyledons were significantly different, by 67 and 38%, respectively.

For root and hypocotyls length, a trend similar to that of the dry mass for the same organs was observed in the stress environment, due to the factors mentioned above. However, observing the data it can be seen that the characteristics related to the root (length and mass) present the lowest average percentage difference between the control and stress conditions, i.e., the root characteristics are least differentiated between the environments, especially when compared to characteristics related to the aerial part of the seedlings, such as hypocotyls and cotyledons.

Table 1. Phenotypic variation for the different germination and growth characteristics of soybean seedlings (*Glycine max* L. Merrill) (n = 97) germinated in a controlled environment without stress (control) and with water stress simulated with the use of PEG 6000 at a potential of -0.2 MPa

Trait	Unit	Control				Stress				CI		MD (%)
		Min	Max	Mean	SD	Min	Max	Mean	SD	Best	Worst	
Ger	%	0.08	0.98	0.72	0.15	0.00	1	0.55	0.31	40%	50%	24.21
DR	mg	0.86	46.2	3.96	1.63	0.00	19.7	3.76	1.72	10%	30%	5.02
DH	mg	8.63	93.73	22.62	5.3	0.00	27.2	7.42	2.72	20%	20%	67.18
DC	mg	7.94	172.52	104.53	17.38	53.85	632.33	144.4	31.25	0%	10%	38.15
RL	cm	1.67	16.54	9.63	2.9	0.00	20.92	9.43	5.51	20%	20%	2.01
HL	cm	1.28	15.34	8.41	2.11	0.00	11.05	1.92	1.02	10%	20%	77.14

Ger—Germination, **DR**—Dry mass of the roots, **DH**—Dry mass of the hypocotyl, **DC**—Dry mass of the cotyledon, **RL**—Length of root, **HL**—Hypocotyl length, **MIN**—minimum, **MAX**—maximum, **SD**—Standard deviation, **CI**—coincidence index, **MD**—Mean difference between environments without and with stress.

Germination was the variable that presented the highest coincidence index among the genotypes. The other variables, even when presenting very similar phenotypic values among the environments, such as for characteristics related to the root, had a low coincidence index, showing that the genotypes behave differently at different stress levels. This observation can also be verified in the analysis of variance (supplementary material Table S2), in which the interaction between the genotypes and the environment is significant for all variables.

The heritability (h^2) of the characteristics when evaluated together, without differentiating the environments, ranged from 69 to 99% (Table 2). In the control condition, the h^2 of DR was close to zero; DH and DC showed the next lowest heritability, 49 and 63%, respectively. The other variables presented h^2 values higher than 76%. Under stress conditions, the variable with lowest h^2 value was DC, 60%. All other characteristics assessed under stress had h^2 values higher than 82%, which represents a major phenotypic variation attributed to genetic effects. In general, the h^2 in each environment (control and stress) presented similar values for the studied variables, except for the dry mass of the root (DR) and hypocotyl (DH). These two variables had h^2 values in the control that were well below those measured under stress conditions, demonstrating that there are fewer loci with greater effects controlling these characteristics under control conditions than in stress conditions (Table 3). This observation was verified by the genomic association analysis, which found more significant SNPs under control conditions than in stress conditions (Table 4).

Table 2. Genetic heritability in the broad sense (h^2g), and genetic ($CV_{gi}\%$) and environmental variation coefficients ($CV_e\%$) for soybean seeds (*Glycine max* L. Merrill) ($n = 97$) germinated in a controlled environment without stress (control) and under stress caused by PEG 6000 at an osmotic potential of -0.2 MPa.

	Ger	DR	DH	DC	RL	HL
Control						
h^2g	0.76	0.07	0.49	0.63	0.86	0.85
$CV_{gi}\%$	24.85	38.19	25.30	18.83	271.38	98.42
$CV_e\%$	8.50	166.22	20.84	10.65	8.27	9.75
Stress						
h^2g	0.82	0.85	0.85	0.6	0.83	0.82
$CV_{gi}\%$	58.82	966.12	973.75	54.50	98.36	118.70
$CV_e\%$	10.83	2.52	1.50	44.56	16.74	22.57
Joint analysis						
h^2g	0.99	0.83	0.79	0.99	0.69	0.99
$CV_{gi}\%$	53.02	55.03	28.31	704.44	11.69	865.46
$CV_e\%$	9.48	49.29	28.63	25.10	15.41	14.28

Ger— Germination, **DR**— Dry mass of the roots, **DH**— Dry mass of the hypocotyls, **DC**— Dry mass of the cotyledon, **RL**— Length of root, **HL**— Hypocotyls length

Genomic association analysis

Through the analysis of genomic association in the control and stress environments, 31 SNPs with significant effects were found. The SNPs were distributed among 12 of the 20 chromosomes for the variables germination, dry mass of root, dry mass of cotyledon, and root length. No SNPs were significant for the variable hypocotyl length. Significant SNPs with a $-\log_{10}(\text{p-value}) > 3$ were considered significant (Table 3).

Table 3. Significance of SNPs associated with germination (Ger), root dry mass (DR), dry mass of cotyledon (DC), and root length (RL) in soybean seeds (*Glycine max L. Merrill*) (n = 97) germinated in controlled environment without stress (control) and with stress caused by the presence of PEG 6000 at an osmotic potential of -0.2 MPa.

Trait	Controle					Estresse				
	SNP id ^a	P.value	MAF	r ²	FDR	SNP id ^a	P.valu e	MAF	r ²	FDR
GER	Gm14_10071491_C_T	2.37E ⁻⁰⁴	0.216	0.17	0.53					
	Gm14_10197799_A_G	2.37E ⁻⁰⁴	0.216	0.17	0.53					
	Gm14_8527621_A_G	2.97E ⁻⁰⁴	0.088	0.16	0.53					
DR	Gm02_15213831_T_G	2.44E ⁻⁰⁴	0.04	0.20	0.15	Gm04_7795928_A_G	6.51E ⁻⁰⁴	0.03	0.15	0.50
	Gm02_15290331_G_A	2.44E ⁻⁰⁴	0.04	0.20	0.15	Gm03_8434431_A_G	3.54E ⁻⁰⁴	0.17	0.16	0.50
	Gm04_2968281_C_T	4.94E ⁻⁰⁵	0.05	0.24	0.05	Gm03_10552078_T_G	9.00E ⁻⁰⁴	0.16	0.14	0.61
	Gm04_2794768_T_C	2.19E ⁻⁰⁴	0.07	0.20	0.15	Gm17_19126049_T_C	5.51E ⁻⁰⁴	0.03	0.15	0.50
	Gm04_3077939_A_G	5.18E ⁻⁰⁴	0.06	0.18	0.23	Gm17_20787666_A_G	5.51E ⁻⁰⁴	0.03	0.15	0.50
	Gm04_3138411_A_G	5.18E ⁻⁰⁴	0.06	0.18	0.23	Gm17_32801147_T_G	5.51E ⁻⁰⁴	0.03	0.15	0.50
	Gm06_5318544_T_C	9.35E ⁻⁰⁴	0.08	0.17	0.36	Gm17_33800042_A_G	5.51E ⁻⁰⁴	0.03	0.15	0.50
	Gm07_17615932_A_G	9.35E ⁻⁰⁴	0.11	0.17	0.36					
	Gm08_10850793_C_A	1.55E ⁻⁰⁶	0.03	0.32	0.01					
	Gm08_13581113_A_C	1.11E ⁻⁰⁴	0.06	0.22	0.10					
	Gm12_38105342_T_C	4.78E ⁻⁰⁴	0.05	0.18	0.23					
	Gm19_38659685_C_T	8.31E ⁻⁰⁶	0.04	0.28	0.02					
	Gm19_38692980_A_G	1.66E ⁻⁰⁵	0.05	0.26	0.02					
	Gm19_38732345_T_G	1.66E ⁻⁰⁵	0.05	0.26	0.02					
	DC						Gm13_40715679_A_G	5.80E ⁻⁰⁴	0.198	0.17
						Gm18_6117413_A_C	9.51E ⁻⁰⁴	0.031	0.16	0.73
RL						Gm13_29265240_A_G	5.04E ⁻⁰⁴	0.447	0.15	0.92
						Gm13_29418256_C_T	5.50E ⁻⁰⁴	0.316	0.15	0.92
						Gm13_29524129_A_C	3.19E ⁻⁰⁴	0.342	0.16	0.92
						Gm13_29739984_C_A	7.57E ⁻⁰⁴	0.458	0.14	0.92
						Gm13_30078140_A_G	8.55E ⁻⁰⁴	0.453	0.14	0.92

^aStart with the version of Joint Genome Institute (JGI 1.01) *G.max* genome sequence followed by chromosome number, physical position of the marker on that chromosome and two alleles of the locus (SCHMUTZ et al., 2010). The first of the two alleles for each locus is the Williams 82 alleles

Among the 31 SNPs, 17 were associated with characteristics when evaluated in the control, and 14 were associated with variables studied under stress. Root dry mass was the only variable with association of significant SNPs in both the control and stress environments, but the significant SNPs in each environment were present in different regions of the genome (Table 3). Germination revealed significant SNPs only in the

control, whereas there were significant SNPs for dry weight of cotyledons and root length only under stress conditions.

