Rpp-Gene pyramiding confers higher resistance level to Asian soybean rust

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Abstract  Asian soybean rust (ASR) causes large reductions in soybean yield, affecting the entire grain market. With low fungicide efficiency, the use of resistant cultivars can be an economical, safe, efficient, and sustainable control alternative. However, the great variability and aggressiveness of ASR and the use of Rpp genes are limited. Thus, gene pyramiding is a promising strategy for the development of cultivars with high resistance to a greater number of isolates. Thus, the objective of this study was to evaluate sister lines, previously evaluated by Meira et al. (2022). https://doi.org/10.1007/s10681-020-02667-x), presenting different Rpp-pyramided genes for resistance to Phakopsora pachyrhizi to clarify the pyramiding effect of two originally developed Rpp-pyramided lines compared to two existing lines or lines possessing only a single Rpp of resistance under field conditions. Rpp-pyramided lines 52117-1 (Rpp2 + Rpp1-b), 52117-57 (Rpp2 + Rpp1-b), 52117-59 (Rpp2 + Rpp1-b) + 52117-60 (Rpp2 + Rpp4) showed high resistance levels compared to resistant sources and resistance control, carrying a single Rpp gene PI 200487 (Rpp5), PI 200492 (Rpp1), PI 230970 (Rpp2), PI 459025A (Rpp4), and PI 506764 (Rpp3 + Rpp5) with significant reductions in sporulation levels (SL), number of uredinia per lesion (NoU), and frequency of lesions with uredinia (%LU). Only, the line 52117-54 (Rpp2 + Rpp1-b), and 52117-63 (Rpp2 + Rpp4) showed resistance level smaller than PI 594723 (Rpp1-b) and similar resistance levels than PI 230970 (Rpp2), respectively. Rpp-pyramided lines carrying Rpp2 + Rpp1-b (52115-64, 52116-74, 52117-21, 52117-59 and 52117-60), and Rpp2 + Rpp4 (52117-60), and single gene Rpp1-b were classified as “highly resistant” and “resistant”. Furthermore, one sister line, 52117-57 (Rpp2 + Rpp1-b), showed immunity under field conditions. The Rpp-pyramided genes are an alternative for achieving high resistance levels against ASR.

Keywords  Phakopsora pachyrhizi · Rpp genes · Genetic resistance · Pyramiding

Introduction

Year after year, soybean farmers face numerous adversities caused by biotic and abiotic stresses, with the potential to damage the crop. Plant diseases are
responsible for huge crop losses and are a threat to global food security and agricultural sustainability. Asian soybean rust (ASR) is one of the most economically important diseases of crops, especially in tropical and subtropical countries, where reductions in grain yield can reach up to 80% (Godoy et al. 2016). In addition, ASR directly impacts the soy market, as it leads to a drop in productivity (grain) and consequently of its derivatives (oil, bran, protein) (Ishiwata and Furuya 2020).

ASR is caused by the fungus Phakopsora pachyrhizi (Sydow and Sydow), an obligatory biotrophic basidiomycete, which has multiple infection cycles in the same crop, with a high capacity to produce uredospores and to easily disseminate (Chander et al. 2019). In addition, it has a high intraspecific variability of isolates and wide geographic distribution and is extremely severe and difficult to control (Darben et al. 2020). These characteristics drive the efforts of scientists, research agencies, and institutions from different countries, who work in a continuous search for management strategies to control the disease (Meira et al. 2020).

Currently, fungicide use is the most commonly used method to manage ASR (Murithi et al. 2021). However, fungicide costs are extremely high ($2.2 billion per harvest), and their ineffectiveness has increased with pathogen insensitivity every cropping season (Godoy et al. 2016). Thus, the use of resistant cultivars may be a promising strategy, being more economical, safe, efficient, and sustainable (Godoy et al. 2016; Ishiwata and Furuya 2020; Murithi et al. 2021).

