VINÍCIUS DE MOURA STOCK

EMS MUTAGENESIS, SELECTION AND MOLECULAR CHARACTERIZATION OF SOYBEAN MUTANTS WITH ALTERED RESPONSE TO *Phakopsora pachyrhizi*

Thesis presented to the Plant Pathology Program of the Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of *Doctor Scientiae*.

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Eduardo Seiti Gomide Mizubuti

Francisco Murilo Zerbini Júnior

João Vítor Maldonado dos Santos

Maximiller Dal-Bianco Lamas Costa

Sérgio Hermínio Brommonschenkel
(Adviser)

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BIOGRAFIA

VINÍCIUS DE MOURA STOCK, filho de Lorildo Aldo Stock e Ângela Regina de Moura Stock, nascido em Viçosa, Estado de Minas Gerais, em 25 de dezembro de 1984.

Em 2005, ingressou na Universidade Federal de Viçosa, MG, graduando-se em Agronomia, em janeiro de 2012. Em novembro de 2012, iniciou o programa de Pós-Graduação em Fitopatologia nesta mesma instituição, e obteve o título de mestre em Fitopatologia em dezembro de 2014. Em março de 2015, ingressou no curso de doutorado em Fitopatologia pela Universidade Federal de Viçosa.

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ABSTRACT

STOCK, Vinícius de Moura, D.Sc., Universidade Federal de Viçosa, March, 2019. **EMS mutagenesis, selection and molecular characterization of soybean mutants with altered response to *Phakopsora pachyrhizi*.** Adviser: Sérgio Hermínio Brommonschenkel.

The susceptibility and resistance of plants to microbial pathogens involves complex molecular interactions between the microbe's effectors and the host's targets. These effectors interact with host's targets altering host cell metabolism, defense response and nutrient supply, favoring the parasitism. Therefore, loss or changes in these targets may limit the pathogen's ability to cause disease. The objective of this study was to identify and characterize mutants from susceptible TMG4182 soybean variety with an altered response to *P. pachyrhizi* aiming to identify genes that encode effector targets. 1,820 M2 families obtained from soybean seeds treated with 0.3% (v / v) EMS for 16 hours were evaluated. Twenty-two seeds from each M2 families were planted in the greenhouse and evaluated for phenotypic variations**.** At the V1 stage, these plants were inoculated with *P. pachyrhizi* isolate PPUFV02 and screened for altered disease development phenotypes. From a total of 30,068 M2 plants evaluated, 2,976 displayed phenotypic variations in growth, plant architecture and leaf morphology, and 101 altered disease development. Plants from two M2 families, GMMT-509-20 and GMMT-504-04, displayed reddish-brown (RB) symptoms with less sporulation, while one, GMMT-496-01 presented necrotic lesions with fewer uredia and little sporulation. F2 segregation indicates that these phenotypes are determined by recessive genes. Whole-genome resequencing of 'TMG4182' and these mutants revealed a mutation frequency of approximately 1 variant nucleotide per 45,647 bases, 50% being base transitions from C to T and G to A. A total of 1,132 non-synonymous SNPs were detected in protein-coding genes. dCAPS assays also confirmed the presence of polymorphisms in plant defense-related genes: Glyma.03G220100, Glyma.18G046300, Glyma.03G049300, and Glyma.01G118900. However, these mutations were not associated with the mutant phenotypes. In future studies, Bulk segregant analysis and MutMap will be employed to identify the mutant genes.

RESUMO

STOCK, Vinícius de Moura D.Sc., Universidade Federal de Viçosa, março de 2019. **Identificação e caracterização de mutantes de soja, obtidos via mutagênese induzida por metanossulfonato de etila (EMS), com respostas alteradas à *Phakopsora pachyrhizi*.** Orientador: Sérgio Hermínio Brommonschenkel.

A suscetibilidade de plantas a patógenos microbianos envolve interações moleculares complexas entre os efetores e seus alvos no hospedeiro. Em interações compatíveis os fitopatógenos empregam efetores para alterar o metabolismo das células hospedeiras e suprimir as suas respostas de defesa. Perda ou mutação dos genes-alvo desses efetores podem limitar o desenvolvimento do patógeno nos tecidos da planta. Esse trabalho teve por objetivo a identificação de mutantes da variedade TMG4182 com resposta alterada à inoculação com o fungo *Phakopsora pachyrhizi* visando a futura identificação de genes que codificam proteínas que são alvo dos efetores. Para tanto, avaliou-se 1.820 famílias M2 geradas a partir do tratamento de sementes de soja com EMS a 0,3% (v / v) por 16 horas. Plantou-se separadamente vinte e duas sementes de cada família em casa-de-vegetação e avaliou-se a presença de variações fenotípicas diversas e também alterações fenotípicas após a inoculação com o isolado PPUFV02 de *P. pachyrhizi.* Das 30.068 plantas M2 avaliadas, 2.976 apresentaram variações fenotípicas no crescimento, arquitetura de planta e morfologia foliar e 101 plantas alterações no desenvolvimento da doença. Alguns desses fenótipos não foram confirmados na geração M3. Não se obteve plantas mutantes com claro aumento de suscetibilidade ou imunes. Duas famílias M2, GMMT-509-20 e GMMT504-04, apresentaram plantas com lesões RB (reddish-brown) com menor esporulação, enquanto uma, GMMT-496-01, apresentou plantas com lesões necróticas, com menor quantidade de urédias e pouca esporulação. Esses fenótipos foram confirmados pela análise na geração M3, que demonstraram que esses fenótipos mutantes são controlados por genes recessivos. A caracterização do genoma de TMG4182 e dos genótipos mutantes por re-sequenciamento relevou uma frequência de ~1 alteração nucleotídica (SNPs)/45kb sendo mais de 50% do tipo transições de C para T e de G para A, comumente observadas em estudos de mutagênese com EMS. Um total de 1.132 SNPs não-sinônimos foram identificados dentro de regiões codificadoras de proteínas. Ensaios de dCAPS também confirmaram a presença de polimorfismos em quatro genes relacionados à respostas de defesa. No entanto, estes não tiveram relação com os fenótipos mutantes. Análises de agrupamentos segregantes baseada em

MutMap será empregada a identificação dos genes mutados nos genótipos identificados.

GENERAL INTRODUCTION

Asian soybean rust (ASR), caused by the biotrophic pathogen *Phakopsora pachyrhizi* Syd., is one of the main soybean diseases (*Glycine max* L. Merr.) In Brazil, yield losses of up to 80% have been reported in untreated areas (Yorinori *et al.*, 2005; Scherm *et al.*, 2009). ASR management is mainly carried out by application of chemical fungicides, use of resistant cultivars, anticipated planting, short cycle varieties and the host-free period called "vazio sanitário" (Del Ponte *et al.*, 2009).

Fungicide application is the primary strategy used to control ASR. In Brazil, an average of three applications per crop are required, which increases the annual cost of controlling the disease by near US$ 2 billion (Godoy *et al.*, 2015). However, the efficiency of the main chemical groups of single-site mode of action fungicides has decreased year after year due to the emergence of resistant populations (Reis *et al.*, 2015; Godoy *et al.*, 2016; Twizeyimana & Hartman, 2017; Childs *et al.*, 2018). The high application costs along with the adverse effects on the environment and fungicide resistance emergence make genetic resistance against *P. pachyrhizi* a highly desirable trait.

Genetic resistance to ASR offers an environmentally-friendly and relatively low-cost alternative to fungicides. Several resistance genes have already been identified and mapped to seven different loci, named *Rpp1* to *Rpp7* (Hyten *et al.*, 2007; Garcia *et al.*, 2008; Silva *et al.*, 2008; Hyten *et al.*, 2009; Kim *et al.*, 2012; Li *et al.*, 2012; Childs *et al.*, 2018). Additional alleles have also been discovered displaying different responses to selected *P. pachyrhizi* isolates and were mapped to previously reported *Rpp* loci (Garcia *et al.*, 2008; Chakraborty *et al.*, 2009; Ray *et al.*, 2011; King *et al.*, 2016; King *et al.*, 2017). *Rpp* genes located at these loci provide pathotype-specific resistance and due to the high pathogenic diversity found in the *P. pachyrhizi* populations, this resistance may quickly lose efficacy, limiting the protection that individual *Rpp* genes provide against different pathotypes (Paul *et al.*, 2013; Akamatsu *et al.*, 2017).

The resistance and susceptibility of plants to microbial pathogens involve molecular interactions between microbial effectors and host targets, which alter the host cell metabolism and inhibit their defense mechanisms. Effectors are any secreted molecule that contributes to the colonization (Rovenich *et al.*, 2014; Snelders *et al.*,

2018), such as cysteine-rich proteins (Qi *et al.*, 2016), secondary metabolites or small RNAs (Wang, Weiberg*, et al.*, 2016).

The vast majority of phytopathogens, especially biotrophic ones, require the collaboration of the host's molecules to establishing a compatible interaction. Plant genes that encode proteins manipulated by pathogens are known as susceptibility genes (S genes) (van Schie & Takken, 2014). Different studies have shown that loss of function or mutations in susceptibility genes may provide durable and broad-spectrum resistance (Pavan *et al.*, 2010; Campbell *et al.*, 2012; Gawehns *et al.*, 2013). Mutation or loss of an S gene may limit the ability of the pathogen to cause disease by impairing requirements such as pre-penetration and penetration or compromise specific infection requirements (e.g., nutrient supply) (van Schie & Takken, 2014).

The *Mlo* gene (Mildew resistance locus) is one of the most studied S gene and is an example of durable resistance gene. The *Mlo* mutation was shown to confer powdery mildew (PM) resistance more than seven decades ago and this mutant continues to confer resistance to PM in the field (Jørgensen, 1992; van Schie & Takken, 2014). *Arabidopsis* resistance to downy mildew caused by *Hyaloperonospora parasitica* was also achieved through the use of artificially induced mutagenesis targeting S genes (Van Damme *et al.*, 2005). However, there is no similar study in soybean.

Different chemical mutagens have been used to induce mutations and variability which are useful for crop improvement as well as for functional studies. Among them, ethyl methanesulfonate (EMS) has been widely used because it can cause allelic mutations in genes through random point mutations that occur at high density (McCallum *et al.*, 2000; Henikoff *et al.*, 2004; Hwang *et al.*, 2014).

Therefore, the objectives of this study were: 1) To generate a large EMS-mutagenized soybean population from the susceptible variety TMG 4182, which was used to identify mutants with altered response to *P. pachyrhizi* (Chapter 1); 2) To characterize by resequencing three identified mutants, aiming the identification of mutations and genes associated with the altered responses (Chapter 2).

LITERATURE REVIEW

Soybean

Soybean [*Glycine max* (L.) Merr.] is a legume of the Fabaceae family, that originated from Southeast Asia, and was domesticated in China around 1100 BC (Qiu & Chang, 2010). In the seventeenth century, soybean plants were taken to Europe where they remained as a botanical curiosity in the gardens of European courts for more than 200 years. Soybean was introduced in the United States in the mid-1890s, and was initially cultivated as a forage crop and later as a succession crop of wheat (Sediyama, 2009). The first report of soybean cultivation in Brazil, in Salvador, Bahia, was made by D'Utra, in 1882 (Cattelan & Dall'Agnol, 2018). Because the variety used was originated from the United States and not well adapted to a region of low latitudes, such as Bahia, this first planting was unsuccessful. More successful results were obtained at Campinas-SP, in 1908 (Bonato & Bonato, 1987). The introduced materials were improved and adapted to low-latitude cultivation by breeders from the Agronomic Institute of Campinas (IAC) and later distributed to growers from São Paulo.

In 1914, American varieties were also introduced in the Rio Grande do Sul State. Better adaptation to the South region was observed, due to the similarities with the North American regions to which they were developed (Sediyama, 2009). In the late 1960s, two internal factors made Brazil begin to see soy as a commercial product, the fact that would later influence the world scenario of grain production. At the time, wheat was the main crop of southern Brazil and soybean emerged as a summer option in succession to wheat. Pork and poultry production also began to grow in Brazil, generating higher demand for protein for animal feed. In 1966, commercial soybean production was already a strategic necessity, and around 500,000 tons were produced in the country (Bonato & Bonato, 1987).

The explosion of soybean prices in the world market in the mid-1970s further awakens farmers and the Brazilian government itself. Since then, the country began to invest in technology to adapt its cultivation for Brazilian conditions, a process led by the Brazilian Agricultural Research Corporation (EMBRAPA). Investments in research led to the "tropicalization" of soybeans, allowing it to be successfully planted in low latitude regions between the tropics and the equator (Bonato & Bonato, 1987).

Until 1960 and 1970, the southern region was the major producer of the country, especially in Rio Grande do Sul and Paraná states, still large producers today. However, currently, they lost in volume to Mato Grosso, which is now the largest national producer (Sediyama, 2009). From the 1980s, soybean has extended to the cerrado, a vast region that covers the so-called acid soil polygon, namely: Triangulo Mineiro, Mato Grosso do Sul, Mato Grosso, Goiás, Tocantins, southern Maranhão, south from Piauí and western Bahia. Thus, the cerrado region is the largest producing region of the country. The expansion to this new agricultural frontier was basically due to studies on the fertilization of the Cerrado soils, its flat topography favorable to mechanization, and the development of adapted cultivars suitable for the region (Bonato & Bonato, 1987).

Being one of the main commodities produced worldwide due to its different uses, soybean is part of the agricultural activities with major emphasis on the world market (Conab, 2019). The United States Department of Agriculture (USDA) estimated in its July 2018 report that the world soybean crop production in 2018/19 will be approximately 359.49 million tons, a value 6.76% higher than estimated in the harvest 2017/18, of 336,70 million tons. Moreover, according to USDA estimates, Brazil (120.50 million tons - 33.52%), the United States (117.30 million tons - 32.63%) and Argentina (57.00 million tons, 15.86%) account for 82.01% of the world's total soybeans production, and China accounts for 61.54% of all world imports (CONAB, 2019). Soybean is also one of the main crops for Brazilian GDP. In 2017, it represented 103,200 billion reais in Brazilian agricultural GDP, with a record volume of 83.6 million tons (40.9 billion dollars) exported in 2018 (Forbes, 2019). Nowadays Brazil is the second-largest soybean producer in the world, occupying an area of 35 million ha to produce 114 million tons of this grain per year (CONAB, 2019).

Asian Soybean Rust

The soybean is host to two species of the genus *Phakopsora*, which cause rust: the American rust, *P. meibomiae*, which is not of great economic importance, and the Asian rust caused by *P. pachyrhizi* (Deslandes, 1979; Yorinori *et al.*, 2005). Considered as the main soybean disease in Brazil, Asian soybean rust or ASR, was first reported in Japan in 1903, from where it spread throughout Asia. After being detected in Paraguay and Brazil in 2001, the pathogen spread rapidly throughout the

continent being reported in Argentina in 2002, Bolivia in 2003 and the United States in 2004 (Schneider *et al.*, 2005; Yorinori *et al.*, 2005).

Unlike rust species that cause disease in gramineous plants, which are highly specialized, *P. pachyrhizi* has a wide range of hosts, being able to infect at least 31 host species from 17 different genera of the *Fabaceae* family. However, there are reports of 152 legumes of 56 genera identified as possible *P. pachyrhizi* hosts (Ono *et al.*, 1992; Slaminko *et al.*, 2008). The life cycle of ASR is complex and may involve different hosts and specialized spores. In the case of *P. pachyrhizi*, not much is known about its sexual form. The production and germination of teliospores have already been reported in the lab (Yeh *et al.*, 1981). However the role of these spores in the pathogen cycle and disease development is still unknown (Bromfield, 1984; Goellner *et al.*, 2010).

Like other rusts, *P. pachyrhizi* is a biotrophic parasite whose life cycle depends on a living host. The fungal cycle begins with the production of uredospores produced in structures called uredia, predominantly located on the abaxial face of the leaves of the host. After the opening of the uredia, the uredospores are easily dispersed by wind. When temperature conditions are between 18 and 26ºC and humidity is high, they germinate, forming the germinative tube that will form a globular appressorium, capable of penetrating directly into the host tissue, with no stomata or injury necessary for its penetration (Bromfield, 1984; Koch & Hoppe, 1988). After appressorium formation, the penetrating hyphae crosses the epidermis of the plant cell and reaches the intracellular space, giving rise to a primary hypha, from which it can branch into secondary hyphae and, finally, form specialized feeding structures, called haustoria (Koch & Hoppe, 1988; Goellner *et al.*, 2010).

Under favorable conditions, infection and colonization of plant tissues can occur between five to seven days, and the production of uredospores, between nine to twelve days, giving rise to a new cycle. Symptoms may manifest at any stage of crop development, depending on the environmental conditions and on the availability of inoculum (Kimati *et al.*, 2005). At the onset of the disease, small chlorotic spots are observed, delimited by the veins that evolve to a dark brown color due to the necrosis of the cells. The lesions increase and, 5 to 8 days after the initial infection, rust pustules (uredia) become visible. These uredia develop mainly in lesions on the abaxial face of the leaves, but can also appear in petioles, pods, and stems. Early defoliation of the

plant can occur in cases of high severity, causing a reduction in the photosynthetic capacity of the plant (Kimati *et al.*, 2005; Goellner *et al.*, 2010).

Depending on the degree of the host plant susceptibility, different types of reactions to the pathogen may be noted. In plants considered to be resistant, the absence of lesions (Immune reaction) or reddish-brown lesions (RB) can be observed, with little or no sporulation. In susceptible plants, lesions of light brown color with abundant sporulation are produced, known as TAN lesions (Goellner *et al.*, 2010; Miles *et al.*, 2011).

Disease control in Brazil occurs by integrating different management strategies: application of fungicides; use of resistant cultivars; cultural practices such as early planting with early varieties, monitoring and elimination of alternative hosts, and by adopting the host-free period (called *vazio sanitário*) (Hartman *et al.*, 2005; Godoy *et al.*, 2015; Langenbach *et al.*, 2016). The host-free period (HFP) is a legislative control measure, adopted to reduce the severity and progress of ASR. This measure comprises a period of at least 60 days without planting soybean to reduce the inoculum of *P. pachyrhizi* in the field (EMBRAPA, 2019).

The use of fungicides is still the most used strategy to control ASR. In Brazil, at least three applications are needed per crop cycle, which increases the cost of disease control up to about 2.8 billion U.S dollars annually (Consorcio Antiferrugem, Consorcio Antiferrugem, Consorcio Antiferrugem, Consorcio Antiferrugem, 2016). However, the emergence of resistant populations to the major chemical fungicide groups emphasizes the need of searching alternative control methods and also of integrating the available strategies (Godoy *et al.*, 2016; Langenbach *et al.*, 2016).

In soybean, several ASR resistance loci, named *Rpp1* to Rpp7, have already been identified (Hartwig, 1986; Hyten *et al.*, 2007; Garcia *et al.*, 2008; Silva *et al.*, 2008; Hyten *et al.*, 2009; Li *et al.*, 2012; Childs *et al.*, 2018). The *Rpp* genes provide resistance to specific strains of *P. pachyrhizi*. Thus, no currently available soybean genotype and cultivars are resistant to all *P. pachyrhizi* isolates (Monteros *et al.*, 2007). For this reason, resistance mediated by *Rpp* genes can be overcome in the field (Yorinori *et al.*, 2005; Akamatsu *et al.*, 2013; Paul *et al.*, 2013). Pyramiding different resistance genes in a single variety can confer broad-spectrum resistance (Mundt, 2018). In soybean, the pyramiding of different *Rpps* in a single genotype has been shown to provide enhanced resistance to a broad range of *P. Phachyrhizi* isolates (Lemos *et al.*, 2011; Yamanaka *et al.*, 2013; Yamanaka *et al.*, 2015; Yamanaka &

Hossain, 2019). Yamanaka and Hossain (2019) suggest that there's a strong synergic effect between *Rpp* genes, and that soybean lines containing combinations of three *Rpp* genes were generally more resistant than those carrying only two. A natural combination of different resistance genes can be found. For example, the soybean cultivar 'Hyuuga,' contains the *Rpp3* and *Rpp5* resistance genes (Kendrick *et al.*, 2011).

Resistance and susceptibility genes

Communication between plants and pathogenic organisms begins immediately upon contact. Biotrophic fungi and oomycetes recognize chemical components and topographic features present on the surface of plants, forming infection structures such as appressoria or hyphopodium (Liu *et al.*, 2011; Chagas *et al.*, 2018; Sun *et al.*, 2019). The plant, in return, recognizes these structures and responds by forming cytoplasmic aggregations, cell wall deposition (papillae) and by reorganizing the cytoskeleton below the appressorium (Koga, 2001; Yi & Valent, 2013; Chang *et al.*, 2019). After penetration, defense mechanisms are activated by the direct or indirect recognition of pathogen molecules, or host molecules released by the pathogen action or damage inflicted during the infection (Yi & Valent, 2013; Zebelo, 2019). The recognition can be made by extracellular pattern recognition receptors (PRRs), which detect molecular patterns associated with microorganisms or pathogens (MAMPs or PAMPs), such as flagellin and chitin, or damage-associated molecular patterns (DAMPs), such as fragments of the plant cell wall (Boller & Felix, 2009; Beck *et al.*, 2012; Zebelo, 2019). The recognition of DAMP / PAMP by PRRs activates a defense response called PTI (PAMP triggered Immunity), that includes the production of reactive oxygen species (ROS), secretion of antimicrobial compounds and hydrolytic enzymes and local cell wall fortification, such as callose deposition (Boller & Felix, 2009; Wang *et al.*, 2019).