To incorporate rare alleles and those with smaller effects in the study, according to (WALLACE et al., 2016), genotypes with a minimum allele frequency (MAF) ≥ 0.025 can be accepted, as adopted by (HATZIG et al., 2015) in a genomic study of brassica seed germination. The MAF ranged from 0.03 to 0.21 in the control, and from 0.03 to 0.45 in stress. DR was the variable that presented the lowest values of MAF (0.03 to 0.17), demonstrating that the genetic control of this variable is carried out mainly by alleles that appear at low frequency in the genome, i.e. rare alleles.

Among the 31 SNPs observed, 11 were segregating together in linkage disequilibrium (LD), resulting in a total of 19 loci, of which nine were comprised by multiple SNPs (Table 4). Unbalanced linkage of SNPs with an $r^2 > 0.7$ was considered. Of the 19 loci, only four had any published gene identified in the regions flanked by the markers.

Table 4. Information on loci identified as being involved in the control of germination (Ger), root dry mass (DR), cotyledon dry mass (DC) and root length (RL) in soybean seeds (*Glycine max* L. Merrill) (n = 97) germinated in controlled environment without stress (control) and under stress induced by PEG 6000 at an osmotic potential of -0.2 MPa.

Trait	Control (0,0 Mpa)				Stress (-0,2 Mpa)			
	Locus	Chr ^a	LD Interval (bp) ^b	r ^{2c}	Locus	Chr	LD Interval (bp)	r ²
Ger	Ger1	14	10071491-10197799	1				
	Ger2	14	8527621-8527621	-				
DR	MSR1	2	15213831-15290331	0.76	MSR10	4	7795928-7795928	-
	MSR2	4	2968281-2794768	0.75	MSR11	3	8434431-10552078	0.91
	MSR3	4	3077939-3138411	1	MSR12	17	19126049-33800042	1
	MSR4	6	5318544-5318544	-				
	MSR5	7	17615932-17615932	-				
	MSR6	8	10850793-10850793	-				
	MSR7	8	13581113-13581113	-				
	MSR8	12	38105342-38105342	-				
	MSR9	19	38659685-38732345	1				
DC					MSC1	13	40715679-40715679	-
					MSC2	18	6117413-6117413	-
RL					CR1	13	29265240-29265240	-
					CR2	13	29418256-29524129	1
					CR3	13	29739984-30078140	0.9

^aChr – Chromosome

^bLD interval – *interval* of linkage disequilibrium in base pairs (bp)

^c Correlation between alleles of two locus

Allelic substitution at each locus contributed to the phenotypic variation of the studied characteristics, and ranged from 0.0 to 60%, with a mean difference of 28% between the major and minor alleles. The variable with lowest allelic effect in the phenotype was root length (RL), and the variable with the largest effect was root dry mass (DR) in the plants evaluated under stress (Table 5). For multiple SNPs, the SNP with the highest p value was used to represent the locus.

Table 5. Allelic contribution of each SNP within the locus in the phenotype of the characteristics germination (Ger), dry mass of the root (MSR), dry mass of cotyledon (MSC) and root length (CR) of soybean seeds (*Glycine max* L (N = 97) germinated in a controlled environment without stress (control) and with stress induced by PEG 6000 at an osmotic potential of -0.2 MPa.

Trait	Unt	Locus	SNP id ^a	Alleles				Difference (%)
				C	T	A	G	
Ger	%	Ger1 ^c	Gm14_10071491_C_T	0.62	0.77			0.19
		Ger2 ^c	Gm14_8527621_A_G			0.75	0.5	0.33
DR	mg	MSR1 ^c	Gm02_15213831_T_G		4.28		6.27	0.32
		MSR2 ^c	Gm04_2968281_C_T	4.27	6.37			0.33
		MSR3 ^c	Gm04_3077939_A_G			4.26	5.85	0.27
		MSR4 ^c	Gm06_5318544_T_C	5.35	4.28			0.2
		MSR5 ^c	Gm07_17615932_A_G			4.27	5.17	0.17
		MSR6 ^c	Gm08_10850793_C_A	4.28		10.2		0.58
		MSR7 ^c	Gm08_13581113_A_C	5.85		4.26		0.27
		MSR8 ^c	Gm12_38105342_T_C	7	4.28			0.39
		MSR9 ^c	Gm19_38659685_C_T	4.28	7.45			0.43
		MSR10 ^e	Gm04_7795928_A_G			3.7	9.25	0.6
		MSR11 ^e	Gm03_8434431_A_G			5.33	3.51	0.34
		MSR12 ^e	Gm17_32801147_T_G			3.54	5.41	0.35
DC	g	MSC1 ^e	Gm13_40715679_A_G			0.14	0.17	0.19
		MSC2 ^e	Gm18_6117413_A_C	0.2		0.14		0.29
RL	cm	CR1 ^e	Gm13_29265240_A_G			9.57	9.48	0.01
		CR2 ^e	Gm13_29418256_C_T	9.41	9.43			0
		CR3 ^e	Gm13_29739984_C_A	9.41		9.43		0

^c Control environment

^e Stress environment

DISCUSSION

Phenotypic analysis

The two stages most susceptible to water stress in the soybean crop are the reproductive phase and the establishment of seedlings in the field, the latter stage comprising germination and emergence (FARIAS et al., 2007). Seed germination is itself comprised of three phases. The first is the accumulation of water, from which a series of metabolic reactions resulting in the second phase are triggered. This causes intense cell division, and finally the emission of the radicle, closing the third phase

(BEWLEY, 1997). The fact that water stress increases the mass of seed cotyledons and, in turn, reduces germination and consequently the emission of the radicle and hypocotyl, is due the seeds do not complete the three stages of germination (HEGARTY, 1978), don't mobilizing the reserves of cotyledon to the radicle protrusion, causing then the cotyledons increase mass. Under stress conditions only partial hydration of the seeds occurs, allowing the initial accumulation of water, with consequent cell division, but without sufficient cell division to emit the radicle and terminate the germination process.

It was evident in this study that stress was more active in the inhibition of hypocotyl than in radical growth, that is, the aerial part of the soybean seedlings was more sensitive to stress than the root. This may be because hypocotyl elongation is associated with cell division, which depends on cell wall expansion, for which high turgor pressure, provided by the full availability of water, is required. Since the plant is exposed to low osmotic potential in the solution or in the soil, there is a reduction in cellular expansion, mainly reducing the growth of aerial components (CHAZEN; NEUMANN, 1994 e CRAMER; BOWMAN, 1991 e MICHELENA; BOYER, 1982 e NONAMI; BOYER, 1989 e VAN VOLKENBURGH; BOYER, 1985 e WESTGATE; BOYER, 1985 e WU et al., 2005). Although the elongation of the radicle is also reduced by low osmotic potential, it is less susceptible to stress (PRITCHARD et al., 1993). Radicle extension presents three types of growth, not all of which are directly linked to cell division by means of turgor pressure (BEWLEY, 1997). Similar results were obtained by (ABENAVOLI et al., 2016) in bean plants and by (KUMARI et al., 2014) in wheat. The fact that the roots are more stable and less sensitive to stress has prompted interest in the study of roots as a possible characteristic for selection in soybean plants to increase tolerance to water stress. Root morphology is being used as a selection instrument, using approaches ranging from classic breeding (FENTA et al., 2014 e KUNERT et al., 2016) to genomic cellular biology (GUO et al., 2011).