Genetic resistance can be characterized as the ability of a plant to prevent and/or delay pathogen entry and development in its tissues. This mechanism can occur through resistance directly or indirectly controlled by genes, which can detect the presence of pathogens and initiate a signal cascade, resulting in resistance mechanism activation (Nelson et al. 2018; Zaidi et al. 2018). Resistance can be considered qualitative, when governed by a gene with a greater effect (major genes), or quantitative when governed by several genes with less effect (Nelson et al. 2018). Seven major ASR resistance genes have been reported in soybean, known as Rpp1 to Rpp7 (Resistance to P. pachyrhizi) (Bromfield and Melching 1982; Childs et al. 2018; Garcia et al. 2008; Hartwig 1986; Hyten et al. 2007; Li et al. 2012; Yu et al. 2015). These genes interact with pathogen avirulence genes, resulting in different resistance reactions, ranging from reddish-brown lesions (RB), with little or no sporulation, to immunity (absence of lesions) according to isolate severity (Godoy et al. 2016; Langenbach et al. 2016).

In Brazil, genetic resistance to ASR has been used in soybean breeding programs through the release of resistant cultivars. Resistant cultivars have a technology named according to the breeding company such as Inox® of TMG (Tropical Melhoramento e Genética), Shield® of Embrapa (Empresa Brasileira de Pesquisa Agropecuária) (Aoyagi et al. 2020), and TF of GDM (Grupo Don Mario) Genética do Brasil S.A. It is known that there is huge variability in P. pachyrhizi isolates with different degrees of severity and aggressiveness, which can increase the chances of breaking down the rust resistance gene (Darben et al. 2020; Yamanaka et al. 2015). Thus, a broad, effective, and long-lasting range of resistance can be developed through gene pyramiding (Mundt 2018).

Gene pyramiding combines multiple resistance genes in a single genotype (Chander et al. 2019; Mundt 2018). Several studies have been successfully used to improve disease resistance, mainly ASR (Lemos et al. 2011; Parhe et al. 2017; Vigano et al. 2018; Yamanaka et al. 2013; Yamanaka et al. 2015; Yamanaka and Hossain 2019). However, there is little information about the effectiveness of Rpp gene combinations and the pyramiding effect in controlling ASR (Yamanaka and Hossain 2019). Furthermore, the genetic bases are individual of each plant, and the interaction of Rpp-pyramided lines with the environment can result in different resistance phenotypes. Thus, to understand the pyramiding effect, it is necessary to develop and evaluate sister lines, mainly under field conditions (Yamanaka et al. 2015).

Thus, the objective of this study was to evaluate sister lines, previously evaluated by Meira et al. (2022), presenting different Rpp-pyramided genes for resistance to P. pachyrhizi and to clarify the pyramiding effect of two originally developed Rpp-pyramided lines compared to two existing lines or lines possessing only a single Rpp of resistance under field conditions.
Material and methods

Plant material

Seven sister lines from three populations, carrying two Rpp-pyramided genes, four resistance sources (PI 594723-Rpp1-b, PI 594538A-Rpp1-b, PI 230970-Rpp2, PI 459025A-Rpp4) and four resistance control (PI 200487-Rpp5, PI 200492-Rpp1, PI 506764-Rpp3+Rpp5, and PI 587880A-Rpp1-b) (Plant introduction: PI), and five resistant and six susceptible commercial cultivars were evaluated in this study (Table 1; Fig. 1). The Rpp-pyramided lines were developed from double crosses between F1 plants, obtained from susceptible Brazilian commercial cultivars (63I64RSF IPRO and 55I57RSF IPRO) and four different ASR resistance sources (PI 594723-Rpp1-b, PI 594538A-Rpp1-b, PI 230970-Rpp2, PI 459025A-Rpp4) (Table 1) obtained from previous studies by Meira et al. (2022). These lines were selected through marker-assisted selection in the F2 and F3 generations to confirm the presence of two Rpp genes. Information on molecular markers of all strains used in the present study is available at Meira et al. (2022), and they can see Supplemental Table 1. The F4 generation of the lines was evaluated under field conditions. These Rpp-pyramided lines were developed by the breeding company GDM Genética do Brasil S.A.

Table 1 Description and pedigree information of Rpp-pyramided sister lines, resistance source and resistance control (PI) and commercial resistant and susceptible cultivars evaluated