The colonization of plant cells by biotrophic fungi and oomycetes is done by a specialized feeding hyphae, haustoria (Khang *et al.*, 2010; Rafiqi *et al.*, 2010; Ried *et al.*, 2018). This specialized structure is very important for the establishment of infection because it is the main site in which effector molecules are synthesized and secreted. These molecules are capable to alter plant cell metabolism and suppress PTI (Dou & Zhou, 2012; van Schie & Takken, 2014). In response to this attack, plants have evolved resistance proteins that have the ability to recognize some of these effectors

directly, or the modification inflicted by them on the host's proteins. These resistance proteins are encoded by resistance genes (R genes) and the immunity mediated by R genes is referred to as ETI (effector-triggered immunity), that is often accompanied by the cell death at the infection sites or hypersensitive reaction (HR) (Dodds & Rathjen, 2010; Hofer, 2018).

The PTI-mediated recognition has a broad spectrum, since pathogen-associated molecules are widely conserved. The recognition step that activates ETI is more specific, due to the effector's polymorphism (Dodds & Rathjen, 2010; Hofer, 2018). Effectors are under intense selection pressure, contributing to the emergence of new races of the pathogen and consequently leading to the breakdown of the plant's resistance (Jones & Dangl, 2006).

In addition to altering host cell metabolism and evading its defense mechanisms, a vast majority of plant pathogens, especially biotrophic fungi, require host cooperation to develop a compatible interaction. Plant genes that facilitate or, otherwise, support this compatibility, are named susceptibility (S) genes (van Schie & Takken, 2014). A mutation or loss of an S gene may limit the ability of the pathogen to cause disease, impairing requirements such as pre-penetration and penetration, or compromising specific infection requirements, such as nutrient delivery. Therefore, S genes can provide broad-spectrum resistance, promoting prolonged or constitutive defenses (van Schie & Takken, 2014).

The durability of a novel S gene is difficult to predict and depends on the ability of the pathogen to adapt and evolve. However, a number of examples of resistance based on S genes indicate that this resistance can be durable (van Schie & Takken, 2014). A widely studied recessive gene in terms of durability is the S gene *eIF4E*. This gene confers resistance against the potyvirus PVY (Potato virus Y). The pepper mutant gene *pvr*1/2 is the first gene that provides resistance identified by mutagenesis and it is still successfully used (Murphy *et al.*, 1998; Moury & Verdin, 2012). Another classic example of a durable S gene is the *mlo* gene in barley, that provides resistance to all powdery mildew species. This gene was discovered over seven decades ago and is still used as a source of resistance in barley breeding programs (Jørgensen, 1992). Nekrasov *et al.* (2017) used gene editing to knock out the *Mlo* gene in the wild-type tomato plant using CRISPR/Cas9 technology to obtain resistant to the powdery mildew in tomato, showing new perspectives of using susceptibility genes.

The concept of susceptibility gene was further defined in 2002, after identification of the *Pmr6* resistance gene in *Arabidopsis*, it was defined as "a new form of resistance to diseases based on the loss of a necessary gene during a compatible interaction" (Vogel *et al.*, 2002).

According to van Schie and Takken (2014) the mechanisms by which S genes mediate resistance can be divided into three classes. The first includes the genes required for the initial stages of infection by the pathogen; genes that allow basic compatibility, facilitating host-plant recognition, pre-penetration, and penetration. The cuticle of the plant and the cell wall represent the first physico-chemical barrier that opposes penetration. The chemical composition of cuticle and wax plays an essential role in the interactions between plants and fungi. Certain fatty acids, long-chain alcohols or long-chain aldehydes in cuticular wax are required for efficient germination and appressoria formation (Serrano *et al.*, 2014). The *glossy11* corn mutant has low levels of long-chain aldehydes in the leaf cuticle, which results in poor germination of powdery mildew (Hansjakob *et al.*, 2011). A mutant of Medicago, *irg1*, with a reduction of primary alcohols in the surface wax, caused reduced spore differentiation in *Phakopsora pachyrhizi and Puccinia emaculata*, as well as spores of *Colletotrichum trifolii* (Uppalapati *et al.*, 2012). These examples indicate that the leaf cuticle contains components used by filamentous pathogens as a signal to activate developmental pathways required for pathogenicity. Plant genes or enzymes involved in the synthesis of such compounds contribute to susceptibility and can be considered as S genes (van Schie & Takken, 2014).

The second class of S genes encodes negative regulators of plant immune signaling. The regulation of defense pathways mediated by hormones such as salicylic acid, jasmonic acid and ethylene can also increase or decrease plant susceptibility to certain pathogens (van Schie & Takken, 2014). Mutations that cause accumulation of salicylic acid lead to pathogenesis-related proteins (PR) production and an increase in the resistance to biotrophic pathogens. However, plants with these mutations may exhibit slow growth and systemic HR (Moeder & Yoshioka, 2008). An *Arabdopsis* mutant, SA3-hydroxylase (S3H), capable of converting salicylic acid into 2,3-DHBA was more susceptible to *Pseudomonas syringae*, indicating that the hydroxylation of AS may contribute to susceptibility (Zhang *et al.*, 2013). Cellulose synthesis enzymes are essential for the formation of the plant cell wall. Mutants of the *CESA3* gene (*cev1*)

were resistant to multiple pathogens by showing constitutive levels of jasmonic acid and ethylene (Ellis & Turner, 2001; Ellis *et al.*, 2002; Hernández-Blanco *et al.*, 2007).

WRKY transcription factors also play an essential role in the transcription and reprogramming of the cell under attack by pathogens. WRKY genes can function as positive or negative regulators of defense pathways and contribute to resistance or susceptibility (Pandey & Somssich, 2009). In A*rabidopsis*, *WRKY8* is essential for susceptibility to *Pseudomonas* and resistance to TMV (*Tobacco mosaic virus*) (Chen, Zhang*, et al.*, 2010; Chen *et al.*, 2013). In rice, the *WRKY45-1* gene is a susceptibility factor for the bacterium *Xanthomonas oryzae*, while its homolog *WRKY45-2* is important for resistance to the same pathogen (Tao *et al.*, 2009).

The third class of S genes includes genes involved in sustaining the pathogen compatible interaction, such as metabolite biosynthesis and nutrient transport. The *DMR1* gene (Downy Mildew Resistant 1) codes for a homoserine kinase (HSK). *Arabidopsis dmr1* mutants accumulated higher amounts of homoserine in their chloroplasts and were more resistant to *Hyaloperonospora parasitica* that causes powdery mildew (van Damme *et al.*, 2009; Huibers *et al.*, 2013). The production of metabolites can also be modified to benefit the pathogens themselves. In barley, the enzyme AFH (alcohol dehydrogenase) is induced by the infection of powdery mildew and plants in which this gene was silenced were less susceptible to the disease (Pathuri *et al.*, 2011). Other examples include genes encoding SWEET-type proteins, identified as susceptibility factors for *Xanthomonas* spp. SWEET proteins are membrane transporters that pump sugars into the cell's apoplast, providing nutrients for the pathogen (Chen, Hou*, et al.*, 2010; Streubel *et al.*, 2013; Sun *et al.*, 2016). In rice, two recessive mutations in the genes *Xxa12 and Xa25*, which encode SWEET11 and SWEET13 proteins, conferred resistance to *Xanthomonas oryzae* (Chen, Hou*, et al.*, 2010). In cassava, mutation of *MeSWEET10* conferred resistance to *Xanthomonas axonopodis* pv. *manihotis* (Cohn *et al.*, 2016), and mutations in *GhSWEET10* provided resistance to *Xanthomonas citri* subsp. *malvacearum* in cotton (Cox *et al.*, 2017).

Viruses are highly dependent on the cell's machinery to replicate and infect (Dimmock *et al.*, 2016). Translation of potyvirus RNA requires interaction with the plant's elF4E protein (Bastet *et al.*, 2017). Multiple recessive alleles of *elF4E* were associated with virus-resistant phenotypes in tomato (Mazier *et al.*, 2011), melon (Rodríguez-Hernández *et al.*, 2012) cucumber (Chandrasekaran *et al.*, 2016, bell

pepper mutants (Ruffel *et al.*, 2006; Chandrasekaran *et al.*, 2016) and *Arabidopsis thaliana* (Duprat *et al.*, 2002).

Susceptibility genes can also encode targets of microbe effectors. The genes *KRBP1, PP1C*, and *NRL1* encode proteins that are targets of *Phytophthora infestans* effectors Pi04089 (Wang *et al.*, 2015), Pi04314 (Boevink, Wang*, et al.*, 2016), and Pi02860, respectively (Yang *et al.*, 2016). Silencing of these genes reduced *P. infestans* infection, while its overexpression increased plant susceptibility (Boevink, Wang*, et al.*, 2016).

Chemical mutagenesis

Mutant organisms exhibit changes in DNA and protein sequences that result in heritable variations, which may be accompanied by visible or measurable phenotypic changes (Guo *et al.*, 2005). These changes can occur naturally or be induced. Induced mutations can be a source of new genetic variations for crop improvement. In addition, mutagenesis may reveal the function and assist in the characterization of genes involved in biological processes (Zhu *et al.*, 2005; Cui *et al.*, 2013; Maghuly & Laimer, 2013; Boevink, McLellan*, et al.*, 2016; Huang *et al.*, 2016).

Artificial mutagenesis can be induced by biological agents such as transposons and T-DNA; physical agents such as fast neutron radiation, UV radiation, and X-rays; and by chemical agents such as N-methyl-N-nitrosourea (MNU), 1,2:3,4-diepoxybutane (DEB) or ethyl methanesulfonate (EMS) (Serrat *et al.*, 2014). Among these compounds, EMS has been frequently used as a chemical mutagen in plants (Brockman *et al.*, 1984).

EMS is an organic mutagenic compound capable of inducing random point mutations in the genetic material due to the alkylation of guanines (McCallum *et al.*, 2000; Kodym & Afza, 2003; Kim *et al.*, 2006). The EMS ethyl group reacts with the guanine (G) of DNA at the 6'-O position, forming a modified base, called O-6-ethylguanine, which can pair with a thymine (T) instead of cytosine (C). During DNA replication, DNA polymerase enzymes often add thymine in place of cytosine at the opposite side of the modified O-6-ethylguanine base, resulting in an unusual pairing of base pairs. After subsequent replications, the original G: C base pair may become an A: T pair, which corresponds to approximately 99% of all EMS-induced nucleotide changes (Greene *et al.*, 2003). However, the depuration of alkylated guanine (G) may

form gaps in the DNA which, during replication, may be filled with a random base or become a deletion, causing a frameshift change in the open reading frame of genes (Krieg, 1963; McCallum *et al.*, 2000; Kodym & Afza, 2003). These nucleotide changes caused by EMS may lead to a complete or partial loss of gene function (Greene *et al.*, 2003; Kim *et al.*, 2006).

Chemically mutagenized populations have been successfully generated in a wide variety of plant species, including *Arabidopsis* (McCallum *et al.*, 2000; Greene *et al.*, 2003), wheat (Slade *et al.*, 2005; Uauy *et al.*, 2009; Ansari *et al.*, 2012; Tian *et al.*, 2012), rice (Gu *et al.*, 2004; Wu *et al.*, 2005; Till *et al.*, 2007; Wang *et al.*, 2013), maze (Till *et al.*, 2004; Weil & Monde, 2007), peanuts (Zhu *et al.*, 1997), barley (Caldwell *et al.*, 2004), sorghum (Xin *et al.*, 2008), tomato (Menda *et al.*, 2004; Minoia *et al.*, 2010) and cabbage (Huang *et al.*, 2016).

Recently, many mutagenesis studies in soybean were also reported. Carroll *et al.* (1985) developed a mutagenized soybean population with 2,500 M2 lines and isolated 15 nitrate tolerant and supernodulating mutants. Soybean seeds of different cultivars, treated with EMS and N-nitroso-N-methyl urea (NMU), showed a high density of mutations (Cooper *et al.*, 2008). Anai (2012) generated a population with more than 10,800 M2 lines, treating soybeans of three cultivars with different concentrations of EMS. A mutant library consisting of 1,477 M3 lines was generated by treating soybean seeds M1 and M2 with EMS. Evaluation by the re-sequencing of the entire genome of independent mutant lines resulted in a mutation density equivalent to 1 mutation / 74kb (Tsuda *et al.*, 2015). Li *et al.* (2017) generated a population of 21,600 independent M2 lines and identified a gene responsible for chlorophyll production. Espina *et al.* (2018) generated 1,820 mutant lines and found mutants that yielded higher protein and oil content.

However, this approach has not been used in soybean to identify genes that are important in plant-pathogen interactions. Ethyl methanesulfonate (EMS) mutagenesis can be used for both forward and reverse genetic studies. Generation of diverse mutant alleles in the same gene provides critical tools to understand the function of these genes in the plant and have the potential to yield useful mutant alleles for soybean breeding for disease resistance.

Chapter 1 - Development of an EMS mutagenized soybean population and identification of mutants with altered response to *Phakopsora pachyrhizi*

Abstract

Ethyl methanesulfonate (EMS) can establish allelic variation in genes due to random point mutations that occur at high density. This genetic variation makes it possible to identify novel traits that can be used in crop improvement, and in studies aiming to determine gene function. The objective of this study was to create a mutant soybean population to screen for mutants with altered responses to *P. pachyrhizi*. Twenty-thousand soybean seeds from cultivar 'TMG4182' were treated with EMS 0.3% (v/v) for 16h and planted in the field and seeds from each plant were individually harvested. Twenty-two seeds from 1,820 M2 families were planted in a greenhouse and evaluated for phenotypic variations. At the V1 stage, these plants were inoculated with *P. pachyrhizi* isolate PPUFV02 and screened for any phenotypic changes in disease development. From a total of 30,068 M2 plants evaluated, 2,976 displayed phenotypic variations such as dwarfism (723), albinism (43), altered leaf morphology (825) and 101 altered disease symptoms development. Progenies from M2 lines GMMT-509-20 and GMMT-504-04 displayed reddish-brown (RB) symptoms, with fewer uredia, and GMMT496-01 necrotic lesions with fewer uredia and less sporulation when compared to susceptible WT 'TMG4182', highlighting the potential of this approach to find novel variation to be used for disease resistance breeding

Keywords: *Glycine max*; Asian Soybean Rust; Ethyl methanesulfonate.

Introduction

Asian soybean rust caused by the biotrophic pathogen *Phakopsora pachyrhizi* is the most important soybean disease in Brazil, controlled by using fungicides that are becoming less effective every year. Resistance to ASR has been identified and mapped in a few germplasm accessions and is conferred by either dominant or recessive genes. However, there is no durable resistance is available in commercial cultivars.

Artificially induced genetic mutations can provide supplementary variation to plant breeding programs and have the advantage over transgenic approaches because is not under legislative and intellectual property restrictions. Among the chemical mutagens, EMS has been widely used to generate new traits (Hwang *et al.*, 2014). EMS can establish allelic variation in genes due to random point mutations altering the amino acid composition of proteins that may lose or gain function (McCallum *et al.*, 2000; Henikoff *et al.*, 2004).

Susceptibility of plants to microbial pathogens involves complex molecular interactions between the microbe's effectors and the host's targets. Pathogens are able to alter host cell metabolism and evade defense mechanism. In addition, biotrophic fungi require the cooperation of the host's genes to establish a compatible interaction. Plant genes that facilitate or support this compatibility may be considered as susceptibility (S) genes. Mutation or loss of an S gene may limit the ability of the pathogen to cause disease by impairing requirements such as pre-penetration and penetration or by compromising specific infection requirements such as nutrient supply (van Schie & Takken, 2014).

The objective of the present study was to create a large mutagenized soybean population using ethyl methanesulfonate (EMS) as a mutation inducer, to identify mutants with altered reaction to *P. pachyrhizi* isolate PPUV02, aiming the identification of susceptibility genes.

Material and Methods

Plant materials and EMS treatment optimization

To access the effects of EMS on soybean seeds and to generate a large mutagenized population, seeds from conventional soybean cultivar TMG4182 were selected. This conventional commercial cultivar has a determined growth habit, high

yield potential and it is susceptible to *P. pachyrhizi* (TMG, 2019). Seeds from this cultivar were also available in large quantities at the laboratory.

The germination rate of seeds treated with EMS is used as a selection method to assess an optimal concentration to be used in large-scale mutagenesis (Meksem *et al.*, 2008). Eight concentrations of EMS (v/v) were selected to analyze the effect on the germination rate of soybean seeds cultivar TMG 4182. Samples of 120 soybean seeds from cultivar 'TMG4182' were immersed in 100 mM phosphate buffer (pH7.5) and EMS in concentrations varying from 0 to 1% (v/v) (0%, 0.3%, 0.4%, 0.5%, 0.6%, 0.7%, 0.8% and 1%), and kept in 50ml tubes in a rotary shaker (60 rpm) at 25 °C for 16h.

Treated seeds were then washed three times with distilled water and sown in 125 cell trays containing Tropstrato HA® growing media in the greenhouse at 25-28 °C. Germination rate was evaluated 10 to 15 days after sowing by counting the number of seedlings that emerged from the soil (Meksem *et al.*, 2008). The concentration of EMS that inhibited 50% of seed germination was then used to develop the mutant soybean population.

It is important to emphasize that mutagenesis breeding involves specific terms that are generally adapted from those used in standard breeding, in which, seeds prior to the mutation treatment are called the M0 seeds, or wild-type seeds. Once mutagenized, the seeds are then known as the M1 generation. The progeny of the M1 (seeds from M1) is the M2, and this population represents the earliest generation in which screening for mutations is carried out. The progeny from M2 is known as M3 and so on.

Developing a mutant soybean population (M1)

A concentration of EMS that inhibited 50% of seed germination calculated from the first trial was used to treat 20,000 seeds of soybean cultivar 'TMG 4182'. The seeds were immersed in 100 mM phosphate buffer (pH7.5) and 0.3% EMS (v/v) in a 2L Erlenmeyer placed on a rotary shaker (60 rpm) at 25 °C for 16h.

Treated seeds were then washed three times with distilled water; this water was collected and then neutralized with a 10% (w/v) sodium thiosulfate solution. The seeds were then sown in 1L plastic pots containing a mix of soil and Tropstrato HA® growing media, inside a greenhouse at 25-28°C. Fifteen days after sown, the seedlings were

transplanted to the field where they received all the necessary cultural treatments to produce M2 seeds through self-pollination. Plants were regularly scouted for signs of phenotypic variants. At the end of the crop cycle, each plant was harvested individually by using a soybean threshing machine.

Assessment of phenotypic variations in the M2 population

Twenty-two seeds from each M2 family were planted in 40 x 60 cm plastic trays (two M2 families/tray) (Figure 1-A-B), containing a mix of soil and Tropstrato HA® growing substrate, and kept in a greenhouse at 25 °C. Survival rates and phenotypic variations of growth behavior, plant architecture and leaf morphology, in comparison to the wild-type 'TMG4182', were recorded and documented (Espina *et al.*, 2018).

***Phakopsora pachyrhizi* spore production**

In order to produce enough *P. pachyrhizi* spores for the screening trials, susceptible soybean cultivar Conquista plants were inoculated at the phenological stages V3-V4 (Fehr & Caviness, 1977). All young and fully expanded trifoliate were sprayed on the abaxial surface with a spore suspension of monouredinal isolate PPUFV02, at 5.0 x10^4 uredospores/ml in distilled water + Tween 20 (0.01%), using a direct air atomizer and placed in a dark moist chamber with spray shifts of 5 seconds every 10 minutes at 22 ± 2ºC for 24 h. The plants were then transferred to a greenhouse under standard growth condition of 12-hour photoperiod at 25-28°C, for 30 days. After the 10th day of inoculation, uredospores were collected with a vacuum spore collector coupled to an air compressor every three days, and stored at -80 °C. After short-term storage, and prior to use, these uredospores were heat-shocked for 10 minutes at 40 ºC in a water bath and rehydrated for 24h to break dormancy (Furtado *et al.*, 2008).

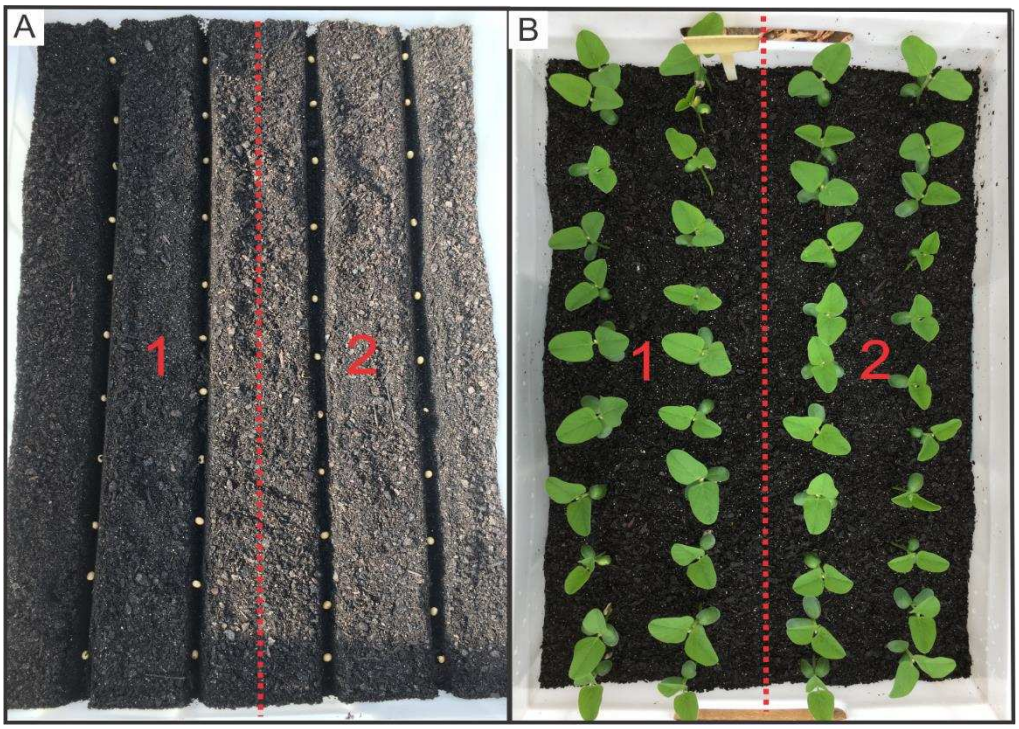

Figure 1 - Details of M2 family screening procedure using plastic trays. (A), 22 seeds per M2 family two M2 families were sown per tray. (B), Aspect of germinated seedlings, eight days after sowing.