Genetic breeding can only be achieved if there is genetic variability to be exploited. The tested genotypes presented different behaviors in the control and stress environments, showing that there is genetic variability to be explored in both environments. Thus, the selection of tolerant genotypes is feasible from the variables studied here. In a working with barley seeds an interaction between genotypes and

different stress levels caused by polyethylene glycol 6000 was also verified (ABDELGHANI et al., 2015); however, the authors found higher values of heritability in the control environment, in contrast to the results obtained in the present study, where the h^2 values were similar in both environments, except for the variables DR and DH.

Although heritability was higher in the stress conditions, for some variables, more significant SNPs were verified in the control in the genomic association study. This may occur because the stress exposes the differences between genotypes to a greater extent; however, as most of these locus have very small association effects, they were not identified in the GWAS study. (WALLACE et al., 2016) in a study of genomic association with *Zea mays* under stress and control conditions, also found similar results where in the variables studied under stress had higher heritability, however, fewer significant SNPs were found.

Genomic association analysis

Seed vigor characteristics such as germination, root and hypocotyl length, and root, hypocotyl and cotyledon dry mass are controlled by several genes, being quantitative in nature (DICKSON, 1980), and influenced by environmental factors during seed maturation, harvesting and storage. This makes genetic analysis of seed vigor very difficult (QUN et al., 2007). The genomic association is an alternative that may help in the genetic analysis of these characteristics, narrowing down the allelic variants involved in the control of these variables.

To obtain greater reliability of results from genomic association, parameters such as MAF, r^2 and FDR are employed. Regarding MAF, the International HapMap Consortium recommends using only SNPs with a $MAF \geq 0.05$ (FRAZER et al., 2007 e XING et al., 2010); however in this study, SNPs with $MAF \geq 0.025$ were retained. The maintenance of these rare locus in GWAS analyses is not recommended because it inflates the significance of the SNPs and increase the false positive rate (FDR) (ARDLIE et al., 2002 e LAM et al., 2007). However, (TABANGIN et al., 2009) observed that although the FDR is increased, the increase is not statistically significant. In the present study, the FDR had a higher mean in the stress condition, independent of MAF, which suggests that a different statistical approach should be adopted in genetic studies for stress conditions, in order to correct this problem.

Among the 19 loci presented in Table 4, only four have a previously published gene identified in the regions flanked by the markers. However, these genes not correspond to a known protein. Confirmation of these results in genomic analyses, with the identification of similar areas in different studies of the same characteristics, is important for the validation of QTLs and candidate genes or locus, which in the future can be cloned or used for assisted selection purposes.

The low allelic contribution of SNPs significant for root length may be related to their high FDR (Table 3), greater than 90%, which means that even though these SNPs are significant, they have a 92% probability of being false findings. This is confirmed by their low allelic influence on the phenotypes, where the difference between the presence and absence of the allele is zero for all loci. For the other variables, the results presented in Table 5 show that germination, dry mass of the root and cotyledon characteristics can be breeding by the pyramiding of favorable alleles.

The use of preliminary genotyping information is necessary to help in assisted selection and gene pyramiding, as it serves as the basis for the formation of genetic maps and for the validation of QTLs. The process of combining characteristics or alleles of interest in the same plant, known as gene pyramiding, can be accelerated by the use of molecular markers. This allows the identification and maintenance in the work collection of the breeding program only those plants that have the allelic combination of interest, discarding the others. In this way, the breeding process is optimized in time and cost. The process also provides a greater probability of achieving durable resistance against stresses, among them abiotic stresses such as drought (JOSHI; NAYAK, 2010).

CONCLUSIONS

The genetic control of seed germination and vigor behaves differently, according to the presence of stress conditions. However, there is genetic variability to be explored among the 97 studied varieties, with heritability values that will enable the improvement of characteristics in both environments, control and stress.

The GWAS study found significant SNPs, which can be mapped to the study of locus related to the control of these traits. However, as studies in this area are still scarce, more research is needed to validate and confirm these locus, and then use them for genetic breeding.

SUPPLEMENTARY MATERIALS

Table S1. List of soybean cultivars (*Glycine max* (L.) Merrill) (n = 97) used in the experiment.

MG BR Conquista	CD 215	EMGOPA 304
CD 235 RR	CD 205	CD 254 RR
BMX Potência RR	CD 250 RR	BRS MT Pintado
Tropical RR	M 6707 RR	FUNDACEP 33
CD 226 RR	CD 217	P98Y70
TMG 1066 RR	NK 8350	CD251 RR
CD 2630 RR	CD 2792 RR	BMX Energia RR
CD 253	BRS 284	CD 242 RR
MSOY 7901	OCEPAR 3	CD 201
CD 2721 RR	CD 202	5G770 RR
CD 229 RR	BMX Titan RR	CD 246
CD 237 RR	FUNDACEP 96 RR	CD 288
CD 225 RR	CD 5807	CD 218
TMG 115 RR	BRAGG	MSOY 8001
CD 204	BRS 283	BRS 213
BRS 184	EMBRAPA 59	BRS 257
EMGOPA 304	MG/BR 48 GARIMPO	CD 215
CD 254 RR	FUNDACEP 58	CD 205
BRS MT Pintado	RA 626	CD 250 RR
FUNDACEP 33	CD 2585 RR	M 6707 RR
P98Y70	CD 226 RR	CD 217
CD251 RR	TMG 1066 RR	NK 8350
BMX Energia RR	CD 2630 RR	CD 2792 RR
CD 242 RR	CD 253	BRS 284
CD 201	MSOY 7901	OCEPAR 3
5G770 RR	CD 2721 RR	CD 202
CD 246	CD 229 RR	BMX Titan RR
CD 288	CD 237 RR	FUNDACEP 96 RR
CD 218	CD 225 RR	CD 5807
MSOY 8001	TMG 115 RR	BRAGG
BRS 213	CD 204	BRS 283
BRS 257	BRS 184	EMBRAPA 59
MG/BR 48 GARIMPO		

Table S2. Summary of variance analyses with the mean squares values for genetic (Gen), environmental (Env) and their interaction (Gen:Env).

FV	GL	GER	DR	DC	DH	RL	HL
Gen	96	0.459 ***	21.647 ***	6238 ***	117 ***	134.09 ***	19 ***
Amb	1	5.962 ***	7.674 ***	308455 ***	44793 ***	7.13	8004 ***
Gen:Amb	96	0.111 ***	10.158 ***	2636 ***	65 ***	45.97 ***	15 ***
Residuals	582	0.004	3.617	1270	14	2.16	1

Ger— Germination, **DR**— Dry mass of the roots, **DH**— Dry mass of the hypocotyl, **DC**— Dry mass of the cotyledon, **RL**— Length of root, **HL**— Hypocotyl length