| Population | Genotype | Gene   | Phenotype | Pedigree                                                                 |
|------------|----------|--------|-----------|--------------------------------------------------------------------------|
| P1*2^b     | 52116-54 | Rpp2+Rpp1-b | gNI       | [(PI 230970×55I57RSF)×(PI 594723×63I64RSF)]                               |
| P1*2       | 52116-74 | Rpp2+Rpp1-b | NI        | [(PI 230970×55I57RSF)×(PI 594723×63I64RSF)]                               |
| P1b2       | 52117-21 | Rpp2+Rpp1-b | NI        | [(PI 230970×55I57RSF)×(PI 594538A×63I64RSF)]                               |
| P1b2       | 52117-57 | Rpp2+Rpp1-b | NI        | [(PI 230970×55I57RSF)×(PI 594538A×63I64RSF)]                               |
| P1b2       | 52117-59 | Rpp2+Rpp1-b | NI        | [(PI 230970×55I57RSF)×(PI 594538A×63I64RSF)]                               |
| P24        | 52117-60 | Rpp2+Rpp4   | NI        | [(PI 230970×55I57RSF)×(PI 459025A×63I64RSF)]                               |
| P24        | 52117-63 | Rpp2+Rpp4   | NI        | [(PI 230970×55I57RSF)×(PI 459025A×63I64RSF)]                               |
| –          | b95R51   | No Rpp gene | Susceptible | –                                                      |
| –          | b95Y72   | No Rpp gene | Susceptible | –                                                      |
| –          | ^BMX Raio IPRO | No Rpp gene | Susceptible | –                                                      |
| –          | ^BMX Zeus IPRO | No Rpp gene | Susceptible | –                                                      |
| –          | BRS 511  | Rpp5      | Resistant  | –                                                      |
| –          | BRS 531  | Rpp1-b    | Resistant  | –                                                      |
| –          | BRS 539  | Rpp1-b+Rpp4 | Resistant  | –                                                      |
| –          | ^NK 6201 | No Rpp gene | Susceptible | –                                                      |
| –          | ^NS 6700 | No Rpp gene | Susceptible | –                                                      |
| –          | ^PI 200487 | Rpp5       | Resistant  | –                                                      |
| –          | ^PI 200492 | Rpp1       | Resistant  | –                                                      |
| –          | ^PI 230970 | Rpp2       | Resistant  | –                                                      |
| –          | ^PI 459025A | Rpp4     | Resistant  | –                                                      |
| –          | ^PI 506764 | Rpp3+Rpp5 | Resistant  | –                                                      |
| –          | ^PI 587880A | Rpp1-b     | Resistant  | –                                                      |
| –          | ^PI 594538A | Rpp1-b     | Resistant  | –                                                      |
| –          | ^PI594723 | Rpp1-b    | Resistant  | –                                                      |
| –          | ^TMG7058 | NI        | Resistant  | –                                                      |
| –          | ^TMG7062 | NI        | Resistant  | –                                                      |

^AGDM genética do Brasil S.A., ^bPioneer seeds, ^BMX: Embrapa – Empresa Brasileira de Pesquisa Agropecuária, ^syngenta seeds, ^US National Plant Germplasm System, ^TMG – Tropical Melhoramento e Genética, ^– no information, ^Population code. For more information view Meira et al. (2022)
Field experiments

The Rpp-pyramided lines, four resistance control, four resistance sources and resistant and susceptible commercial cultivars (Table 1) were evaluated under field conditions at the experimental area at the Federal University of Technology – Paraná (UTFPR), Campus Pato Branco (26° 13’ 43” S; 52° 40’ 14” O; 760-m altitude), in Pato Branco, State of Paraná, Brazil. The climate is classified as Cfa (temperate climate, without a dry season and hot summer) according to the Köppen climate classification (Alvares et al. 2013).

Four weeks before sowing, the border area was sowed to increase pathogen occurrence. Sowing was realized on a non-preferential date (December 1st 2020) to enable the natural occurrence and development of ASR, and no fungicide was used to control the disease. The field experiments were performed using a randomized block design with three replications. Each plot was composed of two 3-m rows spaced 0.5 m apart, totaling 3 m², with a seed density of 14 seeds m⁻¹. Fertilizer management and pest control were performed in accordance with the technical recommendations for soybean crops, and weed control was performed manually.