Screening with *Phakopsora pachyrhizi*

All plants from each M2 family were inoculated at the beginning of the V1 stage, with *Phakopsora pachyrhizi*, monouredinal isolate PPUFV02. The inoculations were done with a direct air atomizer using a suspension of 2.0 x 10^4 uredospores/ml in distilled water + Tween 20 (0.01%) (Figure 2). The inoculation was done late in the evening and the floor of the greenhouse was irrigated to maintain high humidity in the greenhouse overnight. The plants were kept in a greenhouse for 15 days at 25-28°C. One tray (44 plants each) of wild type, non-mutagenized 'TMG4182' and the susceptible cultivar 'Conquista' were also inoculated, as the control treatment.

All mutant M2 plants were evaluated every two days for ASR disease symptoms and other phenotypic changes. Plants that displayed any variation on disease symptoms, as well as lack of symptoms or fungal signs, were selected and transplanted into 2L pots containing a mix of soil and Tropstrato HA® growing media, kept in a greenhouse at 25-28°C, grown to maturity and individually harvested. All selected M2 plants were treated with azoxystrobin + benzovindiflupir (Elatus®, 150 g/ha) after transplant.

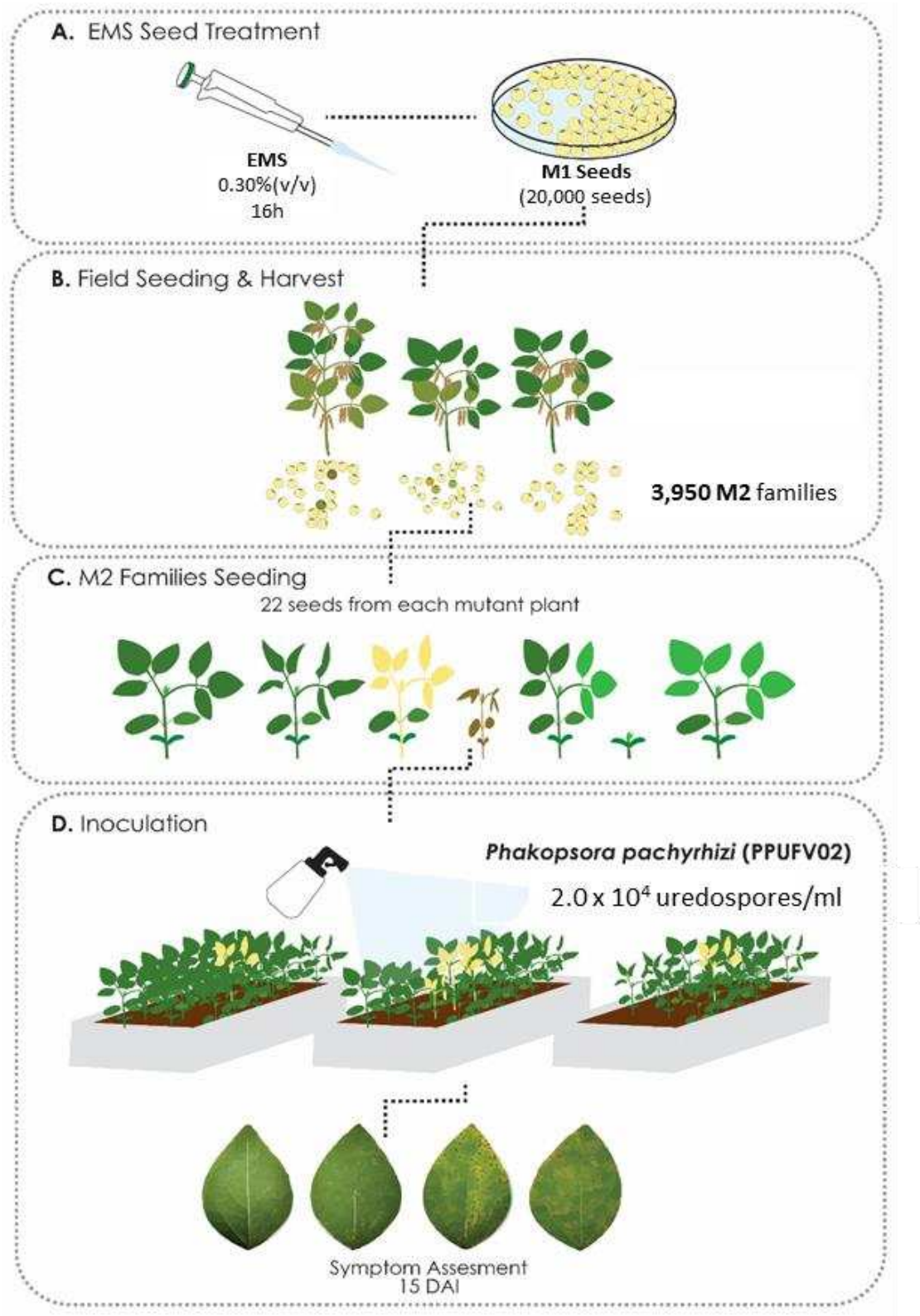

Figure 2 - Procedure used in the development of a soybean ethyl methanesulfonate (EMS) induced mutant population and screening with Phakopsora pachyrhizi isolate PPUFV02 (A) 20,000 soybean seeds from cultivar TMG4182 were treated with EMS at 0.30% (v/v). (B) Germinated seeds (M1 seeds) were planted in the field and individually harvested, generating the mutant population with 3,950 M2 families. (C) Twenty-two seeds from each M2 family, were evaluated for phenotypic variations (D) inoculation at V1 stage with *P. pachyrhizi* isolate PPUFV02 and symptom assessment.

Confirmation of phenotypes observed in selected M2 plants

Mutant M2 plants were selected and transplanted to 2L pots with a mix of soil and Tropstrato HA® growing mix and allowed to reach maturity. Seeds were individually harvested from fertile plants making up different M3 families. To confirm if the altered phenotypes to *Phakopsora pachyrhizi* observed in the large-scale screening was caused by mutations and stable in the next generation, ten seeds of each M3 family along with ten seeds of the wild-type control 'TMG4182' were planted in 2L pots with a mix of soil and Tropstrato HA® growing mix and individually inoculated with *P. pachyrhizi* isolate PPUFV02 at the V2-V3 stage. All young and fully expanded trifoliate were sprayed on the abaxial surface with the spore suspension of 2.0 x 10^4 uredospores/ml in distilled water + Tween 20 (0.01%), through a direct air atomizer and kept in a dark mist chamber with spray shifts of 5 seconds every 10 minutes at 22 ± 2ºC, for 24 h, then transferred to the greenhouse under the conditions of 12 h photoperiod at 25-28 °C. These plants were individually evaluated every two days for phenotypic variations in disease symptoms.

Phenotypic characterization of selected ASR resistant mutants

EMS mutant M2 families GMMT04-30-496-01, GMMT04-30-509-20, GMMT04-30,504-04, GMMT02-50-05-17, GMMG04-30-362-16, and GMMT-05-30-745-16 along with WT 'TMG4182' and 'Williams82' were also inoculated with PPUFV-02 and three additional monouredinal *P. pachyrhizi* isolates, to verify if the phenotypic change were specific to PPUFV-02 and to quantify the disease severity in these mutant phenotypes (Table 1). These mutant M2 families were selected for this assay because the phenotypic alteration observed was consistent in all M3 plants tested.

Table 1 - Nomenclature, geographic origin, and host of monouredinal isolates of *Phakopsora pachyrhizi* selected to characterize the phenotypic reaction of soybean 'TMG4182' mutants.

***P. pachyrhizi* isolate**	**Year**	**Location**	**Host**	**Geographic coordinates**	
				Latitude	**Longitude**
PPUFV02[c]	2002	Viçosa, MG	Soybean	20° 46' 2.338"S	42° 52' 12.047"O
PR 8.4	2014	Tibagi, PR	Soybean	24° 30' 6.480"S	50° 26' 29.760"O
RS 2.4	2014	Marques de Souza, RS	Soybean	29° 15' 3.816"S	52° 9' 42.804"O
PR 183.2	2018	Londrina, PR	Soybean	23° 18' 37.000"S	51° 09' 46.000"O

[c] Control isolate

These isolates were selected because they displayed distinct virulence patterns in soybean plants containing different *Rpp*-resistance genes. Monouredinal control isolate PPUFV02 and PR 8.4 were virulent in soybean accessions carrying the *Rpp*1, and *Rpp*3 genes, RS 2.4 was virulent in *Rpp*1 and *Rpp*1b but displayed an RB reaction to soybean accession PI 462312 carrying the *Rpp*3 gene. PR183.2 displayed an RB reaction to both *Rpp*1 and *Rpp*1b but was virulent in soybean accession PI 471904 carrying the *Rpp*5 gene (Table 2).

Table 2 - Virulence of monouredinal isolates of *P. pachyrhizi* inoculated in soybean accessions carrying different *Rpp* genes.S = susceptible TAN reaction R = Immune or RB type reaction.

Soybean accession	*Rpp gene*	*P. pachyrhizi* monouredinal isolate			
		PPUFV02[C]	PR 8.4	RS 2.4	PR 183.2
PI200492	*Rpp1*	S	S	S	R
PI 587886	*Rpp1b*	R	R	S	R
PI 230970	*Rpp2*	R	R	R	R
PI 462312	*Rpp3*	S	S	R	R
PI 459025	*Rpp4*	R	R	R	R
PI 471904	*Rpp5*	R	R	R	S
PI 567102B	*Rpp6*	R	R	R	R

[c] control isolate

The *P. pachyrhizi* monouredinal isolate PPUFV02 was used in the present study as the standard control treatment. This isolate is derived from the experimental fields of the Federal University of Viçosa, and it is maintained at the LGGIPP-UFV laboratory.

Three plants from each mutant (1 plant = 1 repetition) and three plants of non-mutagenized ‘TMG 4182’and ‘Williams 82’ (susceptible controls) were inoculated with each isolate using the same methodology described above.

The qualitative and quantitative evaluations were done 15 days after the inoculation. The type and color of the lesions, presence or absence of spores were evaluated using the procedures and scale proposed by Yamanaka *et al.* (2010). In short, light brown lesions with little necrosis were labeled as "TAN"; reddish-brown lesions delimitated by necrosis as RB; and small necrotic reactions, fleck or hypersensitive reaction designated by "IF."

To better quantify the severity of the disease, two other parameters were used: sporulation level (SL) and the number of open uredia per lesion (NoU). The intensity of sporulation was evaluated using a number scale, proposed by Yamanaka *et al.*

(2010) using scores from 0 to 3, where: 0 = no sporulation; 1 = little sporulation; 2 = moderate sporulation; and 3 = abundant sporulation. To select the lesions to be evaluated, each leaflet was divided into two areas, following the main central vein. From each subarea, five lesions were randomly marked with a hydrographic pen without the aid of optical instruments to reduce the error and subjectivity of the parameters. The "SL" and " NoU " were evaluated and counted with the aid of a stereomicroscope from 30 lesions per trifoliate (1 rep = 1 trifoliate) for each mutant/isolate combination. The mean comparisons were made by using the software R (R Development Core Team, 2018) and by the Agricolae package (De Mendiburu, 2014).

Results

Optimization of EMS treatment and development of the 'TMG4182' mutant population

Soybean seeds from 'TMG4182' displayed lower germination rates at higher concentrations of EMS (Table 3).

Table 3 - Germination rates of 'TMG4182' seeds after 16 hours soaking in different EMS concentrations.

EMS % (v/v)	Seeds planted	Nº of Germ. seeds	Germination (%)
0	110	89	80.9
0.3	110	63	57.3
0.4	110	42	38.2
0.5	110	26	23.6
0.6	110	30	27.3
0.7	110	10	9.1
0.8	110	1	0.9
1	110	0	0.0

From these results, we calculated the dosage of 0.3% (v/v) to use as the optimum treatment for our bulk EMS mutagenesis of soybean (Figure 3). Presumably, at this concentration, we expect to yield high-frequency mutations and still maintain enough viable seeds to create a mutant collection.

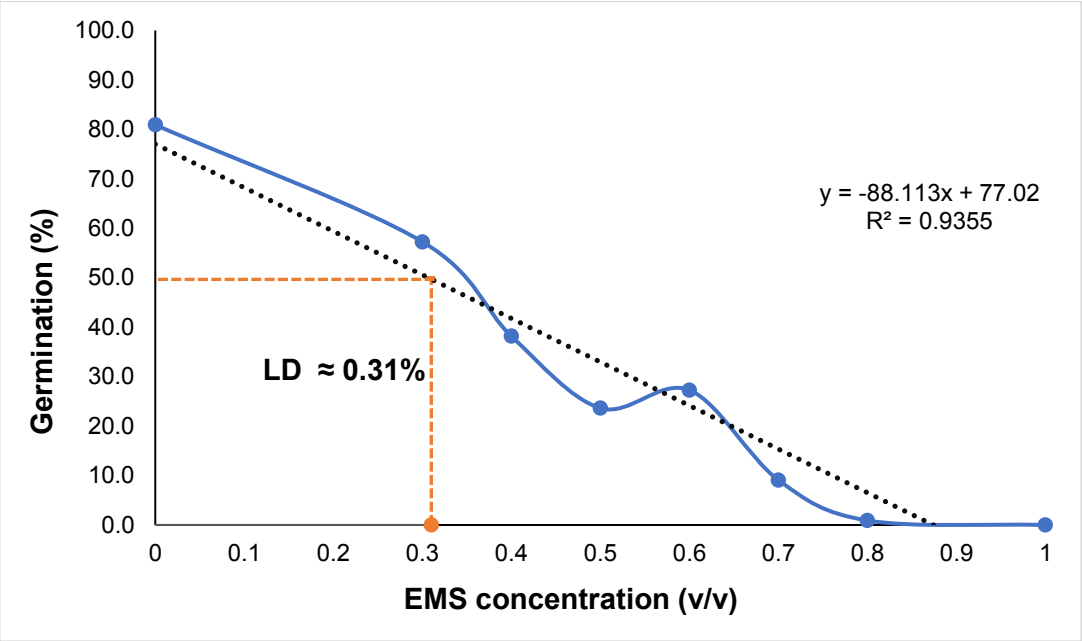

Figure 3 - Germination rates of 'TMG4182' seeds soaked for 16h in different concentrations of EMS. Marked in orange is the concentration of EMS that inhibited 50% of the germination.

Development of EMS mutated 'TMG4182' population

Using the optimal treatment concentration, determined in the first trial, a total of 3,950 M1 were generated from 20,000 treated seeds. In the field, few phenotypic changes were observed including 32 plants with chimeric phenotypes with chlorophyll alterations (Figure 4-A to C), and 19 with changes in leaf texture, such as rugose and tanned leaves (Figure 4-D). We obtained seeds from a total of 3,950 M1 plants (M2 families).

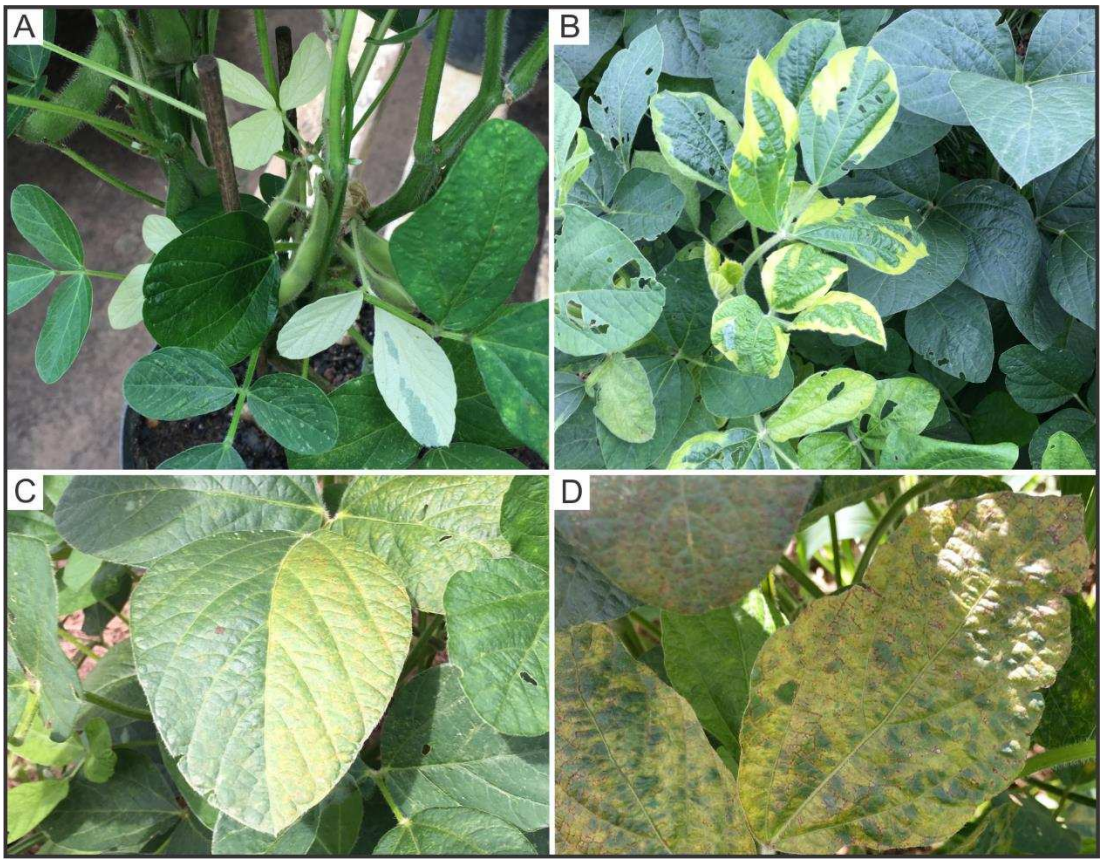

Figure 4 - Overview of plant development and phenotypic changes observed in M1 plants grown in the field. (A), chimeric plant with regular and chlorotic leaves. (B and C), leaves displaying chlorotic sectors. (D), leaf with coriaceous and rugose texture.

Phenotypic variations in the M2 families

We evaluated 1,820 M2 families, a number that corresponds to 46% of all M2 families obtained. The germination rates of the M2 population ranged from 4.5% (1 out of 22 seeds germinated) to 100% (22 planted seeds germinated). The overall germination frequency was 78%; 88% of the families (1,609 M2) had a germination rate above 50%, and 35% (642 M2) above 90% (Figure 5).

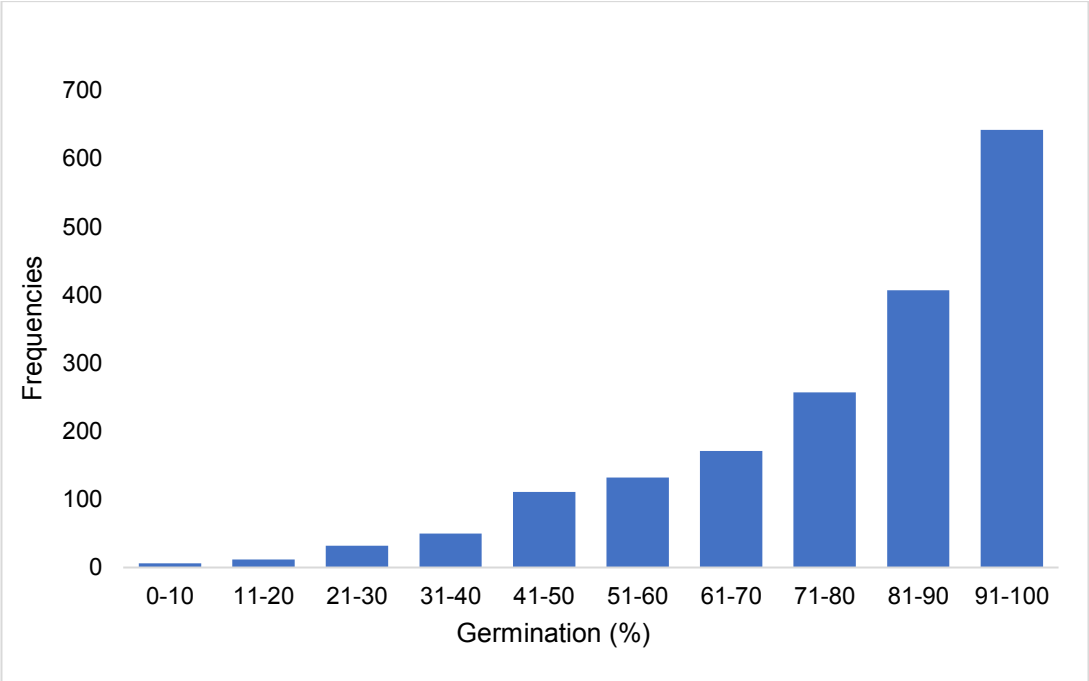

Figure 5 - Frequency distribution of seed germination percentages from 1,820 M2 families. The germination was evaluated ten days after planting.

From 40,040 planted seeds, 31,068 plants were obtained and assessed for phenotypic alterations; 2,976 plants displayed one or more phenotypic variation and 28,092 did not differ from the control non-mutagenized 'TMG4182'. These numbers correspond to 1,156 M2 families with one or more phenotypic alterations and 664 without phenotypic variants.