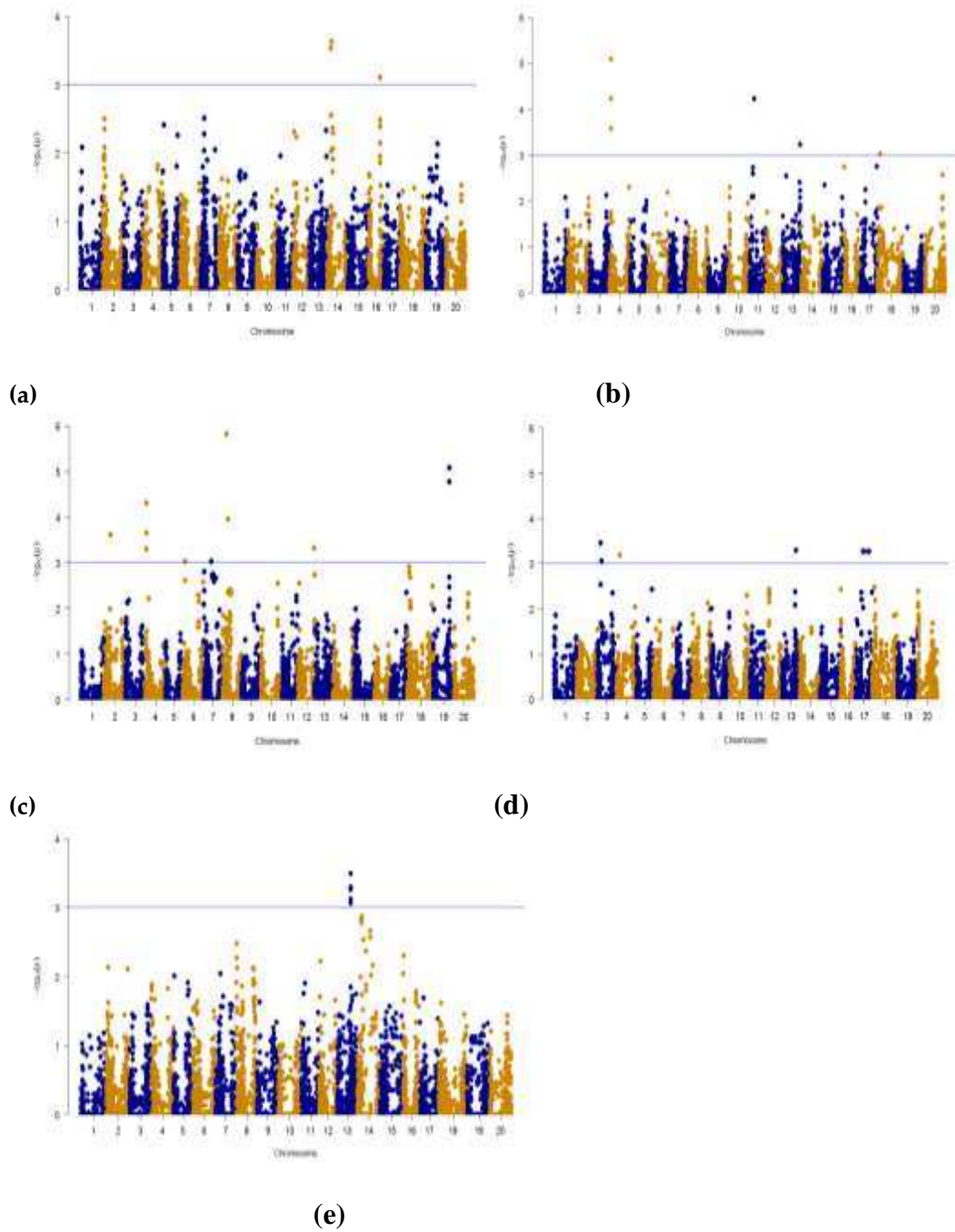


Figure S1. Manhattan plots showing SNPs (single nucleotide polymorphisms) candidates and their respective P-values for genomic association of the characteristics germination in control (a), dry mass of cotyledon in stress (b), dry mass of root in control (c), dry mass of root in stress (d), hypocotyls length in stress (e) using linear compressed linear models (MCML).

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ARTIGO CIENTÍFICO 4 - ASSOCIAÇÃO GENÔMICA NA DINÂMICA DE RESERVAS DE SEMENTES DE SOJA

RESUMO

A dinâmica de reservas das sementes representa sua capacidade em converter carboidratos, proteínas e lipídeos em novos tecidos. O objetivo desse estudo foi verificar a relação entre as características da dinâmica de reservas das sementes de soja e realizar um estudo de GWAS que possa iniciar a elucidação dessas relações, para seu futuro potencial uso no melhoramento genético. Para esse estudo foram genotipadas 97 cultivares, com marcadores tipo SNPs. As sementes foram postas para germinar durante sete dias e ao final desse período a massa seca da radícula, hipocótilo e plântula foram pesadas. Concomitantemente, foi estimada a massa seca das sementes. Os resultados obtidos foram utilizados para se calcular as variáveis relacionadas à dinâmica de reserva das sementes. Não houve correlação significativa entre as variáveis relacionadas à dinâmica de reservas com a massa das sementes e nem da plântula. Todas as variáveis apresentaram variabilidade genética a ser explorada, porém, apenas a variável eficiência de conversão de reservas teve SNPs candidatos associados ao controle da característica. Metade desses SNPs estão em desequilíbrio de ligação, segregando conjuntamente, formando um bloco de ligação o qual sua posição no genoma é similar a outros genes já encontrados, que são todos relacionado à quebra e degradação de reservas de sementes durante o processo germinativo. Concluiu-se que a dinâmica de reservas de soja pode ser melhorada geneticamente, com destaque para a característica eficiência de conversão de reservas.

Palavras-chave: Redução de reservas das sementes (RRS), Eficiência de conversão de reservas das sementes (ECR), Taxa de redução de reservas das sementes (TRRS), GWAS, *Glycine max* (L.) Merrill

ABSTRACT

The dynamics of seed reserves represent their ability to convert carbohydrates, proteins and lipids into new tissues. The objective of this study was to verify the relationship between the characteristics of soybean seed reserves dynamics and conduct a GWAS study that could initiate the elucidation of these relationships for their future potential use in genetic breeding. For this study, 97 cultivars were genotyped, with SNPs markers. Seeds were germinated during seven days and dry mass of the radicle, hypocotyl, cotyledons and seedling were weighed at the end of this period. Concomitantly, the dry mass of the seeds was estimated. The results obtained were used to calculate the variables related to seed reserve dynamics. According to the results, no significant correlation between the variables related to the reserve dynamics with the seed mass or the seedling were found. All variables showed genetic variability and can be exploited. However, only the variable conversion efficiency of seed reserve had candidate SNPs associated with control of the traits. Half of these SNPs are in linkage disequilibrium, segregating together, forming a block whose position in the genome is similar to other genes already found, which are all related to the degradation of seed reserves during the germination process. It was concluded that the dynamics of soybean reserves can be improved genetically, with emphasis on the characteristic of reserve conversion efficiency.

Key-words: Seeds reserve reduction (SRS), Conversion efficiency of seeds reserve (CESR), Seed reserve reduction rate (SRRR), GWAS, Glycine max (L.) Merrill

INTRODUÇÃO

A soja é uma das culturas agrícolas mais importantes do mundo, ocupando aproximadamente 6% de todas as terras aráveis do planeta, e desde a década de 1970 a soja é a cultura que mais registra aumento percentual de área plantada no mundo, ano após ano (HARTMAN et al., 2011).

Por essa razão a cultura vem sendo extensivamente estudada, a fim de se aumentar os rendimentos por área e estabilizar a produção, principalmente em áreas propensas a estresses abióticos. Já foi evidenciado que o estabelecimento inicial de plantas no campo e seu rendimento está diretamente relacionada ao vigor das sementes de soja (DIAS et al., 2011), fato também registrado nas culturas do trigo (ABATI et al., 2017), milho (MONDO et al., 2013) e feijão (MONDO et al., 2016).

Vigor pode ser definido como a soma de todas as propriedades que determinam o potencial nível de atividade e performance de sementes durante a germinação e emergência das plântulas (FINCH-SAVAGE; BASSEL, 2016). A dinâmica de reservas das sementes representam uma parte fundamental no processo germinativo, pois apesar de o embrião iniciar seu desenvolvimento com as próprias reservas, para manter e continuar o seu desenvolvimento é preciso um fluxo contínuo de compostos solúveis sendo enviados aos novos tecidos em formação (HENNING et al., 2010). Dessa forma, a dinâmica de reservas das sementes é de fundamental importância para o seu vigor (CHENG et al., 2013 e SOLTANI et al., 2006).

O controle genético do vigor tem sido relatado em vários trabalhos, como cita Finch-Savage e Bassel (2016). No entanto, a maioria dos trabalhos relatados são de espécies não domesticadas e que não se enquadram no contexto agrícola. Contudo, é amplamente aceito que o vigor de sementes é uma característica de controle genético quantitativo (DICKSON, 1980) influenciada por fatores ambientais durante a maturação da semente, colheita e armazenagem, o que torna a análise genética do vigor de sementes mais difícil (SUN et al., 2007). Perante essa complexidade que envolve o vigor de sementes, uma das alternativas para facilitar o entendimento do controle genético dessa características é o uso da associação genômica (GWAS) e da seleção (GWS) integradas com fisiologia e outras ciências no entendimento das interações existentes (VADEZ et al., 2014).