Resistance evaluation

Ten leaflets from the middle third of the soybean plants in each plot were collected at the R5 growth stage (Fehr and Caviness 1977). Leaflets were analyzed in the laboratory to determine the number of lesions (NL), sporulation level (SL), frequency of lesions with uredinia (%LU), and number of uredinia per lesion (NoU) in 1 cm² of leaf tissue. These evaluations were performed using a binocular stereo microscope with a magnification of 4× and 10× objective lens, resulting in a magnification used of 14×. According to the data obtained from each plot, the

Fig. 1 Phakopsora pachyrhizi sporulation in soybean Rpp-pyramided lines, resistance source and commercial resistant and susceptible cultivars. Photographs of the abaxial leaf segment, with 14 × magnification. S susceptible, R resistant, NI no information
classification criteria to determine the resistance of ASR were by Yamanaka et al. 2020 (Tables 2 and 3). Collected data of NL, %LU, NoU, and SL were submitted to analysis of variance, and when a significant effect to genotype factor was detected using test $F (p<0.01)$, the mean was grouped using Skott Knott test ($p<0.05$). Data analysis was performed using ExpDes.pt package (Ferreira et al. 2014) in R software v. 4.0.3 (R Development Core Team 2020).

ASR lesion type (RB): according to a visual scale adapted from Yamanaka et al. (2010) and Miles et al. (2011), five infected leaves of different plants in the middle third of the plants were visually evaluated. Lesion types were recorded as immune (IM), no sporulation of reddish-brown lesions (RB1), little sporulation (RB2), moderate sporulation (RB3), and reaction for abundant sporulation (TAN).

### Results

The variance analysis showed significant effects on all evaluated resistance characteristics: number of lesions (NL), frequency of lesions per uredinia (%LU), number of uredinia per lesion (NoU), and sporulation level (SL) (Table 4). The heritability ranged from 0.91 to 0.97, showing the highest genetic effects.

The border sowed a few times before the $Rpp$-pyramided lines, resistance sources, and commercial cultivars contributed to the presence of pathogen inoculum in the area. The presence of ASR in the experimental area was confirmed by susceptible lesions with abundant sporulation (TAN) in the susceptible commercial cultivars (NK6201, NS6700, 95R51, 95Y72, BMX Zeus IPRO, and BMX Raio IPRO) (Fig. 1, Table 5). The resistance characteristics to ASR on $Rpp$-pyramided lines, resistance sources, resistance control, and resistant and susceptible commercial cultivars are presented in Fig. 1 and Table 5.

The $Rpp$-pyramided lines 52116-74 [(PI 230970 × 55i57RSF) × (PI 594723 × 63i64RSF)], 52117-21, 52117-57 [(PI 230970 × 55i57RSF) × (PI 594538A × 63i64RSF)], carrying $Rpp2 + Rpp1-b$ genes, showed no sporulation (SL = 0). Furthermore, these lines were statically similar to their parents carrying $Rpp1-b$ (PI 594723 and PI 594538A) (Table 5). However, line 52117-59 [(PI 230970 × 55i57RSF) × (PI 594538A × 63i64RSF)],

### Table 2 Classification criteria to determine resistance of differential genotypes: frequency of lesions with uredinia (%LU), number of uredinia per lesion (NoU), and sporulation level (SL), by Yamanaka et al. (2020)

| Resistance characters | Resistant (R) | Susceptible (S) |
|-----------------------|---------------|-----------------|
| %LU                  | 0.0 ≤ x < 70.0 | 70.0 ≤ x ≤ 100.0 |
| NoU                  | 0.0 ≤ x ≤ 2.0 | 2.0 ≤ x         |
| SL                   | 0.0 ≤ x ≤ 2.0 | 2.0 ≤ x ≤ 3.0   |

### Table 3 Classification criteria of rust resistance of soybean genotypes, by Yamanaka et al. (2020)

| Resistance categories | Criteria |
|-----------------------|----------|
| Immune (IM)           | Having no lesions |
| Highly resistant (HR) | Having lesions showing the resistant phenotype for all three characters and without uredinia |
| Resistant (R)         | Having lesions showing the resistant phenotype in all three resistance characters with uredinia |
| Slightly resistant (SR) | Having lesions showing the resistant phenotype for any of three resistance characters |
| Susceptible (S)       | Having lesions showing the susceptible phenotypes for all three resistance characters |

### Table 4 Analysis of variance to resistance characters number of lesions (NL), frequency of lesions per uredinia (%LU), number of uredinia per lesion (NoU) and sporulation level (SL) of $Rpp$-pyramided sister lines, resistance sources and resistance control and commercial cultivars resistant and susceptible to ASR

| Parameters | NL | %LU | NoU | SL |
|------------|----|-----|-----|----|
| Heritability | 0.97 | 0.91 | 0.94 | 0.97 |
| Genotypic variance | 534.75** | 0.17** | 1.78** | 1.47** |
| Residual variance | 56.43 | 0.05 | 0.31 | 0.15 |
| Mean | 26.23 | 0.35 | 1.63 | 1.50 |
| CV (%) | 28.64 | 34.50 | 34.58 | 25.40 |