Abnormal chlorophyll phenotypes including albino, chlorotic and chimeric plants (Figure 6-A to C), altered plant architecture, including compact and dwarf plants (Figure 6-D), stem alterations such as stem-loops (Figure 6-E), and lateral branching (Figure 6-F), were the most notable phenotypic variations observed. Leaf morphology changes were also very common and included curly leaves (Figure 7-A), folded leaves (Figure 7-B) and narrow leaves (Figure 7-B). Necrotic and leaf lesions were also observed in the mutant population (Figure 8-A to D). M2 plants that died after germination were classified as carrying lethal mutations.

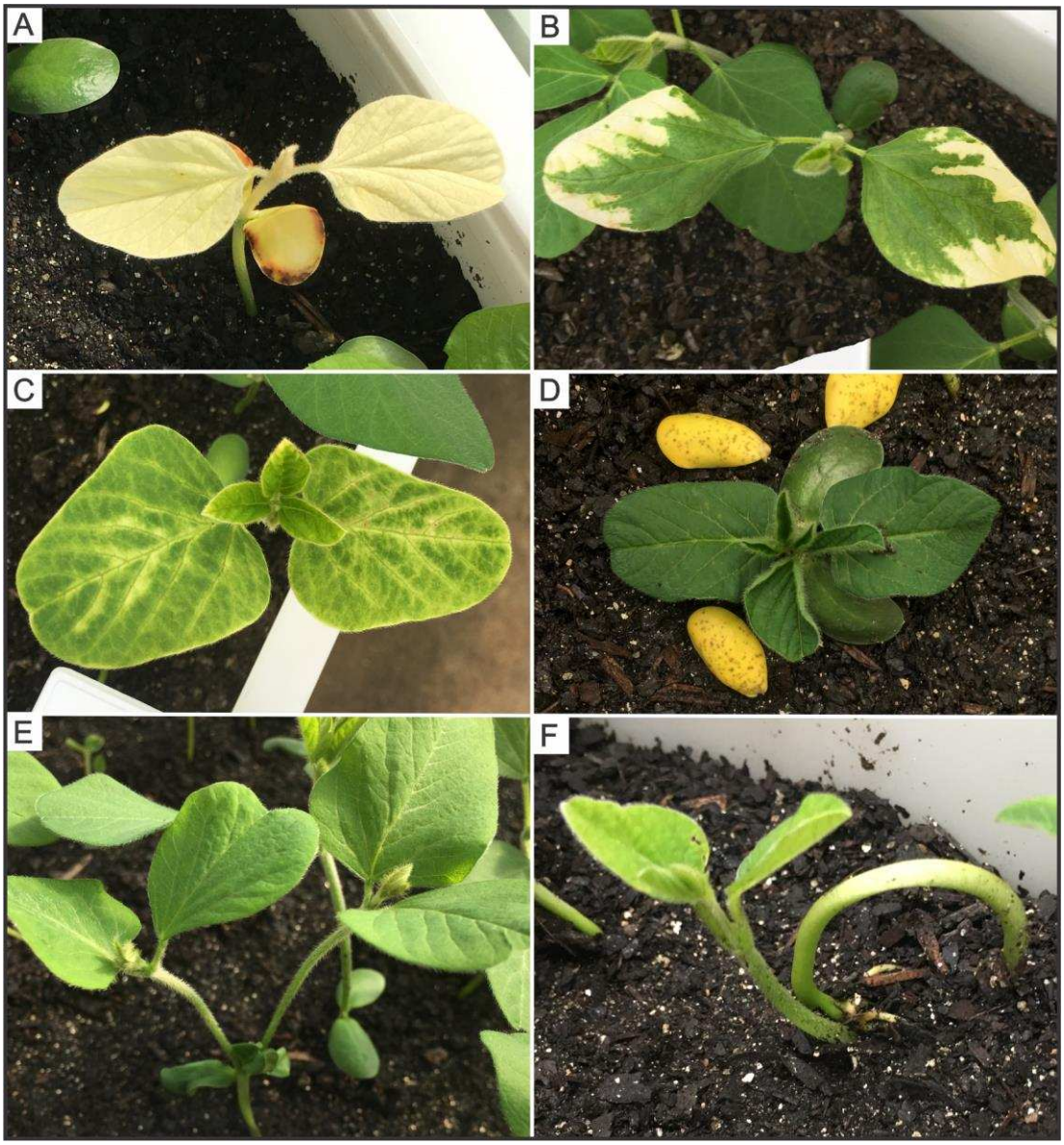

Figure 6 - Different types of phenotypic alterations observed in the M2 population. (A), albino mutation. (B), chlorotic mosaic mutation. (C), chlorotic veins mutation. (D), chlorotic bicolor leaves. Phenotypic alterations in plant architecture and growth observed in M2 plants. (E), dwarf mutation. (F), stem curl abnormality. (G), Lateral branching. (H), lethal mutation.

Figure 7 - Leaf morphology alterations observed in plants from the M2 population screened. (A), Curled leaf. (B), folded leaf. (C) changes in leaf shape (left: 'TMG4182, right: GMMT04-30-745-16).

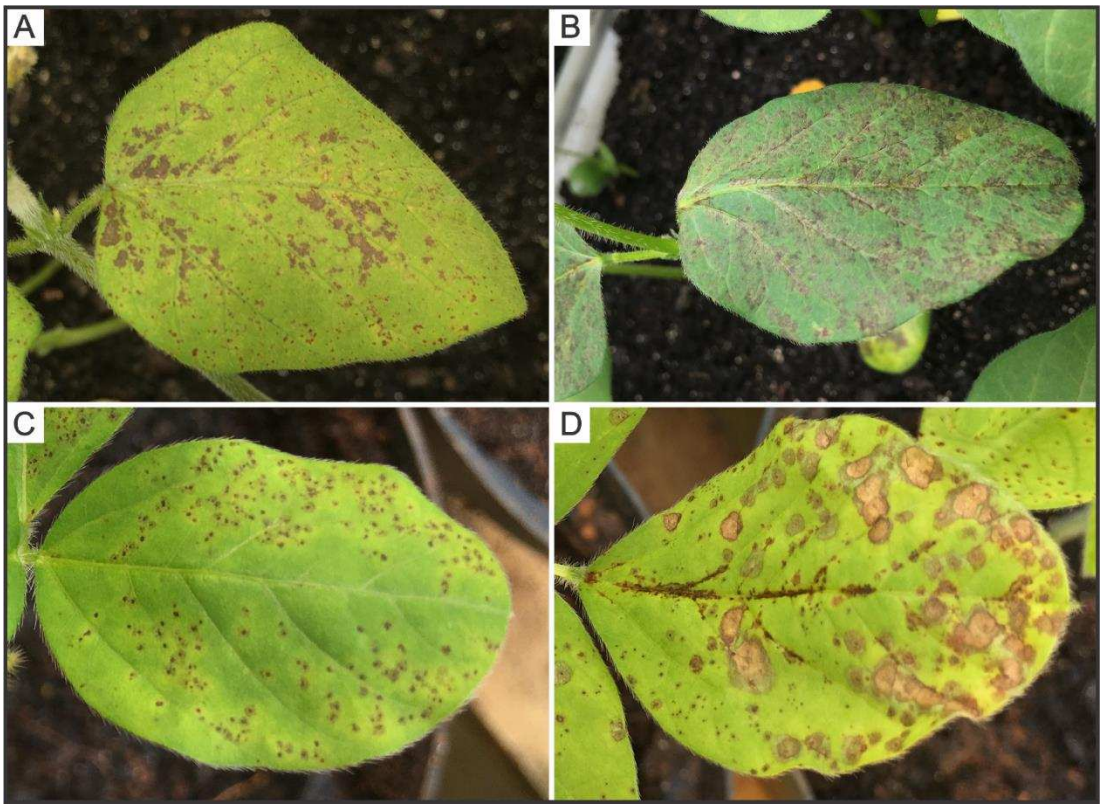

Figure 8 - Different types of necrotic and leaf lesions observed in the M2 population before the inoculation with *P. pachyrhizi* isolate PPUFV-02. (A), GMMT04-30-83-02. (B), GMMT04-30-51-02 (C), GMMT04-30-597-08 (D), GMMT04-30-288-03.

The observed phenotypic variations were divided into four groups: chlorophyll, plant architecture and growth, leaf morphology and lethal mutations (Table 4).

Table 4 - Incidence of different types of phenotypic variation observed in the 31,068 M2 plants.

Phenotypic variants	**No. of mutants**	**Incidence (%)**
Chlorophyll Mutants		
Albino	43	0,14
Chlorotic/chimeric	547	1,76
Plant architecture e growth		
Dwarf	723	2,33
Semi-Dwarf/small	1077	3,47
Thick stem/stem curl	152	0,49
Additional Lateral branching	73	0,23
long internode	3	0,01
Leaf morphology		
Narrow/Curled/Folded	624	2,01
Leaf lesions	20	0,06
Rugose leaves/rough leaves	124	0,40
Necrotic spots	3	0,01
Lethal mutation	54	0,17
Total Phenotypic variant plants*	2976	9,58

Soybean rust reaction screening

All mutant plants were inoculated with *P. pachyrhizi* PPUFV02 and evaluated on a daily basis for any disease symptom development. Two hundred and twenty-one plants that displayed changes in symptom development were selected from 30,068 M2 inoculated mutants. As expected, 99% of all plants tested displayed TAN type lesions with abundant sporulation (Figure 9-A); Eleven M2 plants did not present any ASR symptoms (Figure 9-B); 31 displayed RB-like (Reddish-brown) symptoms (Figure 9-C); 42 displayed necrotic leaf spots and fewer lesions (Figure 9-D); eight with round reddish lesions (Figure 9-E); six with a yellow halo around the ASR lesions (Figure 9-F); 36 were selected for displaying lower than average disease severity or sporulation; three with smaller than the average-size lesions (Figure 9-G); two with white necrotic lesions (Figure 9-H); and one with target spot-like symptoms (Figure 9-I).

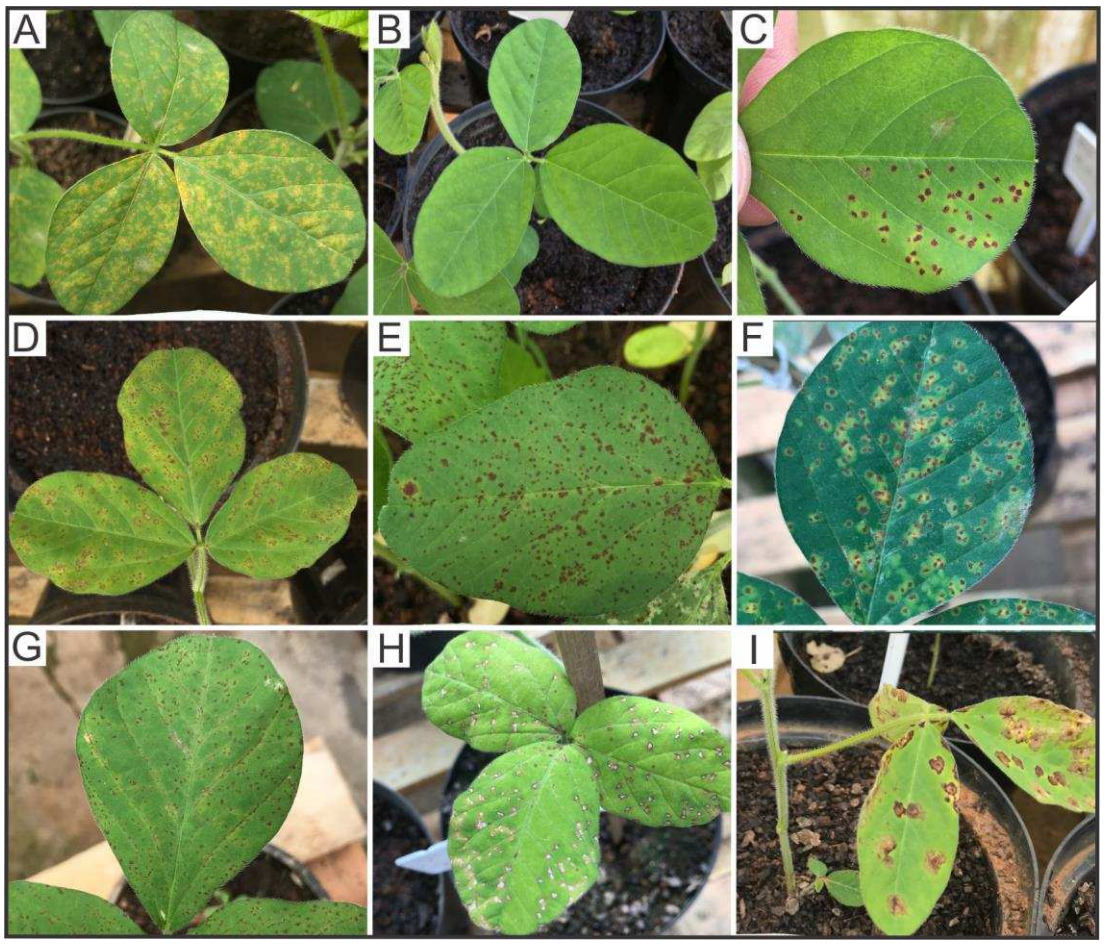

Figure 9 - M2 plants displaying different types of mutant phenotypes 15 days after inoculation with *P. pachyrhizi* PPUFV02. (A), the adaxial face of wild-type 'TMG4182' leaflet displaying TAN lesions. (B), asymptomatic plant GMMT04-30-202-05 leaflet (C), abaxial face of a leaflet from mutant GMMT04-30-504-04 displaying RB (reddish-brown) lesions. (D), necrotic leaf spots on leaflets from

GMMT0-496-01. (E), adaxial face of a leaflet from GMMT04-30-509-20 displaying round reddish spots. (F), yellow halo around lesions observed in GMMT04-30-216-01. (G), GMMT02-50-05-17 plant with small necrotic lesions. (H), GMMT05-30-63-01 mutant displaying necrotic white lesions at the infection sites of *P. pachyrhizi* (I), GMMT05-30-345-17 mutant displaying target spot-like lesion mimic.

Confirmation of mutant ASR phenotypes observed in selected M2 plants

From the 220 M2 mutants selected in the inoculation assay with *P. pachyrhizi*, only 180 produced seeds. Of these, 101 M3 mutant families were chosen for a second inoculation assay to verify if the observed phenotype was maintained in the subsequent generation. These plants were chosen based on the phenotypic variation observed and the number of viable seeds obtained.

All M3 plants from selected asymptomatic M2 families were susceptible in this assay. That the lack of disease symptoms observed in the M2 plants was probably due to inoculation errors and/or escapes. Most M3 families tested did not display the mutant phenotypes of the M2 selected, probably due to the continued segregation of mutations and/or interactions of different mutations (Figure 10).

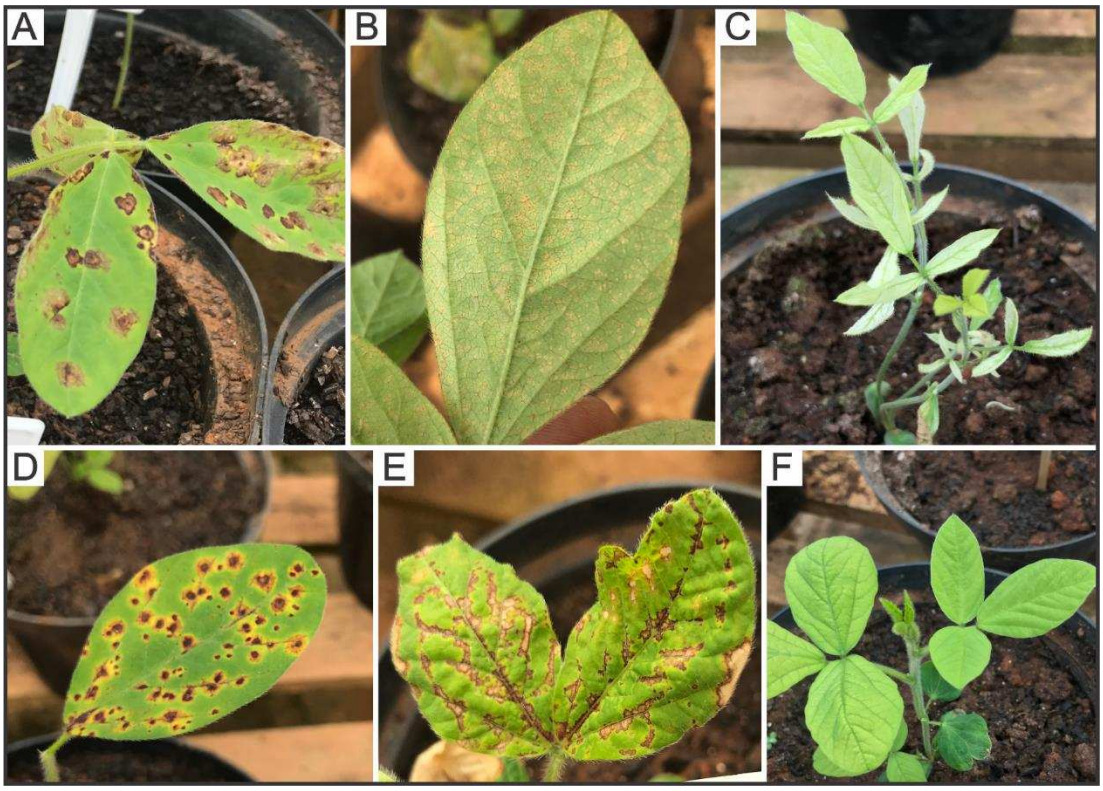

Figure 10 - Altered responses to *Phakopsora pachyrhizi* observed in selected M2 mutants and their M3 progenies, before and after the inoculation with *P. pachyrhizi* PPUFV02. (A), M2 mutant GMMT05-30-345-17 selected for displaying target spot

lesions. (B), M3 progeny from GMMT05-30-345-17 displaying TAN lesions ten days after the inoculation. (C), M3 plant from the GMMT05-30-345-17 family with alterations in plant architecture and chlorotic leaves. (D), Leaflet from M2 mutant GMMT05-30-862-14 displaying a yellow halo around a large reddish lesion. (E), Leaflet from one plant from GMMT05-30-862-14 M3 progeny displaying systemic necrosis before the inoculation with *P. pachyrhizi* PPUFV02. (F), M3 lefaflet from one plant the GMMT05-30-862-14 family with mild chlorosis without necrotic lesions.

Based on this assay, seven M2 mutant families were selected for further study. These families were selected since all M3 plants from these families displayed phenotypic alterations consistent with those observed in their respective M2 family. The selected M2 families were: GMMT02-05-50-17, GMMT04-30-496-01, GMMT04-30-504-04, GMMT04-30-509-10, GMMT04-30-509-10, GMMT05-30-362-16 and GMMT05-30-754-16 (Figure 11).

All seven selected families displayed ASR lesions with spore production, but these reactions differed among these mutants. M2 mutants GMMT04-30-509-10, GMMT04-30-509-20, GMMT04-30-504-04 and GMMT05-30-745-16, showed an RB reaction, but it was stronger in mutants GMMT04-30-509-10, GMMT04-30-509-20, and GMMT04-30-504-04, where sporulation was also lower. The plants from GMMT05-30-362-16 displayed low amounts of sporulation and entered senescence two weeks earlier than the wild type ‘TMG4182’. M3 plants from GMMT02-50-05-17 and GMMT04-30-496-01 families had the lowest disease severity, presenting necrotic spots, fewer uredia and low sporulation (Figure 11).

Other phenotypic alterations were also observed in the selected M2 plants. Mutant GMMT05-30-745-16 had an overall change in plant architecture, with additional lateral branching and small lanceolate leaves; GMMT05-30-362-16 as mentioned before displayed leaf senescence two weeks earlier than WT ‘TMG4182’ (Figure 11).

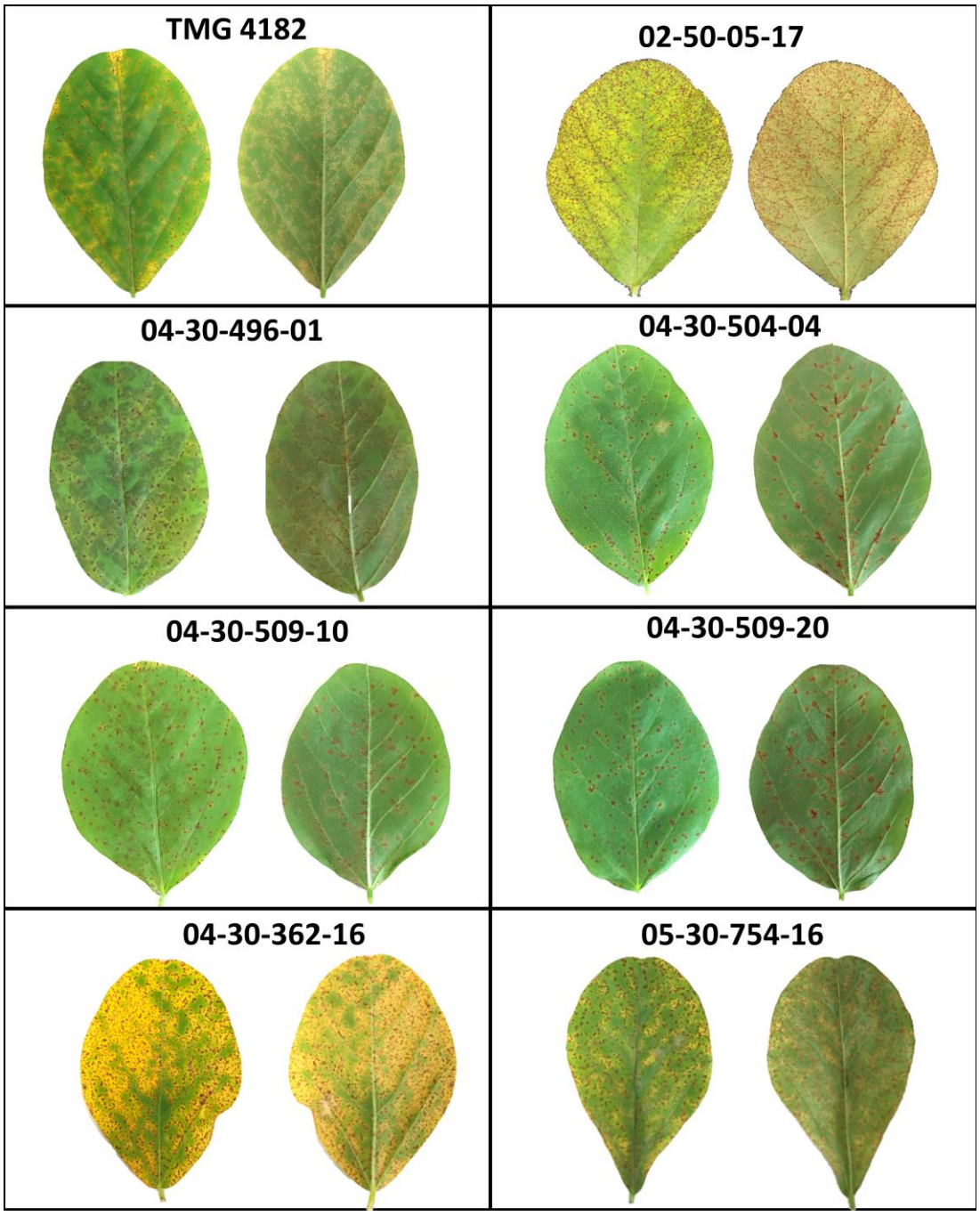

Figure 11 - Symptoms observed in leaflets from 'TMG4182'and from selected M3 mutants 15 days after the inoculation with $2.0x10^4$ spores/ml of *P. pachyrhizi* PPUFV02 isolate. The illustration shows the abaxial (A), and adaxial (B) faces of the leaflets.