GWAS é uma abordagem no melhoramento genético molecular de plantas para a identificação de associação entre marcas moleculares (SNPs) e características de interesse em uma determinada população de indivíduos, sendo um eficiente método para a identificação de regiões genômicas associadas com características complexas de controle genético quantitativo (TAN et al., 2017).

O objetivo desse estudo foi verificar a relação entre as características da dinâmica de reservas das sementes e realizar um estudo de GWAS que possa iniciar a elucidação dessas relações, para seu futuro potencial uso no melhoramento genético.

MATERIAL E MÉTODOS

Obtenção das sementes

As sementes de 97 cultivares de soja (Tabela 1) foram produzidas na safra 2015/2016, na estação experimental de Coimbra, Minas Gerais, Brasil. As plantas foram cultivadas conforme recomendações de tratamentos culturais para lavoura de produção de grãos. Não foi realizada irrigação suplementar.

Tabela1. Lista das cultivares de soja (*Glycine max* (L.) Merrill) (n = 97) utilizadas no experimento.

MG BR Conquista	CD 215	EMGOPA 304
CD 235 RR	CD 205	CD 254 RR
BMX Potência RR	CD 250 RR	BRS MT Pintado
Tropical RR	M 6707 RR	FUNDACEP 33
CD 226 RR	CD 217	P98Y70
TMG 1066 RR	NK 8350	CD251 RR
CD 2630 RR	CD 2792 RR	BMX Energia RR
CD 253	BRS 284	CD 242 RR
MSOY 7901	OCEPAR 3	CD 201
CD 2721 RR	CD 202	5G770 RR
CD 229 RR	BMX Titan RR	CD 246
CD 237 RR	FUNDACEP 96 RR	CD 288
CD 225 RR	CD 5807	CD 218
TMG 115 RR	BRAGG	MSOY 8001
CD 204	BRS 283	BRS 213
BRS 184	EMBRAPA 59	BRS 257
EMGOPA 304	MG/BR 48 GARIMPO	CD 215
CD 254 RR	FUNDACEP 58	CD 205
BRS MT Pintado	RA 626	CD 250 RR
FUNDACEP 33	CD 2585 RR	M 6707 RR
P98Y70	CD 226 RR	CD 217
CD251 RR	TMG 1066 RR	NK 8350
BMX Energia RR	CD 2630 RR	CD 2792 RR
CD 242 RR	CD 253	BRS 284
CD 201	MSOY 7901	OCEPAR 3
5G770 RR	CD 2721 RR	CD 202
CD 246	CD 229 RR	BMX Titan RR
CD 288	CD 237 RR	FUNDACEP 96 RR
CD 218	CD 225 RR	CD 5807
MSOY 8001	TMG 115 RR	BRAGG
BRS 213	CD 204	BRS 283
BRS 257	BRS 184	EMBRAPA 59
MG/BR 48 GARIMPO		

As análises das sementes foram conduzidas no Laboratório de Melhoramento de Oleaginosas, no Departamento de Fitotecnia da Universidade Federal de Viçosa, Minas Gerais, Brasil.

Determinação da umidade e massa seca das sementes

Para determinação da massa seca das sementes (*MSS*), três repetições de 50 sementes foram levadas para estufa de circulação de ar a 105°C por 24 horas e então pesadas em balança analítica. A massa seca das sementes foi calculada a partir dos dados de umidade. Para o propósito deste estudo, a *MSS* foi considerada como o total de reservas da semente disponível para ser mobilizado às plântulas.

Dinâmica de reservas

Para a obtenção dos dados de dinâmica de reservas das sementes, quatro repetições de 10 sementes cada, distribuídas no terço superior do papel *germitest* com suas micrópilas direcionadas para a base do papel foram postas para germinar sobre duas folhas de papel *germitest* umedecidas com água destilada. A quantidade de água utilizada foi uma proporção de três vezes o peso do papel. Sete dias após a semeadura a radícula, o hipocótilo e os cotilédones foram separados e colocados para secar em estufa de circulação de ar forçada a 80 ± 1 °C, por 24 horas. Após esse período, o material vegetal foi pesado em balança analítica, separadamente, para avaliação das massas secas. A massa seca das plântulas (*MSP*) foi formada pela massa seca das radículas (*MSR*) mais a massa seca do hipocótilo (*MSH*). A parte remanescente do cotilédone foi colocada para secar, conforme descrito anteriormente, e a massa seca foi pesada para compor a massa seca remanescente nos cotilédones (*MSC*). Os valores considerados nessas variáveis foram a média das *MS* das plântulas germinadas, sendo assim expressas pelo valor médio individual.

Devido à necessidade de padronizar com os dados da *MSS*, o qual é uma média das 50 sementes postas para secar, o valor obtido individualmente de massa seca (*MS*) para cada variável foi multiplicado por 50, assim estimando um valor aproximado para 50 plântulas (*MSEP* – Massa seca estimada de plântulas), se a germinação tivesse sido 100% (PEREIRA et al., 2015 e SOLTANI et al., 2002, 2006).

A partir da *MSC* e da *MSS* foi possível obter o valor da redução de reservas da semente (*RRS*) pela fórmula: $RRS = MSS - MSC$. Seguindo o mesmo raciocínio e com base nos valores de massa seca estimado das plântulas (*MSEP*), foi realizada a eficiência de conversão de reservas (*ECR*), correspondendo ao quanto de massa seca da semente se tornou massa seca de plântula, pela fórmula: $ECR = MSEP/RRS$. E por último a taxa de redução de reservas da semente (*TRRS*) obtida pela equação: $TRRS = RRS/MSS$, a qual permitiu identificar o genótipo que mais mobilizou massa seca (SOLTANI et al., 2006).

Análise estatística dos fenótipos

As análises fenotípicas foram realizadas no *software* estatístico R Studio versão 3.4.3 e utilizando o pacote integrado base (<https://www.R-project.org/>). Para a análise de normalidade foi utilizado o teste Shapiro Wilk. Para a análise de correlação e a formação do correlograma foi utilizado o pacote corrgram (WRIGHT, 2017).

Genotipagem

A genotipagem foi realizada na Deoxi Biotecnologia Ltda, em Araçatuba/SP, utilizando-se a plataforma Illumina *iScan* e o painel *Infinium iSelect HD Custom Genotyping BeadChips* 6k (Illumina, Inc, San Diego, CA, USA), customizado para soja com 24 amostras por BeadChip. A genotipagem foi realizada de acordo com protocolo descrito pela fabricante (ILLUMINA, 2015).

Associação genômica e desequilíbrio de ligação

Inicialmente os dados genotípicos foram filtrados considerando valores mínimos de frequência alélica no genoma (MAF) e proporção no genoma (*call rate*) para que os SNPs possam ser identificados de forma confiável. Foram adotadas $MAF \geq 0,025$, conforme utilizado por Hatzig et al. (2015) em estudo genômico da germinação de sementes de brássicas e $call\ rate \leq 90\%$, restando 1931 SNPs após a limpeza, realizada utilizando o software Rbio (BHERING; BHERING, 2017)

A análise de associação genômica, bem com a densidade de marcas e desequilíbrio de ligação paterno das marcas, foi realizada com modelos lineares mistos comprimidos (CMLM) (ZHANG; ERSOZ; LAI; TODHUNTER; TIWARI; GORE; BRADBURY; YU; ARNETT; ORDOVAS; et al., 2010) implementadas no pacote GAPIT no R (LIPKA et al., 2012b). Foram considerados significativos SNPs com taxa de falsos descobertos $FDR \leq 0,95$ e $p\text{-value} \leq 0,001$.

O desequilíbrio de ligação entre SNPs localizados em uma mesma região do genoma, formando o bloco de ligação, e a realização do *heatmap* foram realizadas no *software* Haploview (BARRETT et al., 2005).

Análise de contribuição alélica

Para realização dessa análise foi amostrada a média fenotípica de cada alelo de cada SNP significativo, que posteriormente foram agrupados em locos e representados pelo SNP com

maio P-valor. Em seguida foi realizado um cálculo de diferença percentual no valor fenotípico entre o alelo de maior e menor efeito de cada loco.