CV coefficient of variation

**Significant at 1% by F test, respectively
showed higher values of SL = 0.63 compared to its sisters.

The Rpp-pyramided line 52117-60 developed by double crossing [(PI 230970 × 55i57RSF) × (PI 459025A × 63i64RSF)], carrying Rpp2 + Rpp4 presented no sporulation (SL = 0), and shows better results than those of parents PI 230970 (SL = 1.33) and PI 459025A (SL = 3) (Fig. 1, Table 5). Among the evaluated lines, 52117-63 (Rpp2 + Rpp4, SL = 2), followed by 52116-54 (Rpp2 + Rpp1-b, SL = 1), showed the highest sporulation levels.

Higher SL was observed in susceptible commercial cultivars 95R51, 95Y72, BMX Raio IPRO, BMX Zeus IPRO, NK6201, and NS6700 (Table 5). In addition, the resistant commercial cultivars TMG7062 and PI 459025A (Rpp4) showed high SL (SL = 3). It is worth mentioning that the resistant commercial cultivars BRS531 (Rpp1-b) and BRS539 (Rpp1-b + Rpp4) showed no sporulation (SL = 0).

The sister lines 52117-21 and 52117-57, descendants of double crossing [(PI 230970 × 55i57RSF) × (PI 594538A × 63i64RSF)] (Rpp2 + Rpp1-b), 52117-60 [(PI 230970 × 55i57RSF) × (PI 459025A × 63i64RSF)], showed null %LU and NoU (Table 5). These lines showed lower values than their resistant parent, carrying a single Rpp gene PI 230970 (Rpp2, NoU = 1.01; %LU = 76.67), PI 459025 (Rpp4, NoU = 2.88; %LU = 100), and its sister line 52117-59 (Rpp2 + Rpp1-b, NoU = 0.86; %LU = 33.33). On the other hand, the line 52117-74 (Rpp2 + Rpp1-b) showed similar values of NoU and %LU to its genitors, PI 594723 (Rpp1-b, NoU = 0; %LU = 0) and PI

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**Table 5** Phenotypic classification (Class) based in characters’ resistant of Phakopsora pachyrhizi: number of lesions (NL), frequency of lesions with uredinia (%LU), number of uredinia per lesion (NoU), sporulation level (SL) and lesion type (RB) in soybean Rpp-pyramided sister lines, commercial cultivars resistant and susceptible, resistance sources and resistance control