Mutant families GMMT02-50-05-17 and GMMT04-30-496-01, presented regular plant development in the first three weeks after planting. However, after three weeks, they began to show necrotic spots, systemic necrosis, and leaf senescence (Figure 12). In the GMMT02-50-05-17 families, these necrotic spots resembled

symptoms that are caused by *P. pachyrhizi* (Figure 12-A and B). It's important to note that these symptoms occurred before the inoculation with *P. pachyrhizi*.

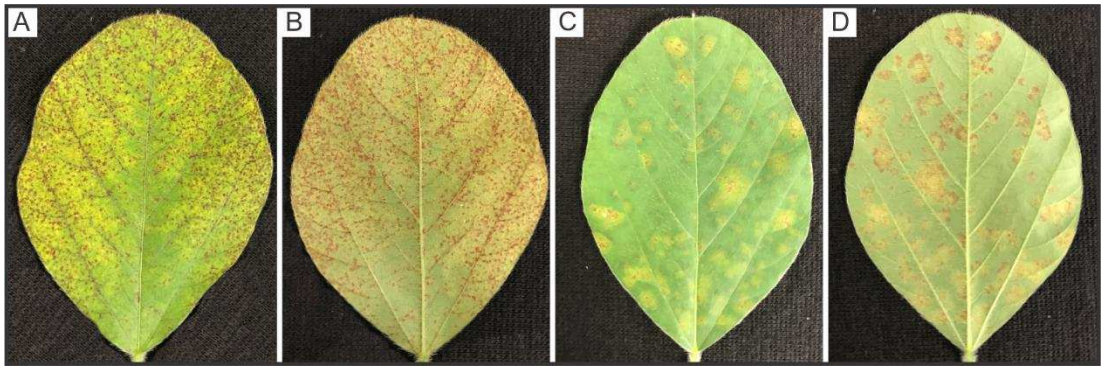

Figure 12 - Non-inoculated M3 mutants displaying necrotic lesions 30 days after planting. (A and B), adaxial and abaxial faces of GMMT02-50-05-17 leaflet displaying small necrotic lesions that mimics ASR symptoms. (C and D), adaxial and abaxial faces of GMMT04-30-496-01 leaflets displaying large round necrotic spots.

Soybean plants kept in greenhouse conditions displayed natural occurring infections of powdery mildew (*Microsphaera diffusa)*. In this condition, we observed that mutant families GMMT02-50-05-17 and GMMT04-30-496-01 were less susceptible to natural occurring powdery mildew (Figure 13). Since *M. diffusa* is also a biotrophic pathogen, the disease resistance mechanisms observed in these mutants for *M. diffusa* and *P. pachyrhizi* could be linked.

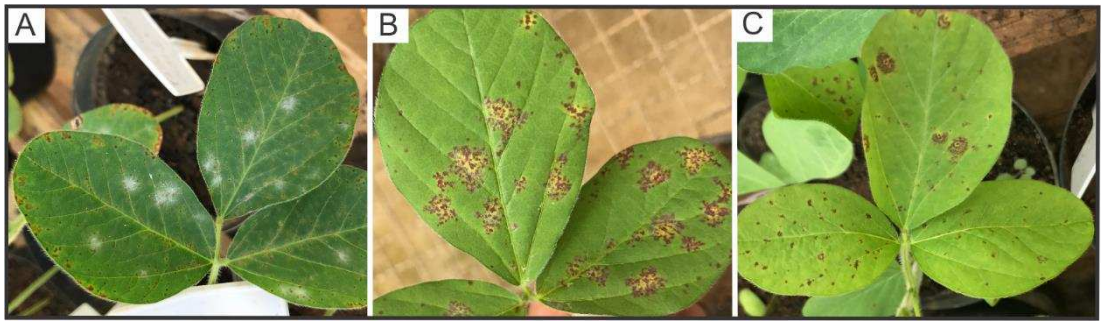

Figure 13 - M2 plants displaying different reactions to the powdery mildew caused by *Microsphaera diffusa.* (A), Mutant plant GMMT04-30-253-02 displaying white powdery patches of fungal mycelium and conidia on the upper surface of the soybean leaves. (B), GMMT02-50-05-17 displaying a necrotic reaction on the infection sites of PM without the production of fungal mycelium on the surface of the leaflet. (C), 04-30-496-01 displaying necrotic reactions with no sign of PM mycelium.

A complete description of the selected M2 plants is listed in Table 5.

Table 5 - Selected M2 plants that displayed different disease symptoms development after inoculation with *P. pachyrhizi* PPUFV02, when compared to wild-type 'TMG4182'

M2 Family	Plant	Selection reason	Seeds	ASR lesions/sporulation	Adult Plant - Phenotypic alterations	ASR symptoms and other diseases reaction
02-50-05	17	Small necrotic spots on leaves, little sporulation	53	Yes/Yes	Senescent leaves, necrotic spots resembling ASR symptoms (lesion mimicry)	Necrotic spots, little sporulation (Disease Lesion Mimic).Resistant to powdery mildew
04-30-496	1	Necrotic spots, little sporulation	26	Yes/Yes	Systemic necrosis, smaller than control 'TMG4182'	Necrotic spots, very little uredia with fewer uredospores, circular lesions on the leaves. Resistant to powdery mildew
04-30-504	4	RB, red spots around lesions, little sporulation	100	Yes/Yes	Non-inoculated plants display small red spots on cotyledonal leaves	RB-like symptoms, little sporulation
04-30-509	10	RB, red spots around lesions, little sporulation	107	Yes/Yes	Non-inoculated plants display small red spots on cotyledonal leaves	RB-like symptoms, little sporulation
04-30-509	20	RB, red spots around lesions, little sporulation	79	Yes/Yes	Non-inoculated plants display small red spots on cotyledonal leaves	RB-like symptoms, little sporulation
05-30-362	16	Reddish spots and leaf senescence	30	Yes/Yes	Small senescent plant, small seeds	Little sporulation, infected leaves may fall before fungal dispersal
05-30-745	16	RB	69	Yes/Yes	Additional lateral branching, small lanceolate leaves	RB-like symptoms, abundant sporulation

Qualitative and quantitative characterization of ASR resistant mutants

The selected mutants GMMT04-30-745-16, GMMT04-30-362-16, GMMT04-30-504-04, GMMT04-30-509-20, GMMT02-50-05-17, and GMMT04-30-496-01, along with WT 'Williams82' and 'TMG4182', were inoculated with monouredinal isolates PR8.4, RS2.4 and PR183.2 to verify if the development of ASR symptoms would vary from our control isolate PPUFV02. However, all isolates caused disease symptom development similar to those observed with *P. pachyrhizi* PPUFV02 (Table 6), indicating that the phenotypic reactions observed might not be isolate-specific.

Table 6 - Reaction type of selected M3 mutants and wild-type control, observed 15 days after the inoculation with four different monouredinal isolates of *P. pachyrhizi.*

Phenotypes	Reaction type/symptoms[a]
Williams 82	TAN
TMG4182	TAN
GMMT04-30-745-16	Weak RB
GMMT04-30-362-16	Weak RB - yellow halo around lesion and leaf senescence
GMMT04-30-504-04	RB - yellow halo around the lesion
GMMT04-30-509-20	RB - yellow halo around the lesion
GMMT04-30-05-17	Necrotic spots (RB)
GMMT04-30-496-01	Necrotic spots

[a] Reaction type (lesion color) and symptoms observed 15 days after the inoculation

The mean number of open uredia per lesion was analyzed by one-way ANOVA and Tukey HSD test ($p < 0.05$) while sporulation intensity calculated using the Kruskal-Wallis test for non-parametric data ($p < 0.05$) to compare the disease development in the mutant families and WT phenotypes. Treatments with the monouredinal isolates PPUFV02, RS2.4, and PR8.4, were significantly different ($p < 0.05$) among the mutant and WT phenotypes, for the mean number of open uredia (Table 7), and for sporulation intensity (Table 8). Treatments with monouredinal isolate PR183.2 did not differ between the mutant and WT phenotypes (Table 7 and Table 8). In this treatment the overall uredia count was below three uredia per lesion in all treatments, indicating that the disease progression was slower when compared to other treatments. Mean comparison of closed uredia was not significant in any of the treatments.

Table 7 - Mean number of open uredia, in mutant and WT phenotypes, 15 days after the inoculation with four different monouredinal isolates. Groups with the same letter are not significantly different ($P < 0.05$).

PPUFV02		
Plants	**Mean Number of Open Uredia**	**Groups**
TMG4182	4.95	a
Williams82	4.09	ab
GMMT-745-16	3.88	abc
GMMT-504-04	3.01	abcd
GMMT-362-16	2.75	bcd
GMMT-509-20	2.61	bcd
GMMT-05-17	1.82	cd
GMMT-496-01	1.49	d

PR183.2		
Plants	**Mean Number of Open Uredia**	**Groups**
TMG4182	2.82	a
Williams82	2.76	a
GMMT-509-20	2.34	a
GMMT-362-16	2.10	a
GMMT-504-04	2.09	a
GMMT-745-16	2.09	a
GMMT-05-17	1.86	a
GMMT-496-01	1.69	a

PR8.4		
Plants	**Mean Number of Open Uredia**	**Groups**
Williams82	3.44	a
TMG4182	2.97	ab
GMMT-509-20	2.76	abc
GMMT-362-16	2.46	abc
GMMT-504-04	2.26	bc
GMMT-745-16	2.08	bc
GMMT-05-17	1.81	c
GMMT-496-01	1.77	c

RS2.4		
Plants	**Mean Number of Open Uredia**	**Groups**
TMG4182	4.07	a
Williams82	3.79	a
GMMT-745-16	3.37	ab
GMMT-504-04	2.69	ab
GMMT-509-20	2.68	ab
GMMT-362-16	2.61	ab
GMMT-496-01	2.06	ab
GMMT-05-17	1.31	b

Table 8 - Mean sporulation intensity evaluated using a number scale. (0 = no sporulation, 1 = little sporulation, 2 = moderate sporulation, 3 = abundant sporulation), in mutant and WT phenotypes, 15 days after the inoculation with four different monouredinal isolates. Groups with the same letter are not significantly different ($P < 0.05$).

PPUFV02		
Plants	**Mean Sporulation intensity (0 to 3)**	**Groups**
Williams82	2.74	a
TMG4182	2.70	a
GMMT-745-16	2.52	a
GMMT-504-04	2.09	ab
GMMT-509-20	1.68	bc
GMMT-362-16	1.59	bc
GMMT-05-17	1.27	bc
GMMT-496-01	0.96	c

PR183.2		
Plants	**Mean Sporulation intensity (0 to 3)**	**Groups**
GMMT-05-17	1.00	a
GMMT-362-16	1.33	a
GMMT-496-01	1.00	a
GMMT-504-04	1.33	a
GMMT-509-20	1.33	a
GMMT-745-16	1.33	a
TMG4182	1.67	a
Williams82	2.33	a

PR8.4		
Plants	**Mean Sporulation intensity (0 to 3)**	**Groups**
GMMT-509-20	2.67	a
Williams82	2.67	a
TMG4182	2.33	ab
GMMT-504-04	2.00	ab
GMMT-745-16	2.00	ab
GMMT-362-16	1.67	bc
GMMT-05-17	1.00	c
GMMT-496-01	1.00	c

RS2.4		
Plants	**Mean Sporulation intensity (0 to 3)**	**Groups**
Williams82	2.94	a
TMG4182	2.58	ab
GMMT-745-16	2.44	ab
GMMT-509-20	1.92	bc
GMMT-496-01	1.38	cd
GMMT-362-16	1.33	d
GMMT-504-04	1.15	d
GMMT-05-17	0.76	d

The variation in and among the treatments was high, but in general, mutant families GMMT04-30-496-01 and GMMT02-50-17-05 had the lowest numbers of open uredia per lesion when inoculated with all four monouredinal isolates. GMMT04-30-496-01 varied from less than 1.4 in PPUFV02 to 2.0 open uredia per lesion in RS2.4 (Table 7) and GMMT02-50-17 from 1.3 in RS2.4 to 1.85 in PR183.2 (Table 7). The sporulation intensity was also lower in those two mutants for all four treatments, with scores ranging from 0.5 to 1.2 (Table 8). Wild type 'TMG4182' and Williams82' both shared higher numbers of open uredia per lesion, being the highest for 'TMG4182' (4.9) in the inoculation with PPUFV02 (Table 7). Sporulation intensity scores were also high for these two WT phenotypes being highest for 'Williams82' ranging from 2.3 in PR183.2 to 2.9 in RS2.4 (Table 8).

Discussion

Using EMS treatment, we have successfully created a large mutagenized soybean population that carried a suitable density of mutations. This genetic variability was confirmed through the high number of observed phenotypic alterations in the M1 plant progenies. Therefore, this population can be used to find new alleles and phenotypes making it an important resource to identify genes related to traits such as Asian soybean resistance or susceptibility.

In this study, our interest was focused on phenotypic alterations of disease development after inoculation with *Phakopsora pachyrhizi.* Although we have not recovered soybean mutants completely immune to *P. pachyrhizi* PPUFV02, we have retrieved 'TMG4182' mutant phenotypes with alterations in ASR disease development from this collection. These phenotypes are a valuable resource that could be employed in breeding programmes in combination with different resistant genes and also to elucidate and identify disease-related genes.

The success of a mutagenesis experiment depends on the efficiency of the mutagen used (Arisha *et al.*, 2015). Mutagenic agents have different effects according to the concentration and material treated. The chemical mutagen methanesulfonate has already

been efficiently applied in several soybean mutagenesis studies (Akao & Kouchi, 1992; Meksem *et al.*, 2008; Tsuda *et al.*, 2015). It is important to note that EMS, at higher doses, generally produces a higher frequency of mutations (Espina *et al.*, 2018). However, a high frequency of mutations may be detrimental to the genome, significantly reducing the survival rate of the mutants (Shah *et al.*, 2015). Thus, in order to guarantee a high frequency of mutations as well as a satisfactory number of viable seeds, it is recommended to carry out tests with different dosages of the mutagen in order to calculate the lethal dose for 50% of the seeds, LD50, which can be used as an optimum concentration for a bulk treatment (Arisha *et al.*, 2015). In the present study the calculated LD50 (where 50% of the seeds were viable), was approximately 0.3% (v/v), close to 0.35% (v/v), used in another mutagenesis by Tsuda *et al.* (2015) to obtain a population of soybean with high-frequency mutants.

Twenty thousand seeds were treated to generate a bulk mutant population of 10,000 to 11,000 adult M1 plants. However, our yield was below the expected, and only 3,950 M1 plants were individually harvested, equivalent to 20% of all treated seeds. Plants might have been lost due to lethal mutation expressed after germination or uncontrolled biotic and abiotic field conditions. This loss, observed in the number of M1 individuals was also verified in the soybean mutagenesis study using EMS in which 10,000 soybean seeds were treated with 0.35% (v/v) EMS obtaining ~ 2,000 M1 individuals, equivalent to 20%. (Tsuda *et al.*, 2015). It should be noted that some plants in the field reached maturity, but did not produce seeds. Sterility or nonproducing pods soybean mutants are phenotypic alterations frequently in mutagenesis studies (Li *et al.*, 2017; Espina *et al.*, 2018). An increase in sterility has been found with the increment of the EMS or gamma rays dosages (Patil & Wakode, 2011).

The frequency of phenotypic changes in M1 plants was low, which was expected since only dominant or cytoplasmatic mutations could be identified in this generation. Indeed chlorophyll mutants, chimeric plants, changes in leaf morphology and texture, and sterility was observed under field conditions. The occurrence of these phenotypes was a preliminary indication of the efficacy of EMS treatment. Other authors, e.g., Arisha *et al.* (2014) have used these phenotypes to estimate the optimum EMS dosage while Pino-

Nunes *et al.* (2008) used the fertility rate of M1 tomato plants producing viable seeds as a parameter to evaluate EMS mutagenic effectiveness.

The chance to observe visual phenotypic changes in the M2 generation is higher due to the segregation of recessive mutations (Page & Grossniklaus, 2002), allowing a better evaluation of the EMS treatment effect. In total, 31,068 plants were evaluated from 1,820 M2 families. The average germination rate was only 79% and may be justified by the fact that recessive lethal alleles entered in homozygosis during M2 seed formation (Espina *et al.*, 2018). As pointed by Meinke and Sussex (1979) lethal mutations caused by single recessive mutations are expected to segregate in a Mendelian ratio of 1:3 (mutant: wild type phenotypes). On the count of that, M2 families carrying recessive alleles are likely to display around 25% decrease in seed germination due to lethality.

Despite this, the overall germination frequency was satisfactory: 88% of the M2 had a germination rate higher than 50%. In a mutagenesis study conducted by Espina *et al.* (2018), the germination rates of M2 soybean seeds were considerably lower: only 2% of the M1 families had more than 61% viable seeds, probably due to the higher dosage (0.62% (v/v) of EMS.

Our choice to use the 0.30% v/v of EMS was conservative in order to get a trade-off between mutation frequency and the number of viable plants as higher dosages of EMS could have compromised the number of M1 plants for the disease screening. We efficiently screened 1,820 M2 families. However, if a higher concentration of EMS had been employed, perhaps a more significant number of mutations could have been screened with the same number of individual families tested.

Although the germination rates were high, it was possible to obtain a high frequency of mutations. It was observed that 1,156 of 1,820 M2, equivalent to 63% of the families, had at least one plant with one or more phenotypic alterations. A total of 2,976 plants with one or more altered phenotypic characteristics averaged to 9% of the total plants.

Phenotypic changes like dwarfism and albinism are very common throughout mutant populations. These alterations are fairly easy to visualize due to the contrast between these plants and plants with no phenotypic alterations. In this study, a total of 723 dwarfs and 43 albino plants, which correspond to 2.33 and 0.14%, respectively of the

total of plants evaluated, respectively. Espina *et al.* (2018) evaluated 6,400 M2 individuals and found 50 dwarfs (0.75%) and 14 albino plants. Tsuda *et al.* (2015) observed 21 dwarfs (2.8%) and 76 albino plants (4.4%) in a mutagenized population of 1,732 plants. Although these findings may vary from one study to another, they are clues of the effectiveness of each mutational treatment and indicate the success of the mutagenesis treatment.

Mutant families GMMT02-50-05-17 and GMMT04-30-496-01 displayed enhanced resistance to both Asian soybean rust and the naturally occurring powdery mildew caused by *Microsphaera diffusa,* when compared to wild-type TMG4182. Campbell *et al.* (2012) describe the wheat mutant MNR220, derived from an EMS mutant collection that exhibited resistance to the powdery mildew caused by *Blumeria graminis* f. sp *tritici* and to rusts caused by *Puccinia triticina*, *P. graminis* f. sp. *tritici*, and *P. striiformis* f. sp. *tritici*. Genetic studies demonstrated that this resistance was conferred by a single semi-dominant gene. In this mutant, defense responses were altered, leading to an overall higher basal defense to these biotrophic fungi. Rice *spl11* mutants were also resistant to both *Magnaporthe grisea* and *X. oryzae* pv. *oryzae* (Zeng *et al.*, 2002). In Arabdopsis, *dnd1* (Clough *et al.*, 2000) and *edr1* (Frye & Innes, 1998) both confer resistance to more than one pathogen including viral, fungal and bacterial pathogens. All these reports support also the possibility of obtaining single mutation events that could confer enhanced resistance to more than one disease (Zhang *et al.*, 2003).

Mutant family GMMT02-50-05-17 also displayed lesions that are normally observed in ASR resistant genotypes: small necrotic spots on the leaves, which eventually undergo senescence, despite the absence of any detectable pathogen. These types of mutants are described as lesion mimic mutants (LMMs). LMMs naturally exhibit unregulated local cell death that resembles a necrotic lesion caused by pathogens, even in the absence of a pathogen (Moeder & Yoshioka, 2008). It is believed that these necrotic injuries may be caused by mutations in genes involved in the programmed cell death (Huysmans et al., 2017) and pathogen resistance pathways (Hu et al., 1998; Lorrain et al., 2003; Moeder & Yoshioka, 2008). As observed in mutant GMMT02-50-05-17 these lesions may have an age-related development, because in this mutant they were observed two to three weeks after planting in older leaves and then progressed to upper younger leaves. Chung *et al.* (1998) observed a similar trend in a *dlm* (disease lesion mimic)

soybean mutant, in which the leaves became more necrotic and chlorotic as they aged, eventually undergoing an earlier than normal leaf senescence.