RESULTADOS

Estatística dos fenótipos e qualidade das marcas

Os dados fenotípicos utilizados no experimento apresentaram distribuição normal significativa, como pode ser observado nos histogramas da Figura 1 e Tabela 2, com p-valores menores que 0,01 pelo teste de Shapiro Wilk. A variação fenotípica dos dados, com valores de média, máximo, mínima e seus respectivos p-valores para o teste de normalidade são apresentados na Tabela 2.

Tabela 2. Variação fenotípica para a variável redução de reservas das sementes (RRS), eficiência de conversão de reservas das sementes (ECR), taxa de redução de reserva das sementes (TRRS), massa seca das sementes (MSS) e massa seca de plântulas (MSP) de soja (*Glycine Max* (L.) Merrill).

	RRS	ECR	TRRS	MSS	MSP
Valor máximo	0,06	0,86	0,39	202,55 mg	15,95 mg
Valor mínimo	0,04	0,51	0,25	103,95 mg	0,0 mg
Média	0,05	0,60	0,32	151,99 mg	2,68 mg
p-valor	1,75e ⁻¹¹	1,08e ⁻⁰⁷	2,686e ⁻¹⁴	0,0008744	2,2e ⁻¹⁶

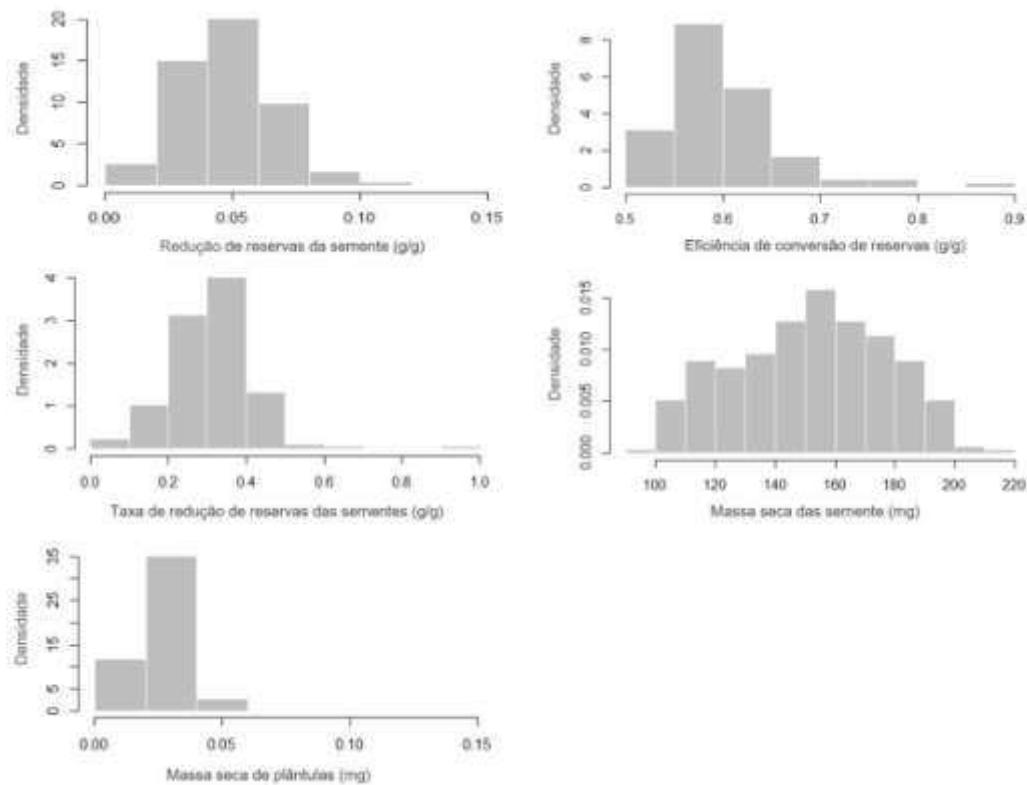


Figura 2. Histograma dos valores fenotípicos da variável redução de reservas das sementes (RRS), eficiência de conversão de reservas das sementes (ECR), taxa de redução de reserva das sementes (TRRS), massa seca das sementes (MSS) e massa seca de plântulas (MSP) de soja (*Glycine Max* (L.) Merrill). .

A massa seca inicial das sementes (MSS) e a massa seca das plântulas (MSP) não apresentaram correlação significativa com nenhuma das variáveis relacionadas à dinâmica de reserva das sementes (Figura 2). Quanto mais intensa a coloração do círculo ou triângulo, maior a correlação entre as variáveis.

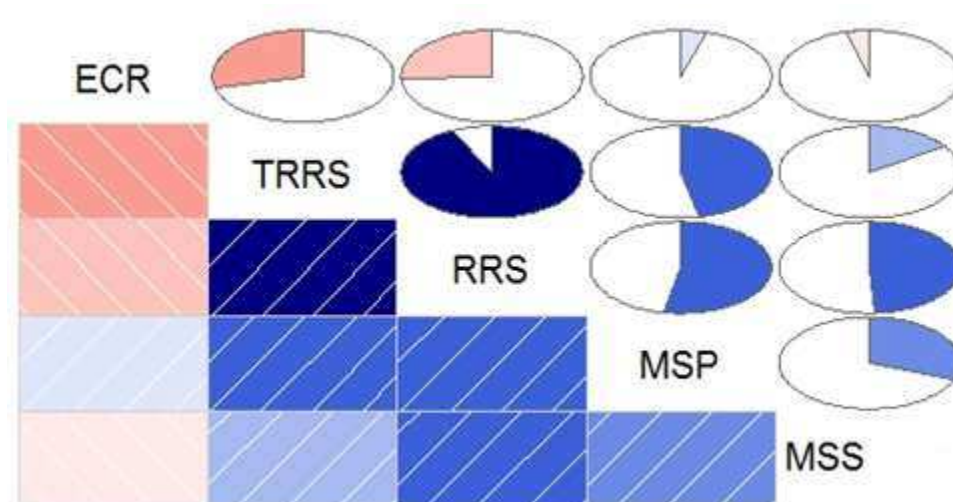


Figura 1. Correlograma entre as variáveis TRRS (Taxa de redução de reservas), RRS (Redução de reservas das sementes), MSS (Massa seca das sementes) e ECR (Eficiência de conversão de reservas) de sementes de soja (*Glycine Max* (L.) Merrill). Correlogramas na cor azul representam correlação positiva, enquanto correlogramas na cor vermelha representam correlação negativa.

Após a limpeza e exclusão de SNPs com frequência alélica mínima de 2,5% e com *call rate* < 90% restaram 1931 SNPs. Esses SNPs apresentaram qualidade para análise, como pode ser verificado na Figura 2A. Na figura observa-se que a densidade das marcas diminui à medida que se aumenta a distância entre os pares de base, indicando que as marcas são densas o suficiente para se ter uma boa cobertura do desequilíbrio de ligação. Isso é comprovado na Figura 2B, onde à medida que se aumenta a distância das marcas reduz o valor do desequilíbrio de ligação (r^2) entre os mesmos, evidenciando que o maior desequilíbrio de ligação se encontra onde há a maior densidade de marcadores. Esses resultados asseguram que mesmo após a limpeza, o número de SNPs avaliados é denso o bastante para não haver intervalos muito grandes no genoma que comprometam as análises genômicas, e que ainda há desequilíbrio de ligação a ser explorado no banco de dados.

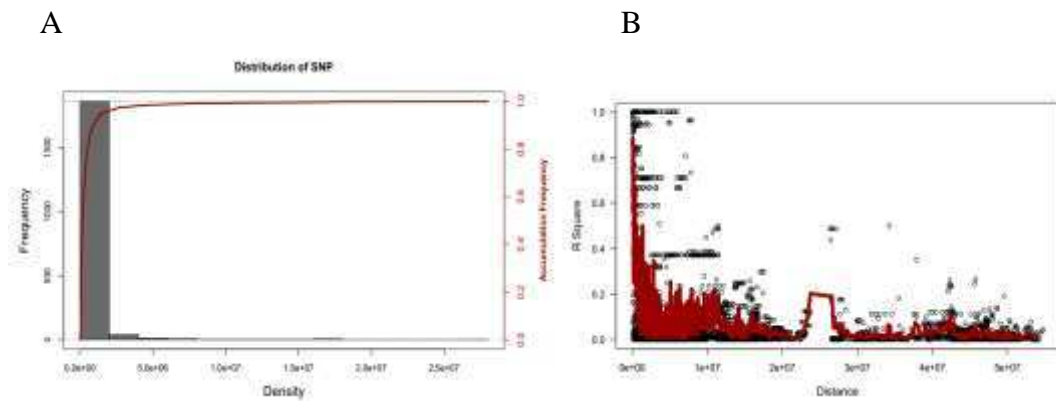


Figura 2. Frequência e frequência acumulada da densidade de marcas (A). Redução do desequilíbrio de ligação com o aumento da distância entre marcas genéticas (B).