| Genotype      | Gene        | SL    | %LU    | NoU    | NL  | Class¹ | RB   |
|---------------|-------------|-------|--------|--------|-----|--------|------|
| 52116-54      | Rpp2 + Rpp1-b | 1.00c | 18.79b | 0.33d  | 14d | R      | RB1  |
| 52116-74      | Rpp2 + Rpp1-b | 0.00d | 0.00b  | 0.00d  | 5e  | HR     | RB1  |
| 52117-21      | Rpp2 + Rpp1-b | 0.00d | 0.00b  | 0.00d  | 1e  | HR     | IM/RB |
| 52117-57      | Rpp2 + Rpp1-b | 0.00d | 0.00b  | 0.00d  | 0e  | IM     | IM/RB |
| 52117-59      | Rpp2 + Rpp1-b | 0.67c | 33.33b | 0.86c  | 7e  | R      | IM/RB |
| 52117-60      | Rpp2 + Rpp4   | 0.00d | 0.00b  | 0.00d  | 1e  | HR     | IM/RB |
| 52117-63      | Rpp2 + Rpp4   | 2.00b | 100.00a| 1.65c  | 27d | SR     | RB3  |
| 52117-60      | Rpp2 + Rpp4   | 0.00d | 0.00b  | 0.00d  | 1e  | IM/RB  | IM/RB |
| 52117-60      | Rpp2 + Rpp4   | 0.00d | 0.00b  | 0.00d  | 1e  | IM/RB  | IM/RB |
| 52117-60      | Rpp2 + Rpp4   | 0.00d | 0.00b  | 0.00d  | 1e  | IM/RB  | IM/RB |
| 52117-60      | Rpp2 + Rpp4   | 0.00d | 0.00b  | 0.00d  | 1e  | IM/RB  | IM/RB |
| 52117-60      | Rpp2 + Rpp4   | 0.00d | 0.00b  | 0.00d  | 1e  | IM/RB  | IM/RB |
| 52117-60      | Rpp2 + Rpp4   | 0.00d | 0.00b  | 0.00d  | 1e  | IM/RB  | IM/RB |
| 52117-60      | Rpp2 + Rpp4   | 0.00d | 0.00b  | 0.00d  | 1e  | IM/RB  | IM/RB |
| 52117-60      | Rpp2 + Rpp4   | 0.00d | 0.00b  | 0.00d  | 1e  | IM/RB  | IM/RB |
| 52117-60      | Rpp2 + Rpp4   | 0.00d | 0.00b  | 0.00d  | 1e  | IM/RB  | IM/RB |
| 52117-60      | Rpp2 + Rpp4   | 0.00d | 0.00b  | 0.00d  | 1e  | IM/RB  | IM/RB |
| 52117-60      | Rpp2 + Rpp4   | 0.00d | 0.00b  | 0.00d  | 1e  | IM/RB  | IM/RB |
| 52117-60      | Rpp2 + Rpp4   | 0.00d | 0.00b  | 0.00d  | 1e  | IM/RB  | IM/RB |
| 52117-60      | Rpp2 + Rpp4   | 0.00d | 0.00b  | 0.00d  | 1e  | IM/RB  | IM/RB |
| 52117-60      | Rpp2 + Rpp4   | 0.00d | 0.00b  | 0.00d  | 1e  | IM/RB  | IM/RB |
| 52117-60      | Rpp2 + Rpp4   | 0.00d | 0.00b  | 0.00d  | 1e  | IM/RB  | IM/RB |
| 52117-60      | Rpp2 + Rpp4   | 0.00d | 0.00b  | 0.00d  | 1e  | IM/RB  | IM/RB |
| 52117-60      | Rpp2 + Rpp4   | 0.00d | 0.00b  | 0.00d  | 1e  | IM/RB  | IM/RB |
| 52117-60      | Rpp2 + Rpp4   | 0.00d | 0.00b  | 0.00d  | 1e  | IM/RB  | IM/RB |
| 52117-60      | Rpp2 + Rpp4   | 0.00d | 0.00b  | 0.00d  | 1e  | IM/RB  | IM/RB |
| 52117-60      | Rpp2 + Rpp4   | 0.00d | 0.00b  | 0.00d  | 1e  | IM/RB  | IM/RB |
| 52117-60      | Rpp2 + Rpp4   | 0.00d | 0.00b  | 0.00d  | 1e  | IM/RB  | IM/RB |
594538A (\textit{Rpp1-b}, NoU = 0.33; %LU = 33) (Table 5, Fig. 1).

The lines 52116-54 (\textit{Rpp2 + Rpp1-b}) [(\textit{PI 230970 × 55i57RSF}) × (\textit{PI 594723 × 63i64RSF})], and 52117-59 [(\textit{PI 230970 × 55i57RSF}) × (\textit{PI 594538A × 63i64RSF})] showed lower values of %LU, by 18.79 and 33.33, and NoU 0.33 and 0.86, respectively. Although these lines present lesions with uredinia these values are lower than the observed values for resistance sources and resistance control carrying a single \textit{Rpp} gene such as PI 200487 (\textit{Rpp5}, %LU = 94.87; NoU = 1.33), PI 200492 (\textit{Rpp1}, %LU = 100.00; NoU = 2.36), PI 230970 (\textit{Rpp2}, %LU = 76.67; NoU = 1.01), PI 459025A (\textit{Rpp4}, %LU = 100.00; NoU = 2.88), PI 506764 (\textit{Rpp3 + Rpp5}, %LU = 100.00; NoU = 2.31), and PI 587880A (\textit{Rpp1-b}, %LU = 63.14; NoU = 1.14).

Higher %LU values were observed for commercial cultivars 95R51, 95Y72, BMX Raio IPRO, BMZ Zeus IPRO, BRS511, NK6201, NS6700, TMG7058, and TMG7062 and to resistant control PI 200492, and PI 506764, and resistance source PI 459025A, which showed 100.00. Genotypes BRS539 (%LU = 66.67), PI 200487 (%LU = 94.87), PI 230970 (%LU = 76.67), and PI 587880A (%LU = 63.14) and the \textit{Rpp}-pyramided line 52117–63 (%LU = 100.00) also showed higher values of %LU and did not differ from susceptible commercial cultivars. Higher values of NoU (>3.00) were detected in 95R51, 95Y72, BMX Raio IPRO, BMZ Zeus IPRO, BRS511, NK6201, NS6700, and TMG7062.