Over the last twenty years, a substantial number of lesion-mimicking mutants (LMM) were isolated and an increasing number of associated genes cloned in *Arabidopsis*, rice, maize and barley (Lorrain *et al.*, 2003). Few LMMs have already been characterized in soybean and the corresponding genes have not yet been cloned. Moreover, little information is available about the molecular mechanism underlying the lesion mimics development as well as how PCD is regulated. T363 was the first LMM soybean mutant characterized as a *dlm* (disease-lesion mimic). This mutant displayed small necrotic spots surrounded by chlorotic halos on leaves (Chung *et al.*, 1998). Later, the *dlm* phenotype was found to be light-dependent and associated with chloroplast dysfunction (Kim *et al.*, 2005). Al Amin *et al.* (2019) describe the *dlm* mutant *spl-1,* derived from soybean cultivar NN11382-0 treated with ethyl methanesulfonate (EMS). In plants carrying this recessive mutation, the formation of the necrotic lesions was associated with PCD and reactive oxygen species (ROS) overaccumulation.

LMMs have been associated with disease resistance. In rice, at least 37 out of 49 LMMs have been reported to show enhanced resistance to at least one pathogen (Huang *et al.*, 2011). In these mutants, over-expression of plant defense-related genes that led to improved disease resistance was observed (Takahashi *et al.*, 1999; Mackintosh *et al.*, 2007). In wheat, *lm3* displayed improved resistance to wheat powdery mildew (*Blumeria graminis* f. sp. *tritici*) infection, which was accompanied by the increased in the expression of seven pathogenesis-related (PR) and two chemically induced (WCI) genes involved in the defense-related reactions (Wang, Wu*, et al.*, 2016).

From a total of 220 mutant plants selected in the M2 population, 101 were reevaluated for disease resistance. All eleven asymptomatic plants, selected in the first screening were susceptible to ASR when reevaluated. Probably the absence of disease in these plants occurred due to escape during our greenhouse and tray inoculation procedure. It was observed that these were generally smaller than the neighboring plants and located at the corner of the tray. Nevertheless, the very low frequency of these escapes demonstrates the efficiency of our screening procedure.

Although there a high pathotypic variation in *P. pachyrhizi* populations (Bromfield *et al.*, 1980; Akamatsu *et al.*, 2017), we decide to use only one *P. pachyrhizi* isolate (PPUFV02) in the screening process. Due to the specific host-pathogen interactions, screening using a different *P. pachyrhizi* isolate may result in different reactions in this same mutant population.

Dominant soy rust resistance responses are divided into (i) immune response (IR); (ii) partial resistance, reddish-brown lesions (RB); or (iii) susceptible, tanned lesions (TAN) (Bromfield, 1984; Miles *et al.*, 2011). These qualifications are further divided according to the sporulation level of the lesion, being RB divided into three subclasses, and TAN, in two, characterized by "few" or "many" uredia (Bromfield, 1984; Miles *et al.*, 2011). The authors pointed out that these lesions color may range from light to dark brown and, therefore, the color of the lesion by itself may not be a reliable means to assess resistance or susceptibility to ASR (Bonde *et al.*, 2006; Yamanaka *et al.*, 2010; Miles *et al.*, 2011).

So far, the IR has been conferred only by the *Rpp1* gene, originating from soybean accession PI 200492, and the immunity response is specific to a few *P. pachyrhizi* isolates. None of the soybean mutants used in the present study produced an IR type response to any of the four isolates tested. Also, the reaction types IR, TAN, and RB were not sufficient to assess the disease symptom development of all mutants , due to the wide range of observed phenotypes which did not meet these specifications like the ones observed in 04-30-496-01. This mutant had neither a TAN nor RB phenotype. Instead, it displayed necrotic lesions with very few or no uredia (Figure 13). These symptoms may, for example, be associated with resistance mediated by recessive genes that can display different disease development phenotypes, alternative to those proposed by Bromfield (1984).

Rust severity was also not associated with lesion type. For example, mutants that had RB type lesions, GMMT04-30-504-04 and GMMT04-30-509-20 had higher uredia counts and sporulation intensity ratings than GMMT04-30-496-01 and GMMT02-50-05-17, which were among the mutants with the lowest severity ratings. However, there was a positive correlation between the number of open uredia and sporulation intensity; mutant genotypes with a higher number of open uredia also displayed more intense sporulation.

Many plants in the first screening with an RB phenotype may have been mistakenly selected due to the subtle difference between the RB and TAN reactions related to the age of the lesion (Bromfield *et al.*, 1980). It was observed that older ASR lesions in WT 'TMG4182' were darker. Therefore, may be possible, to some extent, this susceptible lesion was classified as RB resistant reaction.

Moreover, due to the large number of plants screened, all of the M2 evaluations were done qualitatively by the naked eye. In this way, leaf spots, necrotic lesions, and RB type reactions were easier to detect. Quantitative and subtle changes such as reduced sporulation and lesion diameter might have been ignored because they are not easy to score visually.

It is important to note that the yield of the selected mutants has not been measured at any instance. These mutants carry multiple genomic mutations that will continue to segregate and interact in their progeny causing innumerous pleiotropic effects that alter overall plant development and consequently yield. Purification of selected mutant lines through backcrosses with wild-type cultivar 'TMG4182 is required to purge unwanted mutations and develop stable and homozygous lines.

Plant defense is also tightly integrated into pathways that regulate growth and development. Therefore, mutations in genes related to defense networks often have numerous pleiotropic effects (Brown & Rant, 2013). The increment in disease resistance can be negatively associated with another desirable trait, such as yield, quality or other traits related to the agronomic performance of a cultivar (Foulkes *et al.*, 2007). The mutant allele *mlo* in barley confers strong and durable resistance to the mildew and it is still used as a source of resistance for breeding programs (Jørgensen, 1992; Büschges *et al.*, 1997). Genetic engineering using the CRISPR/cas9 technology has been successfully applied to knock out the *Mlo* gene in tomato (Nekrasov *et al.*, 2017), and grapevine (Pessina *et al.*, 2016), providing durable resistance to powdery mildew in both species. However, the use of *mlo* resistance alleles in barley breeding is limited because of a yield penalty ranging from 5 to 15% of loss (Bjørnstad & Aastveit, 1990; Jørgensen, 1992). This reduction occurs due to a pleiotropic effect of the *mlo,* that's associated with the spontaneous necrotic flecking on barley leaves that negatively correlates with grain size and total gain yield (Kjær *et al.*, 1990). However, this broad-spectrum disease resistance

is still highly valuable for breeders, and its benefit outweighs the undesirable effects (Brown & Rant, 2013).

Chapter 2 - Genetic and molecular characterization of three EMS-mutants from soybean 'TMG4182' with enhanced resistance to *Phakopsora pachyrhizi*

Abstract

From an ethyl methanesulfonate (EMS) soybean mutant collection three mutants with altered response to *Phakopsora pachyrhizi* were selected. The goal of this work was to develop segregant populations from these selected mutants to determine the inheritance pattern of these mutant phenotypes and to identify genes underlying these mutations. The F2 segregation analysis indicated that recessive alleles determine the phenotypes in all three mutants. The whole-genome resequencing and sequence analysis of these individuals revealed a mutation frequency of approximately one variant per 45,647 bases, in which about 50% were base transitions from C to T and G to A, which are the types of nucleotide changes commonly observed in EMS mutagenesis studies. A total of 1,132 nonsynonymous SNPs were detected in a broad variety of protein-coding genes. Derived cleaved amplified polymorphic sequences (dCAPS) assays confirmed four identified SNPs by using a bioinformatic pipeline with different filters. Bulk segregant analysis coupled with MutMap will be used in the future to identify the SNPs responsible for the observed phenotypes.

Keywords: *Glycine max*; Asian Soybean Rust; Next Generation Sequencing.

Introduction

Soybean rust caused by the biotrophic fungus *Phakopsora pachyrhizi* is one of the most destructive foliar diseases of the soybean. Yield losses over 50% are typical when the environmental conditions are conducive for the disease development (Yorinori *et al.*, 2005; Scherm *et al.*, 2009; Hartman *et al.*, 2015). The disease is ubiquitously present in the soybean growing areas of Brazil, which is the second largest producer, contributing with 30% of the world's soybean production (EMBRAPA, 2018). In Brazil, 30% yield losses were observed in 2018 in uncontrolled fields due to ASR (Godoy *et al.*, 2018).

Genetic resistance to ASR in soybean was identified and mapped to seven different loci, named *Rpp1* to *Rpp7* (Hyten *et al.*, 2007; Garcia *et al.*, 2008; Silva *et al.*, 2008; Hyten *et al.*, 2009; Kim *et al.*, 2012; Li *et al.*, 2012; Childs *et al.*, 2018). Different alleles displaying different responses to selected *P. pachyrhizi* isolates were also mapped to previously reported *Rpp* loci (Garcia *et al.*, 2008; Chakraborty *et al.*, 2009; Ray *et al.*, 2011; King *et al.*, 2016; King *et al.*, 2017). These resistance genes provide isolate-specific and not durable resistance, under field conditions (Yorinori *et al.*, 2005; Akamatsu *et al.*, 2013; Paul *et al.*, 2013). Therefore, there is a need for finding new strategies or new genes that could overcome this bottleneck. Pyramiding different combinations of resistance genes in a single variety can confer a higher level of broad-spectrum resistance (Mundt, 2018). In soybean, the pyramiding of different *Rpps* in a single genotype has been shown to provide enhanced resistance to a broad range of *P. Phachyrhizi* isolates (Lemos *et al.*, 2011; Yamanaka *et al.*, 2013; Yamanaka *et al.*, 2015; Yamanaka & Hossain, 2019).

Mutant populations are an important resource of new variation for both crop improvement and basic plant science research. Among the chemical mutagens, ethyl methanesulfonate (EMS) has been widely used to generate new traits (Hwang *et al.*, 2014). EMS can establish allelic variations in genes through random point mutations that generate nucleotide substitutions or deletions that can alter their functions (McCallum *et al.*, 2000; Henikoff *et al.*, 2004; Cui *et al.*, 2013; Boevink, McLellan*, et al.*, 2016; Espina *et al.*, 2018). Forward genetic analysis using EMS mutagenesis has proven to be a powerful tool in biological research (Kim *et al.*, 2006; Addo-Quaye *et al.*, 2017), but identification and cloning of causal mutations by conventional genetic mapping approaches is a time-consuming process (van Schie & Takken, 2014).

The past decade has witnessed several advancements in high-throughput genotyping. High throughput next-generation sequencing (NGS) technologies have demonstrated the capacity to sequence DNA at unprecedented speed, and have made a striking impact on genomic research. This ability to interrogate populations and individuals at sequence level in a cost-effective way has also resulted in a resurgence in experimental mutagenesis as a means of determining gene function. Once the whole-genome draft sequences of a given species become available, resequencing of multiple individuals of the same species allows quick identification of genomic variations (Schneeberger *et al.*, 2009). However, our ability to determine gene function depends on associating genes with their end products, i.e., phenotypes. Several approaches such as bulked segregant analysis (BSA) (Abe *et al.*, 2012; Fekih *et al.*, 2013; Takagi *et al.*, 2015; Song *et al.*, 2017) and transcriptome sequencing (Trick *et al.*, 2012; Hussain *et al.*, 2018; Klein *et al.*, 2018) have been used to accelerate the identification of natural or induced mutations in *Arabidopsis* (Ashelford *et al.*, 2011; Hartwig *et al.*, 2012) rice (Abe *et al.*, 2012), soybean (Song *et al.*, 2017), wheat (Hussain *et al.*, 2018) and other species. These approaches combine a high-throughput whole-genome re-sequencing with a bulked-segregant analysis to identify the target gene. Therefore, their success depends upon the availability of segregating populations with a reliable phenotypic assessment.

In the previous study, we identified three 'TMG 4182' EMS-mutants (GMMT04-30-509-20, GMMT04-30-504-04, and GMMT04-30-496-01) with enhanced resistance to *P. pachyrhizi* isolate PPUFV02. Mutants GMMT04-30-509-20 and GMMT04-30-504-04 displaying strong RB-type resistance reaction and GMMT04-30-496-01 necrotic lesions with fewer uredia when compared to the non-mutagenized wild-type 'TMG4182'. The objectives of this work was to study the inheritance of these mutants and to characterize the mutants lines using NGS resequencing aiming at the identification of the causative mutation underlying the phenotypes.

Materials and Methods

Segregation analysis

Three mutants GMMT04-30-509-20, GMMT04-30-504-04, and GMMT04-30-496-01, selected in the previous study (see chapter 1), were crossed with non-mutagenized wild-type 'TMG 4182' and 'Williams 82' to generate F1 hybrids that were allowed to self-pollinate and produce F2 seeds. All mutant F2 seeds derived from crosses, along with ten seeds from each mutant parental line and the wild-types 'TMG4182' and 'Williams 82', were planted in 1L pots with a mix of soil and Tropstrato HA® growing media and individually inoculated with *P. pachyrhizi* isolate PPUFV02 at V2-V3 stage. All young and fully expanded trifoliate plants were sprayed on the abaxial surface of the leaves with the spore suspension of 2.0 x 10^4 uredospores/ml in distilled water + Tween 20 (0.01%), through a direct air atomizer and kept in a dark mist chamber with spray shifts of 5 seconds every 10 minutes at 22 ± 2ºC, for 24 h and, then, transferred to a greenhouse under a standard condition of 12 h photoperiod at 25-28 °C. These plants were individually evaluated every two days for disease symptoms and compared to the parental groups, and the lesions scored as TAN lesion with abundant sporulation similar to those observed on the wild-type 'TMG482' and 'Williams 82', Reddish-brown (RB) reactions similar to those observed on mutants GMMT04-30-509-20, and GMMT04-30-504-04 and necrotic lesions with few uredia in similar to those observed on mutant GMMT04-30-496-01. A Chi-square test of the hypothesis of Mendelian inheritance of the mutant phenotypes was performed using the software GENES (Cruz, 1998).

Whole genome sequencing and SNP detection

High-quality genomic DNA was extracted from 400mg of fresh macerated leaf samples from mutants GMMT04-30-496-01, GMMT04-30-509-20, GMMT04-30-504-04 and wild-type 'TMG4182' using the NucleoSpin® Plant II Midi Kit (Macherey-Nagel), according to the manufacturer's instruction. The quality of the DNA was assessed by gel electrophoresis and its concentration adjusted to 25ng/µl, by using a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific). The samples were sent to the Beijing

Genomics Institute (BGI) for whole-genome sequencing using the BGISEQ-500 platform to obtains 20x coverage.

Sequence data from wild-type 'TMG4182' were mapped to a public soybean reference genome 'Williams 82' Gmax 508 wm82.a4.v1 (http://phytozome.jgi.doe.gov) using the software Bowtie 2 (Langmead & Salzberg, 2012). Alignment files were converted to BAM or SAM files using SAMtools (v.1.9). Mpileup and BCFtools (v.1.9) (Li *et al.*, 2009) were used to generate a list of variant sites found in the alignment. The variant sites were filtered for reliable SNPs. This filter was used to maximize accuracy on SNP detection and also minimize false SNP calling by: (i) removing variant sites with depth lower than five reads; (ii) removing INDELs; and (iii) removing multiallelic and heterozygous sites. A variant list was then used to generate a 'TMG 4182' reference genome by replacing nucleotides from the public reference's Gmax 508 wm82.a4.v1 with filtered SNPs from 'TMG4182'.

Paired-end sequence reads from mutants GMMT04-30-496-01, GMMT04-30-509-20 and GMMT04-30-504-04 were then aligned to this new reference sequence using Bowtie and SNPs were scored. The SNPs were custom filtered using six steps: (i) removing all INDELs; (ii) removing all common SNPs shared by all three mutant lines; (iii) removal of multi-allelic sites ; (iv) removal of Phred-scaled quality scores lower than 30 (QUAL); (v) removed SNPs with sequence depth lower than 3; and (vi) selection of SNPs that exhibit G → A or C → T transitions, which are the most frequent changes caused by EMS mutagenesis (Takagi *et al.*, 2015). All the SNP positions were annotated using the software snpEff and snpSift (Cingolani *et al.*, 2012) and all non-synonymous mutations in protein-coding genes, and SNPs affecting the transcripts start and stop codons were selected for further analysis.

Polymorphism confirmation using dCAPs assays

DNA extraction from the segregating populations was performed according to Doyle's (1987) protocol, with modifications. The foliar tissue of each plant, approximately 100 mg, was collected separately in 2.0 mL microtubes, which were kept on ice until the beginning of the extraction. Three stainless steel beads were added to each microtube, and the material was macerated by using a Tissue Lyser (QIAGEN®). In the

macerated tissue was added: 1.0 ml of extraction buffer (2.0% (v/v) (pv-1) CTAB, 100 mM Tris-HCl (pH 8.0), 20 mM EDTA (pH 8.0), 1.4 M NaCl, 2.0% (pV-1) PVP 40) prewarmed at 65°C and the tubes were vortexed before incubation at 65°C for ten minutes, inverting every five minutes. After five minutes at room temperature, it was added 700µl of cold CIA (chloroform: isoamyl alcohol, in the ratio 24:1), reversing the microtubes several times to form an emulsion. The samples were, then, centrifuged for five minutes at 13,000 rpm and the aqueous phase transferred to a new microtube. On these samples were, then, added 480µl of cold isopropanol and 65µl of 10% CTAB (w/v), by inverting the tubes a few times for mixing it before incubating at -20°C for thirty minutes. After the incubation, the samples were centrifuged at 13,000 rpm for seven minutes. The supernatant was discarded, and the pellets washed with 70% ethanol. After a wash, the microtubes were placed upside down over a paper towel to dry. After 2 to 3 hours they were resuspended in 50µl TE (10 mM Tris-HCl pH 8.0, 1 mM EDTA) with RNase (50µg/ml) and incubated at 37 °C for one hour. The concentration, as well as quantity and quality of the extracted material, were verified using the NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific). The DNA was diluted to a final concentration of 25ng/µl and stored in a freezer at -20°C.

From the annotated list obtained after the SNP filtering, we determined the candidate gene's function using the software PhytoMine, available at the JGI website (https://phytozome.jgi.doe.gov/phytomine/begin.do) and selected five SNPs located in plant defense genes, previously reported in the literature, to be validated using cleaved amplified polymorphic sequence (CAPS) (Konieczny & Ausubel, 1993) and derived cleaved amplified polymorphic sequences (dCAPS) (Neff *et al.*, 1998) assays (Table 9). Primers and restriction enzymes were selected using dCAPS Finder 2.0 (Neff *et al.*, 2002) based on 500-700bp fragment containing the selected SNPs. The primers were used to amplify the genomic region containing the selected SNPs using with the following cycling conditions: 94°C for 5 minutes, followed by 30 cycles of 95°C for 30 seconds, along with 30 seconds at the respective binding temperatures of each pair of oligonucleotides: 72°C for 40 seconds and a final step of 72°C for 5 minutes. After the PCR, the amplicons were digested with appropriate enzymes and the fragments analyzed using the QIAxcel® Advanced System.

Table 9 - SNPs and oligonucleotides used in the CAPS and dCAPS assays aiming the validation studies.

Mutant	SNP ID	Primer Sequence	Enzyme	Gene ID
496-01	Gm03:43500036	AAAATTTTCATTACTTGCAATCTC AAAATGTTCCTCGTAAAGTGCAACAAGGAT	FokI	Glyma.03G220100
	Gm18:3984240	CGATAGAACAGAAATGGAGATAGT TATTATGCACTCAAGCATCAGCTGCAACTA	DdeI	Glyma.18G046300
504-04	Gm01:6051100	GCTTGGTTGCAAAGGTAGTACACAATTAGAT CTTTCAATTTTCTCCTTACAAAAA	RsaI	Glyma.01G051300
	Gm03:6421810	CTCGCCGGAGAATTTGTTGCCGGCGAGGTCG AGAACCTTGACCTCTCCGAAAAC	TaqI	Glyma.03G049300
509-20	Gm01:41424168	AATTTAAAAATGTTTTCGGACTAA AGCATGATATATTACATGTTGACG TTGACGAGATTTAAGGTTCCCAATTGCTTA	DdeI	Glyma.01G118900

Results

Segregation analysis

Several F2 populations were obtained from the crosses between mutant lines GMMT04-30-509-20, GMMT04-30-504-04, GMMT04-30-496-01 and wild-type 'TMG4182 and Williams82'. Four populations derived from each mutant parent were randomly selected for the segregation studies reported here.

F2 plants from mutant parents GMMT04-30-509-20, and GMMT04-30-504-01 displayed both the wild-type (TAN) and mutant (strong RB) reaction within the F2 populations after the inoculation with *P. pachyrhizi* isolate PPUFV02 (Figure 14-A to D). F2 populations derived from crosses with mutant parent GMMT04-30-496-01, also displayed both the WT (TAN) and mutant (necrotic lesions, with low sporulation) reactions after the inoculation with *P. pachyrhizi* (Figure 14-E and F), confirming the successful development of the segregation populations for inheritance and mapping studies.