Análise de associação genômica

Dentre as variáveis analisadas, somente a ECR apresentou SNPs com efeitos significativos, sendo observados oito SNPs distribuídos ao longo dos cromossomos 11, 12, 14 e 18, com MAF variando de 0.01 a 0.04 e FDR entre 1 e 21%. Os resultados estão expressos na Figura 3 e Tabela 3.

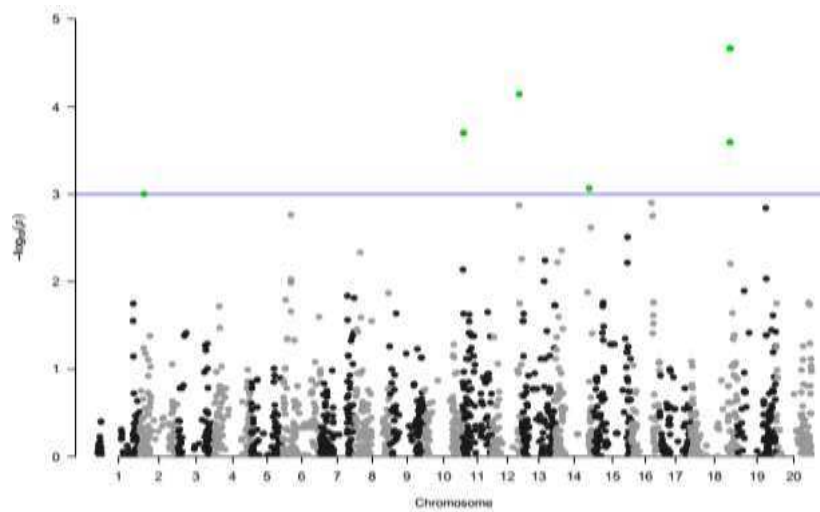


Figura 3. Manhattan plot mostrando SNPs candidatos e seus respectivos p-valores para a associação genômica da característica ECR em sementes de soja (*Glycine Max* (L.) Merrill) usando MCML (*linear compressed linear models*).

Tabela 3. SNPs candidatos, cromossomo, posição em pares de base (bp) no genoma, e P-valores, MAF e FDR para a associação genômica da característica ECR de sementes de soja (*Glycine Max* (L.) Merrill).

SNP ^a	Cromossomo	Posição	P valor	MAF	FDR
Gm18_51214883_A_C	18	51214883	2,16E-05	0,01	0,01
Gm18_51525500_A_G	18	51525500	2,16E-05	0,01	0,01
Gm18_51403980_T_G	18	51403980	2,16E-05	0,01	0,01
Gm12_35876375_T_C	12	35876375	7,19E-05	0,08	0,03
Gm11_1224933_T_C	11	1224933	1,99E-04	0,10	0,07
Gm18_51128392_G_A	18	51128392	2,56E-04	0,02	0,07
Gm18_51659540_A_G	18	51659540	2,56E-04	0,02	0,07
Gm14_44899572_G_T	14	44899572	8,58E-04	0,04	0,21

^a Inicia com a versão do Joint Genome Institute (JGI 1.01) *G.max* genome sequence, seguido pelo número do cromossomo, posição física da marca naquele cromossomo e os dois alelos do loco (SCHMUTZ et al., 2010). MAF – frequência alélica mínima; FDR – Taxa de falsas descobertas

Dentre os oito SNPs candidatos, quando realizada a análise de desequilíbrio de ligação, foram formados quatro locos (Tabela 4). A exceção do loco ECR 4, todos os demais locos tiveram algum gene já observado nessa região, sendo os intervalos flanqueados pelos locos ECR 2 e ECR 3, genes que correspondem a proteínas ainda desconhecidas e não relacionadas ao estudo da dinâmica de reserva das sementes. Os locos que são compostos por um único SNP tiveram 2000 pares de base adicionados à sua posição, para mais e para menos, objetivando com isso aumentar a amplitude do intervalo do SNP no genoma, para realizar a procura por outros genes candidatos já encontrados, ou proteínas que ajudem a explicar o comportamento da característica. No intervalo de desequilíbrio do loco ECR 1 foram encontrados cinco genes já observados em outros estudos, sendo três desses relacionados à enzima acetil CoA carboxilase e os outros dois relacionados à fosfatase e fosforização oxidativa.

Tabela 4. Locos da variável ECR de sementes de soja (*Glycine Max* (L.) Merrill) com seus respectivos SNPs com maior efeito, intervalo do desequilíbrio de ligação em pares de base e genes candidatos observados no intervalo do desequilíbrio.

Loco	SNP	Intervalo do desequilíbrio (bp)	Genes candidatos ^a	Anotação
ECR 1	Gm18_51214883_A_C	51128392-51659540	Glyma18g43000.1 Glyma18g42280.1 Glyma18g42310.1 Glyma18g42350.1 Glyma18g42220.1	acetyl-CoA carboxylase acetyl-CoA carboxylase acetyl-CoA carboxylase Fosfatase Fosforização oxidativa
ECR 2	Gm12_35876375_T_C	35874375-35878375		
ECR 3	Gm11_1224933_T_C	1222933-1226933		
ECR 4	Gm14_44899572_G_T	44897572-44901572		

^a Genes observados no GmGDB Glyma1/1 v181 (<http://www.plantgdb.org>) foram utilizados como fonte para os genes candidatos.

O loco ECR 1 é formado por um bloco de ligação com 531 kb que engloba cinco SNPs (Figura 4). Todos os outros locos são formados por marcas únicas.

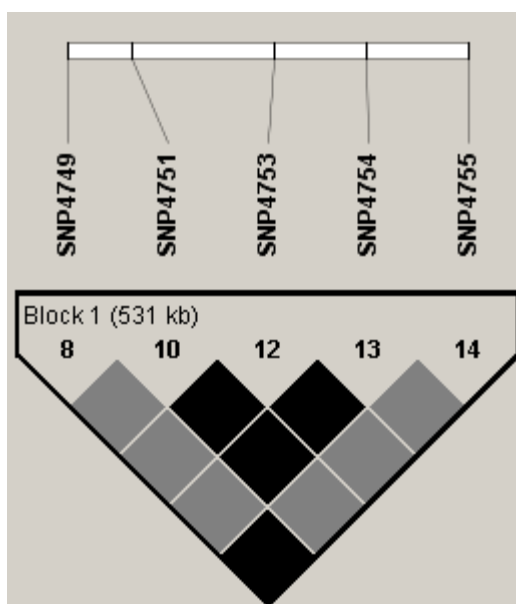


Figura 4. Desequilíbrio de ligação, representado pela correlação (r^2), entre SNPs candidatos formando um bloco de ligação ($r^2 > 50\%$) para provável controle da variável eficiência de conversão de reservas ECR de sementes de soja (*Glycine Max* (L.) Merrill) no cromossomo 18.

A substituição alélica em cada loco contribuiu para a variação fenotípica da ECR dos locos candidatos, com variação no efeito do fenótipo entre 10 e 30, conforme a presença/ausência do alelo de maior e menor efeito (Tabela 5).

Tabela 5. Contribuição alélica de cada SNP dentro do loco no fenótipo da variável eficiência de conversão de reservas (ECR) de sementes de soja (*Glycine max* L. Merrill).

Variável	Unidade	Loco	SNP id	Alelos				Diferença percentual
				A	C	T	G	
ECR	mg/mg	ECR 1	Gm18_51214883_A_C	0,86	0,60			30%
ECR	mg/mg	ECR 2	Gm12_35876375_T_C		0,71	0,59		17%
ECR	mg/mg	ECR 3	Gm11_1224933_T_C		0,65	0,59		10%
ECR	mg/mg	ECR 4	Gm14_44899572_G_T			0,72	0,60	17%

^aFoi utilizado o SNP com o maior P.valor dos múltiplos SNPs para representar o loco.

DISCUSSÃO

Germinação de sementes é um processo complexo, que se inicia com a absorção de água e termina com a protrusão da radícula (BEWLEY, 1997), alterando o estado de vida da planta de heterotrófica para autotrófica. Durante esse período, logo após a embebição de água pela semente é iniciado um conjunto de reações metabólicas, com o intuito de fornecer nutrientes para o desenvolvimento inicial da nova planta, com a mobilização de amidos, proteínas e lipídeos para os novos tecidos a serem formados. Esse processo é fundamental para o estabelecimento de plantas no campo, por isso, tem sido extensivamente estudado nos últimos anos em várias espécies como soja (MOHAMMADI et al., 2012 e PEREIRA et al., 2015) , girassol (ERBAŞ; ŞANLI, 2016) e arroz (CHENG et al., 2015, 2013).

Para a abordagem da dinâmica de reservas em sementes de soja, nesse estudo foram utilizadas as características MSS, MSP, RRS, ECR e TRRS. De acordo com (SOLTANI et al., 2006), sementes com maiores tamanhos possuem a vantagem de produzirem plântulas mais vigorosas. No entanto, como apresentado na Figura 3, não há correlação entre a massa MSS e a sua eficiência em converter massa seca em tecidos de plantulas (ECR) nem com MSP propriamente dito. Mesmos resultados foram observados também por (PEREIRA et al., 2015) e por Dantas et al. (2017) em estudo realizado com sementes de soja. Cheng et al. (2013), em estudo sobre a dinâmica de reservas de sementes de arroz verificaram que a ECR não apresenta correlação com a MSS, no entanto, os mesmo autores relataram existência de correlação entre a MSP e MSS. Isso demonstra que a eficiência das sementes de soja em converter matéria seca em plântulas não esta relacionada com seu tamanho e massa.

Quando realizada a análise de variância, foi verificada diferença estatística entre os genótipos, com variabilidade genética a ser explorada. A variabilidade possibilita seu uso de melhoramento genético para aumentar a utilização de reservas das sementes durante o processo germinativo, e assim produzir genótipos superiores. No entanto, é sabido que características relacionadas à produtividade e performance agrônômica são tipicamente controlada por vários genes (TANKSLEY, 1993) e altamente afetadas por fatores genéticos e ambientais, dificultando o melhoramento dessas características (CHENG et al., 2013). Uma alternativa portanto utilizar as análises genômicas para facilitar, direcionar e acelerar a seleção de genótipos superiores.

Com a redução dos custos de genotipagem e o constante aperfeiçoamento dos métodos estatísticos, estudos de associação genômica ampla (GWAS) e de seleção genômica ampla (GWS) são ferramentas promissoras no melhoramento genético de características complexas em espécies agrícolas (ZHANG et al., 2016). Características relacionadas ao vigor e dinâmica de reservas de sementes, como a ECR, RRS e TRRS são consideradas características complexas e de difícil entendimento (SUN et al., 2007).

Os SNPs candidatos associados ao controle da característica ECR durante a análise de GWAS foram agrupados em locos (Tabela 4), dos quais o loco ECR 1 apresentou genes já identificados e caracterizados na região flanqueada por seus SNPs. Esse genes encontrados apresentam anotações relacionadas a proteínas que atuam na digestão e quebra das reservas armazenadas na semente, principalmente as enzimas de acetilCoa carboxilase, fosforilação oxidativa e fosfatases.

O armazenamento de reservas das sementes consiste em moléculas complexas de carboidratos, proteínas e lipídeos. Durante a fase inicial da germinação das sementes as reservas armazenadas são quebradas em moléculas solúveis como sacarose, podendo ser transportadas para todos os novos tecidos em formação da plântula. Em sementes oleaginosas, como a soja, a maior parte das reservas é armazenada na forma de lipídeos, encontrados na forma de triacilgliceróis (TAG) (BEWLEY; BLACK, 1994). TAG é inicialmente clivado por lípases, formando ácidos graxos, que são subsequentemente quebrados pelas enzimas da B-oxidação do ciclo de glioxalato (BEEVERS, 1961 e KOORNNEEF et al., 1982 e PRITCHARD et al., 2002).

O ciclo de glioxalato é uma parte do ciclo de ácido cítrico, que permite gerar glicose a partir de ácidos graxos, utilizando sua principal enzima, que é a Acetil CoA carboxilase (ACCase). Devido sua importância na quebra e liberação de energia para o desenvolvimento do embrião, essa enzima desempenha um papel central na germinação e dinâmica de reservas (BAUD et al., 2003), podendo por esse motivo ter sido identificado na região flanqueada pelo loco ECR 1.

Os outros dois genes encontrados na região associados com os locos ECR também estão relacionados à produção de energia para o desenvolvimento da plântula, mais especificamente ao processo de respiração e produção de energia. Metabolicamente a germinação de sementes ocorre em três etapas principais: absorção de água pela semente; reativação de organelas e macromoléculas preexistentes; e, respiração das reservas, gerando ATP (trifosfato de adenosina) como fonte de energia para o crescimento (BELTRÃO; OLIVEIRA, 2007)

Dentre as organelas reativadas estão as mitocôndreas, que são as organelas responsáveis pela síntese de ATP e onde ocorre a fosforização oxidativa. Fosforização oxidativa é a síntese de ATP a partir de ADP acoplado à transferência de elétrons para moléculas de oxigênio, comoceptor final da cadeia transportadora de elétrons (COOPER, 2000). Nesse processo a energia obtida a partir da quebra de ácidos graxos no ciclo de glioxalato e de ácido cítrico é utilizado na fosforização oxidativa para sintetizar ATP (BERG et al., 2002).

As fosfatases são enzimas que hidrolisam ligações fosfodiéster em proteínas fosforiladas, retirando fósforo das moléculas («Phosphatase - an overview | ScienceDirect Topics», [S.d.]). Essas enzimas realizam uma função essencial na sinalização celular, com o balanço entre a fosforilação e desfosforilação de proteínas, que é a base para o controle de vários eventos biológicos iniciados por efeitos extracelulares, como a sinalização celular, produção de hormônios e outros (AOYAMA et al., 2003), incluído os eventos que desencadeiam o processo germinativo. Ou seja, um processo é complementar ao outro, o que justifica esses locos terem sido encontrados em regiões bem próximas e segregando juntamente no genoma.

CONCLUSÃO

As características relacionadas à dinâmica de reservas das sementes de soja atuam isoladamente, independente inclusive da massa inicial da semente. Além disso, podem ser exploradas e aprimoradas através do melhoramento genético, com destaque para a variável eficiência de conversão de reservas.

Conforme resultados da GWAS, a característica ECR é controlada por vários locos, muitos dos quais localizados em regiões do genoma que possuem genes já observados em outros estudos como responsáveis pela quebra e degradação de reservas das sementes.

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CONSIDERAÇÕES FINAIS

Existem métodos viáveis e baixo custo para a seleção de plantas de soja tolerantes a estresse hídrico em casa de vegetação em diferentes estádios fenológicos, podendo ser adaptados conforme a necessidade do projeto.

O nível de estresse recomendado para se selecionar genótipos de soja tolerantes a estresse hídrico durante a fase de germinação é o potencial osmótico $-0,2$ Mpa.

Existem diferentes locos, em diferentes regiões do genoma, que estão associados à germinação e vigor de sementes de soja, que variam conforme o ambiente, com e sem estresse hídrico, para a mesma característica.

A análise de GWAS encontrou SNPs candidatos significativamente associados ao controle das características vigor e germinação, com grande potencial de uso dessas informações no melhoramento genético.

A dinâmica de reservas de sementes de soja durante a germinação é independente do tamanho da semente, com possibilidade de ser uma característica incrementada através do melhoramento genético, com destaque para a característica eficiência de conversão de reservas das sementes.