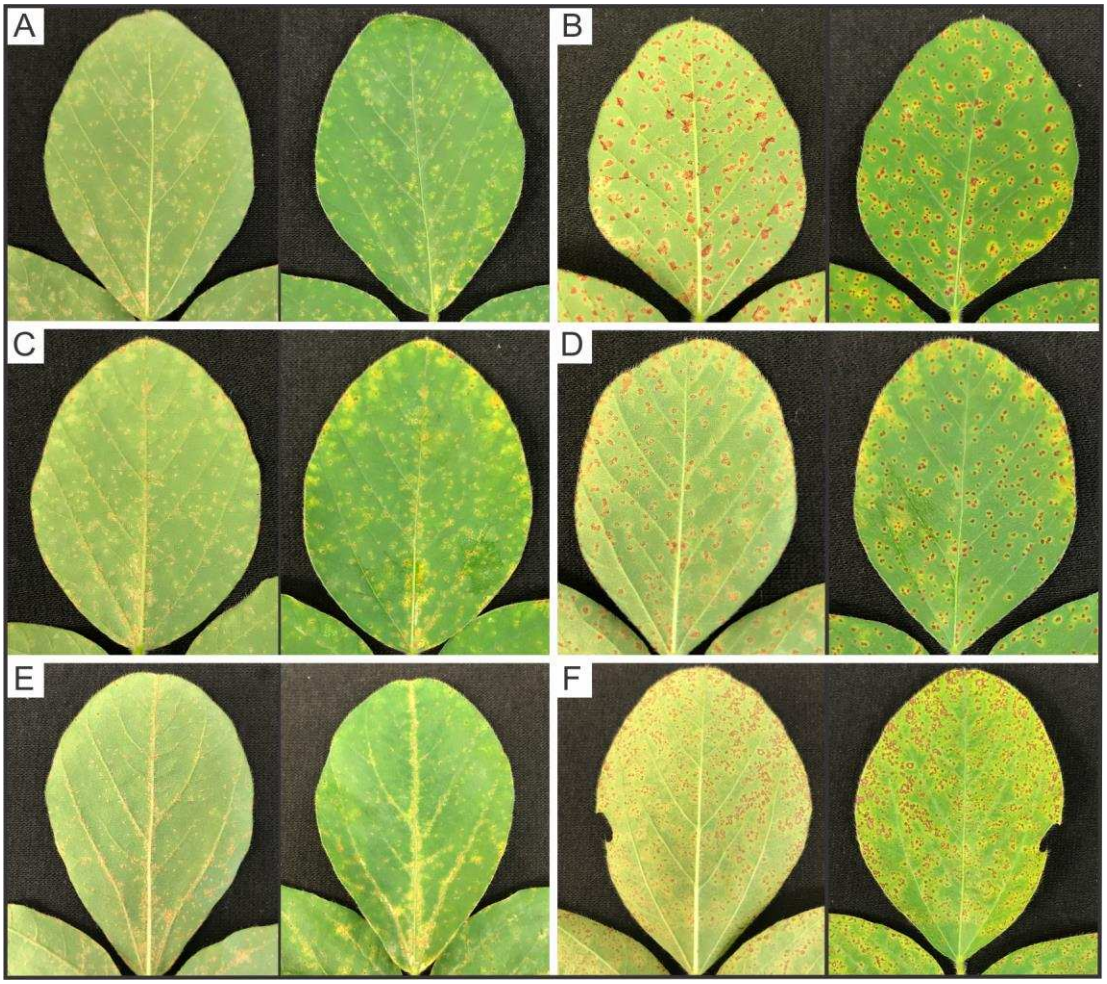

Figure 14 - Different phenotypes observed in the segregating F2 population derived from crosses between WT 'TMG418' and 'Williams82' with mutant GMMT04-30-509-20, GMMT04-30-504-04, and GMMT-04-30-496-01, observed 15 days after inoculation with 5 x 10^4 spores of *P. pachyrhizi* PPUFV02. (A), abaxial and adaxial faces of TMGx509-20-0301-37 leaflets displaying light TAN lesions. (B), abaxial and adaxial faces of TMGx509-20-0301-38 leaflets displaying strong RB symptoms with round reddish spots. (C), abaxial and adaxial faces of TMGx504-04-0302-04 leaflets displaying light TAN lesions. (D), abaxial and adaxial faces of TMGx504-04-0302-48 leaflets displaying strong RB type reaction. (E), abaxial and adaxial faces of W82x496-01-0302-09 leaflets displaying light TAN lesions. (F), abaxial and adaxial faces of W82x496-01-0302-07 leaflets displaying small necrotic lesions with fewer uredia.

Three F2 populations: 'William82' x 509-20-0502, 'Williams82' x 509-20-0801 and 'TMG4182' x 509-20-0301 showed a segregation pattern that fits the 3;1 (wild type: mutant phenotype) ratio expected for a recessive character. In the F2 population derived from 'TMG4182' x 509-20-0102 the number of mutant plants displaying the RB

phenotype was lower than expected, and did not fit the model for a recessive trait at (α= 5%) (Table 10).

Table 10 - Mutant phenotype (RB) segregation observed in the F2 populations derived from crosses 'Williams82' x GMMT-04-30-509-20' and 'TMG4182' x GMMT-04-30-509-20, 15 days after the inoculation with *Phakopsora pachyrhizi* isolate PPUFV02.

F2 Population	Phenotype				χ^2	Probability
	# of plants observed		Expected (3:1)			
	TAN	RB	TAN	RB		
Williams82 x 509-20-0502	35	11	34,5	11,5	0,028	86,48%
Williams82 x 509-20-0801	27	13	30	10	1,2	27,33%
TMG4182 x 509-20-0102	50	7	42,75	14,25	4,92	2,65%
TMG4182 x 509-20-0301	32	11	32,25	10,75	0,0077	92,98%
Total	144	42	139,5	46,5	0,5806	44,60%

*χ^2 Obs. < χ^2 (α 5%), we accept the hypothesis that the segregation is in accordance to the Mendelian ratio of 3:1.

F2 plants from 'Williams82' x 504-04-0703, 'Williams82' x 504-04-0202 and 'TMG4182' x 504-04-0302 crosses displayed segregation of wild (TAN) and mutant (RB) phenotypes that fitted the Mendelian rate of 3:1, indicating that a recessive gene was also responsible for GMMT04-30-504-04 mutant phenotype (Table 11). One mutant F2 family showed no sign of segregation and was eliminated because this lack of segregation indicates that the seeds were derived from the self-pollination of the parental plant.

Table 11 - Mutant phenotype (RB) segregation observed in the F2 populations derived from crosses 'Williams82' x GMMT-04-30-504-04' and 'TMG4182' x GMMT-04-30-504-04, 15 days after the inoculation with *Phakopsora pachyrhizi* isolate PPUFV02.

F2 Population	Phenotype				χ^2	Probability
	# of plants observed		Expected (3:1)			
	TAN	RB	TAN	RB		
Williams82 x 504-04-0703	29	9	28,5	9,5	0,035	85,14%
Williams82 x 504-04-0202	32	10	31,5	10,5	0,317	85,85%
TMG4182 x 504-04-0302	39	9	36	12	1,0	31,73%
Total	100	28	96	32	0,66	41,42%

*χ^2 Obs. < χ^2 (α 5%), we accept the hypothesis that the segregation is in accordance to the Mendelian ratio of 3:1.

Two F2 populations derived from crosses of 'TMG4182' and GMT04-30-496-01 displayed only the wild-type TAN phenotype and were also eliminated. Two remaining F2 populations 'Williams82' x 496-01-0302 and 'Williams82' x 496-01-0602 segregated

into wild-type and mutant phenotypes in a proportion that fitted the expected 3:1 Mendelian for a recessive gene controlling the mutant phenotype (Table 12).

Table 12 - Mutant phenotype (necrotic lesions) segregation observed in the F2 populations derived from crosses 'Williams82' x GMMT-04-30-496-04', 15 days after the inoculation with *Phakopsora pachyrhizi* isolate PPUFV02.

F2 Population	Phenotype				χ^2	Probability
	# of plants observed		Expected (3:1)			
	TAN	Nec. Lesions	TAN	Nec. Lesions		
Williams82 x 496-01-0302	27	5	24	8	1,5	22,06%
Williams82 x 496-01-0602	27	6	24,75	8,25	0,81	36,57%
Total	54	11	48,75	16,25	2,26	13,26%

*χ^2 Obs. < χ^2 (α 5%), we accept the hypothesis that the segregation is in accordance to the Mendelian ratio for recessive genes (3:1).

Whole genome sequencing and identification of SNPs

Seventy-seven gigabytes of raw data was obtained from mutant lines GMMT04-30-496-01, GMMT04-30-504-04, 04-30-509-20 and wild-type 'TMG 4182'. Considering the size of the soybean genome, containing roughly 978Mb, this represents approximately 20x coverage per sample (Table 13).

Table 13 - Number of clean reads, overall read quality and expected coverage for each sequenced genotype sequenced.

Genotype	Clean Reads (100b)	Clean bases (Mb)	Q20%	Coverage
TMG4182	192,527,504	192,528	97.07	19.685
04-30-496-01	192,835,202	192,835	97.16	19.717
04-30-504-04	190,468,042	190,468	96.98	19.475
04-30-509-20	193,209,126	193,209	97.27	19.755

Alignment of reads from 'TMG4182' to the reference genome 'Williams 82' Gmax 508 wm82.a4.v1 allowed to call 1,193,677 SNPs after removing INDELs, low-quality bases, multiallelic and heterozygote sites. This high number of SNPs are mostly due to natural variations that differentiate these two cultivars. This variant information was then incorporated into the 'Williams 82' Gmax 508 wm82.a4.v1 reference genome and used to build a reference genome for 'TMG4182'. Using this new reference, SNPs from mutants GMMT04-30-496-01, GMMT04-30-504-04 and GMMT04-30-509-20

were called. To reduce the occurrence of false SNPs and sequencing errors a pipeline of filters was applied (Table 14).

As a result, we discovered that sequenced mutants GMMT04-30-496-01, GMMT04-30-504-04 and GMMT04-30-509-20 harbor 9,900, 8,824 and 10,158 SNPs indicating G to A and C to T base transitions respectively, presumably caused by the EMS treatment. From these transitions, 737 are non-synonymous, homozygous mutations in protein-coding genes and 68 SNP's affecting the start and stop codons that may alter the protein's structure and function (Table 14).

Table 14 - Number SNPs called at different steps of the variant filtering pipeline used.

Filters	Number of SNPs			
	TMG4182	**496-01**	**504-04**	**509-20**
Raw VCF (all SNPs and INDEL's)	676,110	872,005	842,019	861,915
Removed INDEL's	421,212	574,913	563,009	572,227
Removed SNP's common to all samples	36,931	71,551	66,608	71,219
QUAL >30 and DP > 2	13,492	22,494	20,322	24,473
Multiallelic sites > 2 alleles	8,881	22,792	18,678	22,985
All base-pair substitutions				
Gene annotation	8,881	22,792	18,678	22,985
SNPs in protein coding genes	3,240	11,298	9,440	12,468
Protein coding - Non synonymous	97	611	491	773
Homozygote only	46	348	319	465
Gained Start codon	0	28	17	26
Gained Stop codon	0	18	13	20
Only C to T and G to A substitutions				
Gene annotation	8,881	22,792	18,678	22,985
Only C to T and G to A substitutions	3,148	9,900	8,824	10,158
SNPs in protein coding genes	1,133	4,879	4,409	5,322
Protein coding - Non synonymous	25	352	380	437
Homozygote only	9	217	225	295
Gained Start codon	0	11	9	12
Gained Stop codon	0	10	12	14

DP = Total Depth, number of reads that aligned to a variant site.

Sequenced mutant line GMMT04-30-509-20 had the highest frequency of mutations, being one variant per 42,326 bases, followed by GMMT04-30-496-01 with 1 variant for every 42,647 bases and GMMT04-30-504-04, the least frequent with one

variant every 51,968 bases (Figure 15). In addition, as expected from the EMS mutagenesis, all sequenced mutants displayed two to three times more C to T and G to A transitions, when compared to all other possibilities (Figure 15).

Sequence reads from WT 'TMG4182 were also subjected to the same filtering parameters to access the SNP filtering quality for comparison purposes. It is important to note that most of these SNPs detected in the WT 'TMG4182' are probably due to sequencing and alignment errors, low sequencing coverage, or naturally occurring mutations that were not correctly identified and incorporated in the new TMG4182 reference genome.

TMG4182
1 variant every 108,648 bases

	A	C	G	T
A	0	342	1,333	508
C	450	0	222	1,589
G	1,566	237	0	441
T	521	1,316	364	0

GMMT04-30-496-01
1 variant every 42,647 bases

	A	C	G	T
A	0	807	2,649	1,640
C	942	0	483	4,878
G	5,029	531	0	901
T	1,481	2,655	804	0

GMMT04-30-504-04
1 variant every 51,968 bases

	A	C	G	T
A	0	776	2,698	1,399
C	963	0	554	5,053
G	5,112	554	0	959
T	1,390	2,756	779	0

GMMT04-30-509-20
1 variant every 42,326 bases

	A	C	G	T
A	0	641	2,036	1,113
C	733	0	378	4,311
G	4,520	422	0	760
T	1,047	2,121	604	0

Figure 15 - Mutation density and distribution of base transitions in mutants GMMT04-30-509-20, GMMT04-30-504-04, and GMMT04-30-496-01 and wild-type 'TMG4182'. Rows in gray: reference bases, Columns: alternative bases.

We further analyzed the effects of the EMS mutagenesis on the overall amino-acid transitions, and found that mutagenized lines followed a biased transition pattern when compared to WT 'TMG4182'. Non-mutagenized wild-type 'TMG4182' had an overall low frequency of amino acid transitions, with only 20 changes in N (asparagine) to S

(serine), and 13 L (leucine) to R (arginine) (Figure 16-A). Hence, these transitions could be artifacts of sequencing and alignment errors, low sequencing coverage or naturally occurring mutations. In all three mutant genotypes, these specific transitions were not significant. However, all three mutagenized genotypes shared significantly higher values of amino acid transitions: A (alanine) to T (threonine), A (alanine) to V (valine), E (glutamic acid) to K (lysine), G (glycine) to E (glutamic acid) and P (proline) to S (serine) (Figure 16-B to D).

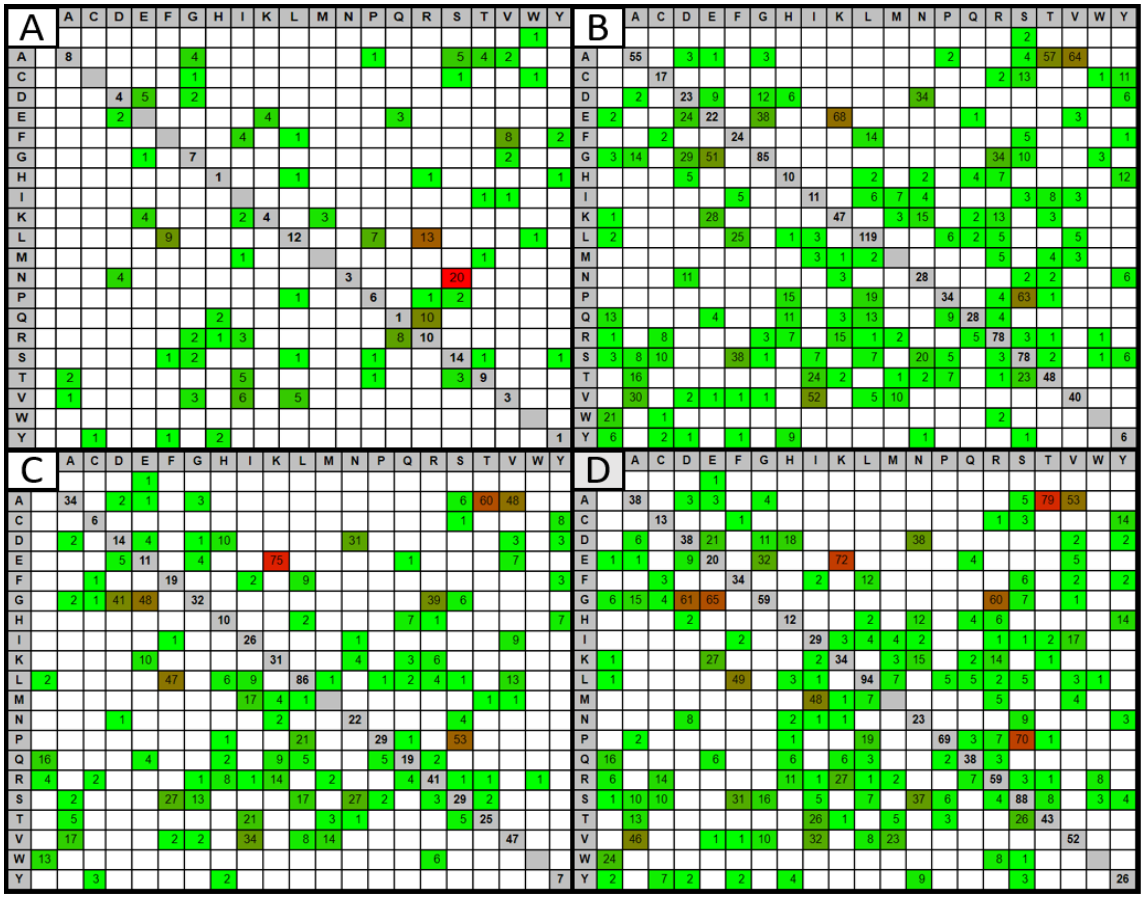

Figure 16 - Heat-maps of amino acid changes from wild-type 'TMG4182' and mutants genotypes. Rows indicate reference genome amino acids and Columns variant amino acids. The red background colors indicate that a higher number of changes happened. (A), wild-type 'TMG4182'. (B), GMMT04-30-496-01. (C), GMMT04-30-504-04. (D), GMMT04-30-509-20.

The distribution of SNPs in the nuclear genome of mutant genotypes indicated that the EMS-induced mutations were random, however chromosomes 15 and 18 displayed a higher number of variants when compared to the other chromosomes (Table 15). Chromosomes 15 and 18 from sequenced mutant genotypes GMMT04-30-496-01,

GMMT04-30-504-04 and GMMT04-30509-20 displayed a high number of SNPs, representing 28 to 31% of all identified SNPs. Moreover, wild-type non-mutagenized 'TMG4182' also displayed 7,092 SNPs in chromosome 18, which counted for 81% of all variation found in this sample (Table 15), almost 5x the variation found in all the other 19 chromosomes put together.

Table 15 - Number of SNPs identified per soybean chromosome for the wild-type 'TMG4182' and mutants GMMT04-30-496-01, GMMT-04-30-504-04, and GMMT-04-30-509-20.

Chr	Length	# of Variant Sites 'Williams82.a4.v1 / TMG4182*	Number of Variant Sites per Chromosome			
			TMG4182	04-30-496-01	04-30-504-04	04-30-509-20
Chr01	57.9	67,834	105	1,531	910	1,061
Chr02	50.4	54,410	67	853	751	883
Chr03	47.0	61,523	93	1,113	750	919
Chr04	51.2	96,299	115	849	771	914
Chr05	42.3	32,555	48	756	602	779
Chr06	50.9	59,225	76	924	705	1,032
Chr07	44.9	52,215	88	846	731	842
Chr08	47.2	20,652	77	638	352	456
Chr09	50.6	72,334	74	896	639	916
Chr10	51.6	36,835	75	819	779	785
Chr11	39.6	16,699	41	513	429	542
Chr12	41.5	29,448	45	534	638	573
Chr13	45.2	73,830	102	1,130	823	1,202
Chr14	49.9	21,774	323	929	566	778
Chr15	53.8	116,868	98	1,451	2,859	3,451
Chr16	38.1	24,001	43	917	406	808
Chr17	41.7	92,885	101	903	632	806
Chr18	58.3	110,461	7,092	5,032	2,951	3,361
Chr19	51.3	99,556	105	839	1,422	1,431
Chr20	47.8	45,411	60	943	712	906

* Number of base substitutions incorporated in the public reference genome 'Williams 82' Gmax 508 wm82.a4.v1 used to build the new reference for 'TMG4182'.

Mutation frequencies were also considered by dividing the number of mutations (SNPs) found in each chromosome by the total chromosome length for each mutant. Mean comparison analysis indicated that, there was no significant statistical difference between the mutation frequencies found among the three mutant lines GMMT04-30-496-01, GMMT04-30-504-04 and GMMT04-30-509-20. However, a high statistical difference

(P-value < 0.001) was found between chromosomes, in which, chromosomes 15 and 18 showed significantly higher mutation frequencies when compared to the other 18 chromosomes (Figure 17).

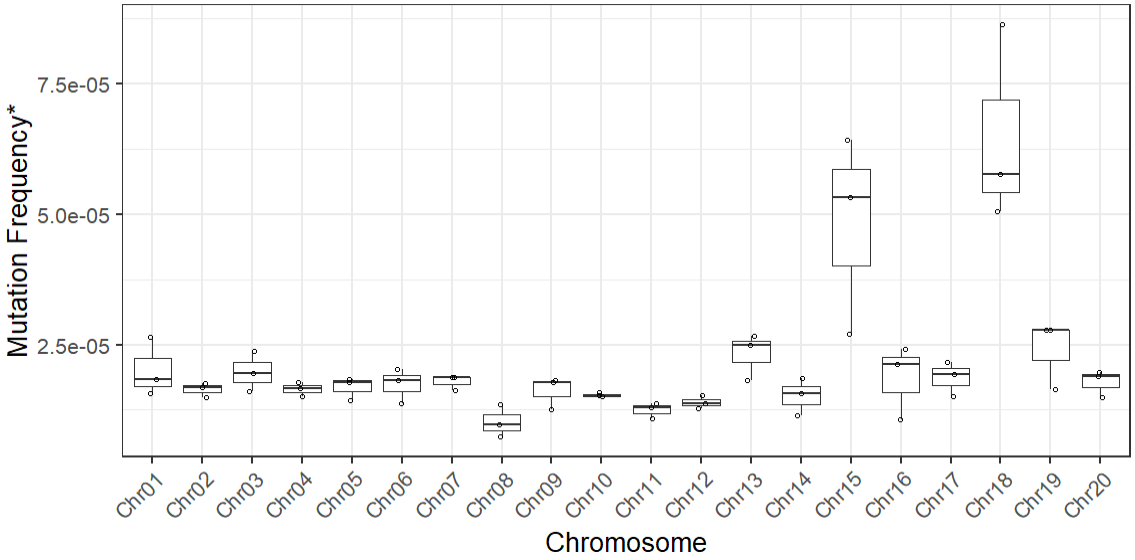

Figure 17 - Mutation frequencies found for each chromosome from mutant lines GMMT04-30-496, GMMT04-30-504-04 and GMMT04-30-509-20. *Mutation frequencies were calculated by dividing the number of mutations (SNPs) found in each chromosome by the total chromosome length.

To further investigate the higher number of variant sites detected in chromosomes 15 and 18, we analyzed the variant information (SNPs) from the sequence alignment of WT 'TMG4182' to the public reference genome 'Williams 82' Gmax 508 wm82.a4.v1 used this information to generate the new reference for 'TMG4281', and found that chromosomes 15 and 18 had the highest number of variant sites (116,868 and 110,461 SNPs respectively) when compared to the other chromosomes (Table 15).

Statistical analysis revealed a positive correlation between the number of SNPs detected in mutant lines GMMT04-30-496-01, GMMT04-30-504-04 and GMMT04-30-509-20 and natural variant sites among cultivars TMG4182 and Williams82 for each chromosome (Figure 18).

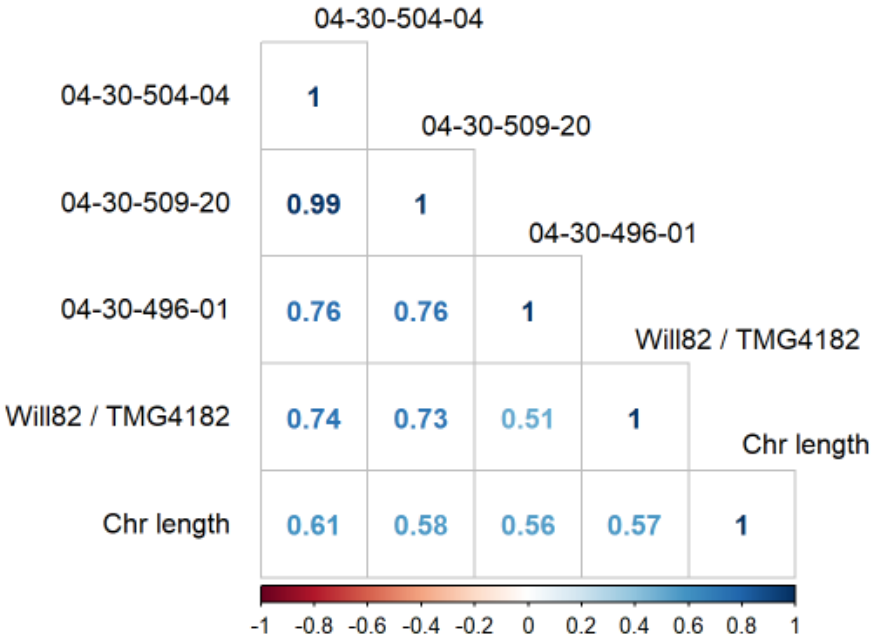

Figure 18 - Correlation analysis between the number of SNPs detected from the alignment of WT 'TMG4182' and the public reference genome 'Williams 82' Gmax 508 wm82.a4.v1 (Will82 / TMG4182), chromosome length (Chr length) and the number of SNPs detected for mutants lines GMMT04-30-496-01, GMMT04-30-504-04 and GMMT04-30-509-20 per chromosome ($\alpha = 0,05$).

These results indicate that along with sequencing and alignment errors, low coverage zones and INDELS, there is also a natural variation among the chromosomes of cultivars 'TMG4182' and 'Williams82' and part of this variation was not incorporated in our new 'TMG4182' reference genome.

From the SNPs found in mutant lines GMMT04-30-496-01, GMMT04-30-504-04 and GMMT04-30-509-20 we compiled a list of protein-coding and non-synonymous SNPs. This selection was made because, non-synonymous mutations lead to amino acid changes that could alter protein function. This alteration could be associated with the observed phenotypes in the mutant lines analyzed. Using the annotation software SnpEff (Cingolani *et al.*, 2012), we identified the soybean genes (Glyma) in which these SNPs occurred. Gene function was also investigated using the software PhytoMine available at the JGI website (https://phytozome.jgi.doe.gov/phytomine/begin.do).

Identified SNPs were found to occur in many different gene families, and instead of testing one by one, we prioritized the ones present in protein-coding genes related to pathogen recognition, signal transduction pathways and plant defense response genes.

Mutant GMMT04-30-496-01 displayed two SNPs, (Gm03:43500036 and Gm05:35676231) in genes containing the WRKY domain, three SNPs (Gm05:35676231, Gm08:41507848 and Gm15:12633332) affecting leucine-rich repeat (LRRs) genes, one SNP in a disease resistance-related protein (Gm09:2454326), one SNP in a laccase gene (Gm18:47664134) and one SNP (Gm13:39605738) in a gene associated with salt stress and antifungal proteins (Table 16). Four SNPs were selected from mutant GMMT04-30-504-04, one (Gm01:6051100) located in a NAC transcription factor and three SNPs (Gm03:6421810, Gm08:19924039, Gm16:6286705) in LRR genes (Table 16). Mutant GMMT04-30-509-20 displayed one SNP in a Laccase related gene (Gm01:37485075), LRR (Gm10:45915850), NB-ARC domain Gm16:37426198), salt stress response and antifungal genes (Gm16:36577399), and one SNP located in a disease resistance protein (Gm06:49518099) (Table 16).

Table 16 - Selected protein-coding genes related to pathogen recognition, signal transduction pathways and plant defense responses with non-synonymous SNPs identified in mutant genotypes GMMT04-30-496-01, GMMT04-30-504-04 and GMMT04-30-509-20 that were used for SNP validation using CAPS or dCAPS assays.

Mutant	SNP ID	AA Change	Gene ID	Gene Description (PhytoMine)
496-01	Gm03:43500036	S233L	Glyma.03G220100	WRKY DNA-binding domain
496-01	Gm05:35676231	T478A	Glyma.05G165800	WRKY DNA-binding domain/NB-ARC domain/TIR domain/Leucine-rich repeat
496-01	Gm08:41507848	Q170E	Glyma.08G302900	Leucine-rich repeat Receptor-like Serine/Threonine-protein Kinase BAM3
496-01	Gm09:2454326	P48L	Glyma.09G029800	Disease resistance Protein-like-related
496-01	Gm13:39605738	Q53R	Glyma.13G305900	Salt stress response/antifungal
496-01	Gm13:44894563	A223V	Glyma.13G370000	Protein kinase domain/NAF domain/Protein tyrosine kinase
496-01	Gm15:12633332	S202F	Glyma.15G152400	Leucine-rich repeat-containing protein
496-01	Gm18:3984240	H128Y	Glyma.18G046300	Protein kinase domain/Salt stress response/antifungal
496-01	Gm18:47664134	S574L	Glyma.18G197400	Laccase-16-related
496-01	Gm19:5527392	A79V	Glyma.19G039100	Protein NSP-Interacting Kinase1
496-01	Gm20:1747653	P246S	Glyma.20G017800	CLAVATA3/ESR -Related protein 16-Related
504-04	Gm01:6051100	E21K	Glyma.01G051300	NAC transcription factor 29
504-04	Gm03:6421810	L174H	Glyma.03G049300	Protein kinase domain/Leucine-rich repeat N-terminal domain
504-04	Gm08:19924039	L403F	Glyma.08G235800	Leucine-rich repeat transmembrane protein kinase
504-04	Gm16:6286705	P903S	Glyma.16G064100	Protein kinase domain/Leucine-rich repeat N-terminal domain
509-20	Gm01:37485075	V512I	Glyma.01G108200	Laccase-7-related
509-20	Gm01:41424168	P120L	Glyma.01G118900	Protein tyrosine kinase/Leucine-rich repeat
509-20	Gm06:49518099	S165F	Glyma.06G311200	Disease resistance protein RPM1 (RPM1, RPS3)
509-20	Gm10:45915850	E100D	Glyma.10G228000	Leucine-rich repeat N-terminal domain
509-20	Gm16:36577399	S260F	Glyma.16G202200	Salt stress response/antifungal/Protein tyrosine kinase
509-20	Gm16:37426198	D159N	Glyma.16G214300	NB-ARC domain/Peptidase of plants and bacteria/TIR domain
509-20	Gm18:57485888	T73I	Glyma.18G294400	Cysteine-rich receptor-like protein kinase 25-related

* Amino acid change and position in the transcript.

Polymorphism validation

From the list of SNPs in protein-coding genes related to pathogen recognition, signal transduction pathways and plant defense responses, five (Gm03:43500036, Gm18:3984240, Gm03:6421810, Gm01:41424168 and Gm01:37485075) were selected for polymorphism confirmation.

CAPS and dCAPS assays confirmed the presence of the polymorphic site Gm03:43500036 and Gm18:3984240 in the mutant line GMMT04-30-496-01, Gm03:6421810 in GMMT04-30-504-04 and Gm01:37485075 in GMMT04-30-509-20, when compared to the WT 'TMG4182'. These four SNPs were further tested in the F2 segregant population, derived from crosses between WT 'TMG4182 and mutant lines GMMT04-30-496-01, GMMT04-30-504-04 and GMMT04-30-509-20, but they were not directly associated with the segregation of altered disease-development phenotypes observed in the F2 populations (Figure 19).

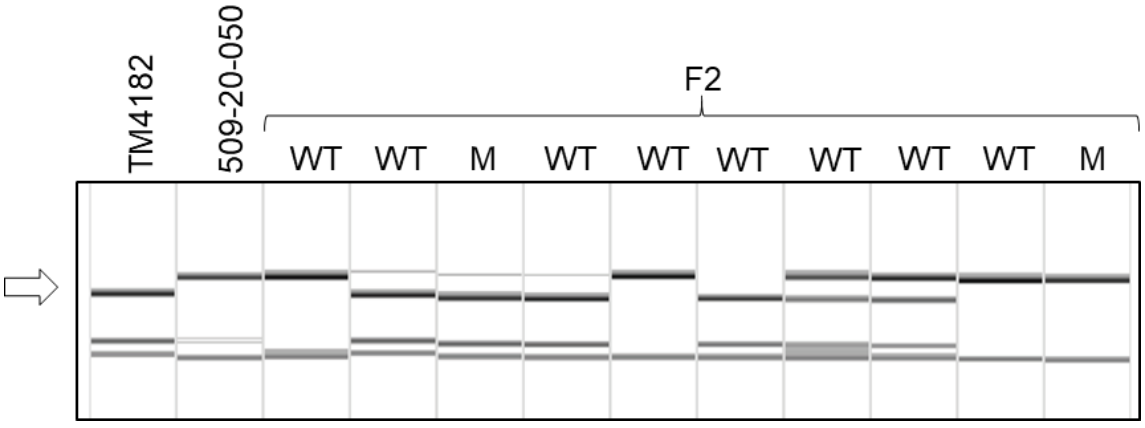

Figure 19 - Confirmation of the EMS-induced SNP Gm01:37485075 identified in mutant GMMT04-30-509-20 using a dCAPS assay, and segregation of the polymorphism in F2 plants from the TMG4182x509-20-0301 cross. The arrow indicates the polymorphism observed after digestion with HpaII. WT = wild-type (TMG4182) phenotype, TAN, susceptible phenotype; M = mutant (GMMT04-30-509-20) phenotype, RB-type resistance phenotype. It's possible to observe that the polymorphism is not linked to the mutant phenotype.

Discussion

Segregation ratios from F2 plants derived from crossings between wild-type 'TMG4182' and selected mutants showed that mutant phenotypes are controlled by single recessive genes. The same segregation pattern was observed in the F2 derived from crossings with 'Williams 82', demonstrating that this phenotype can be recovered even when the mutant genes are transferred to genotypes with different genetic backgrounds. Recessive mutations are usually the kind of mutations associated with EMS mutagenesis, although less common dominant mutations can also be found (Meinke, 2013).

Using whole-genome *de novo* re-sequencing, hundreds of mutations caused by EMS were identified in GMMT04-30-496-01, GMMT04-30-504-04 and GMMT04-30-509-20 mutants. Considering all three mutant genotypes a total of 64,455 SNPs were detected, with an average of 21,485 SNPs per mutant, the overall frequency of base change was around ~1/45,6 kb, which is in the range described in other studies. Cooper *et al.* (2008) using two different mutagens (EMS and N-nitroso-N-methylurea (NMU)) obtained a frequency that varied from ~1/140 to 550kb. However, a higher mutation frequency of ~1/11.8kb was found by Li *et al.* (2017) using 50mM EMS treatment.

Mutations induced by EMS treatment are usually random (Greene *et al.*, 2003). However, we detected a higher number of mutations on chromosomes 18 and 15 when compared to other chromosomes. This variation comes in part, from the natural differences between the reference genome, derived from Williams 82' and the cultivar TMG4182, that were not adequately integrated into our new 'TMG4182' reference. In this case, part of the variation detected between the mutant lines GMMT04-30-496-01, GMMT04-30-504-04 and GMMT04-30-509-20 and WT 'TMG4182 are actually due to false SNPs. In addition, the number of SNPs/False SNPs found for each mutant line chromosome correlates positively with the natural differences observed between these cultivars. In short, chromosomes with higher natural variation between cultivars displayed higher amounts of SNPs/False SNPs, where the more homogeneous chromosomes displayed, the lower the number of SNPs/False SNPs. Chromosome 18 has proven to be of significant importance in disease resistance. Three different *Rpp* loci that confer resistance to Asian soybean rust have been mapped to this chromosome: *Rpp1* (McLean & Byth, 1980; Garcia *et al.*, 2008; Chakraborty *et al.*, 2009), *Rpp4* (Hartwig, 1986; Meyer

et al., 2009) and *Rpp6* (King *et al.*, 2016). The soybean cyst nematode resistance loci *Rhg1* is also on chromosome 18 (Webb *et al.*, 1995), and TMG4182 is cyst nematode resistant variety (TMG, TMG, TMG, TMG, 2019). It is possible the polymorphisms detected are in the introgressed segment harboring the *Rhg1* gene. A higher sequencing coverage (>20X) along with different SNP filtering parameters could generate a better genomic reference for 'TMG4182', providing an even distribution of SNPs generated by EMS among chromosomes reducing the false EMS-related SNP calling.

Frequency and number of mutant sites found in soybean studies can be underestimated. The soybean is a paleopolyploid with 75% of the predicted genes present in multiple copies due to two duplication events that occurred 13 and 59 million years ago (Schmutz *et al.*, 2010). These duplications might restrict the numbers of SNPs found. Sequences reads from different duplicated genes can align to the same gene generating multiallelic variation as well as false heterozygosity, which does not allow to correctly call the SNPs associated with these duplicated genes. In our case, this may be more relevant, because most resistance loci contain fast-evolving paralog genes (Hulbert *et al.*, 2001). However, if the phenotype is the result of a mutation in members of gene families, such as typical resistance genes, the density of mutations found in this study may allow us to find SNPs in single-copy genes that are possibly tightly-linked to the resistance loci. These SNPs can then be used to clone the mutated gene by positional cloning.

It has been described that more than 90% of base changes induced by EMS and N-Nitroso-N-methylurea (NMU) are G to A and C to T transitions. This is expected due to the alkylation of guanine residues, forming O6-ethylguanine, which pairs with T but not with C. As a result, replication repairs the unpaired bases and effectively replacing G/C with an A/T. This mechanism predicts a strong bias in EMS-induced mutations (Greene *et al.* (2003). As expected, these transitions were the most frequently observed in our sequenced mutant lines, GMMT04-30-496-01, GMMT04-30-504-04 and GMMT04-30-509-20. However, the rates found in our study were lower, ranging from 44 to 47% of G to A and C to T transitions. Our findings corroborate with the lower rates of 50 to 70% described by Tsuda *et al.* (2015) in an EMS soybean mutagenesis study. This indicates that most of the variation detected in these mutants was, in fact, due to the effects of the mutagenesis treatment with EMS. However, it is important to note that some of these

SNPs might be derived from sequencing and alignment errors, or naturally occurring variations that distinguish 'Williams 82' and 'TMG4182' that were not eliminated in the first step of our pipeline.

The soybean genome has an approximate size of 1,115Mb. The assembly used in this study as a reference genome 'Williams 82' Gmax 508 wm82.a4.v1 carries approximately 978Mb, (88%) captured in 20 chromosomes, with only a small amount of mostly repetitive sequence in unmapped scaffolds. Comparisons with the soybean Expressed Sequence Tags (ESTs) suggest that more than 98% of known soybean protein-coding genes are represented in this assembly. The majority of these ESTs aligned to the *Glycine max* genome with near 100% identity, suggesting that the annotation of the Glyma1 genes is highly accurate in genic regions (Schmutz *et al.*, 2010).

Non-synonymous mutations were found in a broad diversity of genes, such as genes involved in primary and secondary metabolism, pathogen recognition, and defense transduction pathways. Since the phenotypes of mutants GMMT04-30-504-04 and GMMT04-30-509-20 after inoculation with *P. pachyrhizi* resembles the resistant RB-type of reaction observed in soybean with *Rpp* genes (Miles *et al.*, 2011), we initially focused our SNP validation and preliminary linkage-analysis on candidate genes normally associated with this type of resistance.

Most proteins encoded by plant *R* genes contain a nucleotide-binding site and leucine-rich repeat (NBS-LRR) domains. Based on composition and domain analysis of identified *R*-genes, they can be categorized into at least five major classes: TNL (TIR-NBS-LRR); RLP (Receptor-Like Proteins); RLK (Receptor-Like Kinases); CNL (CC-NBS-LRR); and the KIN class grouping proteins containing only a kinase domain (Sanseverino *et al.*, 2010; Dufayard *et al.*, 2017). Although many aspects of R protein function remain to be clarified, there is evidence for both indirect and direct interactions of these proteins with microbial effectors (Jones & Dangl, 2006; Dodds & Rathjen, 2010).

We identified at least seven SNPs located in genes that encode NLR proteins with leucine-rich repeats (LRR) domains, along with SNPs occurring in kinase and NB-ARC domain. The NB-ARC domain is a functional ATPase domain, and its nucleotide-binding state is proposed to regulate activity of the R protein (Van Ooijen *et al.*, 2008). We have also identified SNPs in genes that could be associated with defense responses such as

laccases (Benhamou, 1996; Miedes *et al.*, 2014; Hu *et al.*, 2018) and WRKY transcription factors (Pandey & Somssich, 2009). This selection represents an important starting point to determine the exact SNPs responsible for the mutant phenotypes, since mutations in these genes could lead to amino acid changes altering protein function. Harris *et al.* (2013) showed that a single-amino-acid mutation near the NB pocket of the potato NLR protein Rx caused an enhanced resistance to Poplar mosaic virus. Stirnweis *et al.* (2014) identified two positions in the NB-ARC domain of the wheat powdery mildew resistance gene Pm3 that, when mutated, enhances the ability of the protein to trigger cell death in *N. benthamiana* and also results in expanded resistance of the *Pm3f* allele in wheat. Using targeted mutagenesis Giannakopoulou *et al.* (2015) recovered a mutant in the NLR I2 protein, $I2^{I141N}$, that showed enhanced response to races of *P. infestans* and *F. oxysporum* f. sp. *lycopersici*.

We successfully confirmed four SNPs identified in candidate genes using dCAPS assays, which demonstrates the robustness of the SNP identification pipeline used. However, when analyzed in the F2 segregant population these SNPs were not linked to the observed phenotype. Given the total of 1,132 SNPs in protein-coding genes that have been identified across all three mutants, a more robust approach, such as bulked segregant analysis coupled with re-sequencing will be used to reduce the number of candidate SNPs and identify the casual mutations linked to the observed phenotypes (Abe *et al.*, 2012; Fekih *et al.*, 2013; Takagi *et al.*, 2015; Song *et al.*, 2017). Once SNPs responsible for the disease resistance phenotype observed in mutant lines GMMT04-30-496-01, GMMT04-30-509-20 and GMMT04-30-504-04 are identified, the mutant gene can be transferred into elite germplasm using SNP-assisted introgression, or by genetic engineering methods such as CRISPR/cas9 (Borrelli *et al.*, 2018). Studies are underway to verify if these mutations have an additive effect on ASR resistance. Pyramiding multiple resistance genes in a single soybean has been shown to provide a higher level of resistance to the ASR pathogen (Lemos *et al.*, 2011; Maphosa, 2012; Mundt, 2018; Yamanaka & Hossain, 2019).

General conclusions

- By using EMS mutagenesis of TMG4182, a susceptible soybean variety, we generated 3,950 M2 families;

- Three families with mutant plants with altered response to *Phakopsora pachyrhizi* PUFV02 were found after the screening of 1820 M2 families. Mutant GMMT04-30-496-01 displayed necrotic lesions with fewer uredia whereas GMMT04-30-504-04 and GMMT04-30-509-20, RB type resistance reaction;

- The phenotype of these three selected mutants is controlled by recessive genes;

- Whole-genome resequencing of TMG4182 and these three mutants revealed a mutation frequency of approximately one variant per 45,647 bases, in which about 50% were base transitions from C to T and G to A, the type of nucleotide changes commonly observed in EMS mutagenesis studies. A total of 1,132 nonsynonymous SNPs were detected in a broad variety of protein-coding genes.

- Derived cleaved amplified polymorphic sequences (dCAPS) assays validated four SNPs identified using a bioinformatic pipeline containing several filtration steps to avoid false-positive SNPs.

- Bulk segregant analysis coupled with MutMap will be used to identify the genes responsible for the phenotypes.

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