All susceptible commercial cultivars, as controls, showed susceptible phenotypic reactions characterized by TAN lesions (Table 5). Immunity associated with few reddish brown lesions without sporulation (IM/RB1) was observed in \textit{Rpp}-pyramided lines 52117-21, 52117-57, 52117-59, 52117-60, and BRS531. In addition, reddish brown lesions without sporulation (RB1) were present in \textit{Rpp}-pyramided lines 52116-54 and 52116-74 in PI 594538A and PI 594723, and in the commercial cultivar BRS539. Only the line 52117-63 showed RB3 type lesion.

The NoU and %LU values for each genotype enabled the classification of ASR resistance (Table 5). Only the \textit{Rpp}-pyramided line 52117-57 [(\textit{PI 230970 × 55i57RSF}) × (\textit{PI 594538A × 63i64RSF})] carrying \textit{Rpp2 + Rpp1-b} was classified as immune (IM) because of the absence of lesions, no uredinias, and no sporulation (NL, NE, NoU, and %LU = 0). \textit{Rpp}-pyramided lines 52116-74, 52117-21, and 52117-60 were classified as highly resistant (HR), along with the commercial cultivar BRS531 and resistance source PI 594723. The genotypes 52116-54, 52117-59, BRS539, PI 587880A, and PI 594538A were phenotypically classified as resistant (R) (Table 5). The \textit{Rpp}-pyramided line 52117-63, commercial cultivars BRS511 and TMG7058, and resistance control PI 200487 and resistance source PI 230970 were classified as slightly resistant (SR) (Table 5).

Genotypes that showed values of NoU and NE ≥ 2.0 and %LU > 70.0 were classified as susceptible (S). The commercial cultivars, susceptible controls (95R51, 95Y72, BMX Raio IPRO, BMZ Zeus IPRO, BRS511, NK6201, and NS6700) presented highest values for NE, %LU, and NoU. Furthermore, the resistant commercial cultivar TMG7062 was phenotypically classified as S, together with resistance source PI 459025A, and resistance control PI 506764, and PI 200492 (Table 5).

The number of genotypes by pyramids and by phenotypic classification is presented in Fig. 2. \textit{Rpp}-pyramided gene combinations resulted in IM genotype 52117–57, carrying \textit{Rpp2 + Rpp1-b}, developed by double crosses among the parents [(\textit{PI 230970 × 55i57RSF}) × (\textit{PI 594538A × 63i64RSF})]. \textit{Rpp}-pyramided lines carrying \textit{Rpp2 + Rpp4}, two lines carrying \textit{Rpp2 + Rpp1-b}, two genotypes carrying \textit{Rpp1-b} were classified as highly resistant (HR) to ASR. Two PIs carrying a single \textit{Rpp1-b} gene were classified as R. Two \textit{Rpp}-pyramided lines carrying \textit{Rpp1-b} were classified as highly resistant (HR) to ASR. Two PIs carrying a single \textit{Rpp1-b} gene were classified as R (Fig. 2, Table 5).

The resistant source carrying single gene \textit{Rpp2} and resistance control carrying single gene \textit{Rpp5}, and the \textit{Rpp}-pyramided population carrying \textit{Rpp2 + Rpp4} were classified as slightly resistant (SR) to ASR. Furthermore, the susceptible commercial cultivars (carrying no \textit{Rpp} genes) and resistance control carrying \textit{Rpp4}, \textit{Rpp3 + Rpp5}, and \textit{Rpp1} showed susceptible (S) phenotypic classification under field conditions.
Pyramidation consists of the combination of several genes in the same genotype, resulting in their simultaneous expression in the host (Chander et al. 2019). This provides broader, longer lasting, and higher-level resistance because of the effects of multiple genes against Phakopsora pachyrhizi (Yamanaka et al. 2015; Yamanaka and Hossain 2019; Chander et al. 2019).

The lines evaluated in the present study were developed and validated by molecular markers by Meira et al. (2022). In their study, Meira et al. (2022) identified lines with different resistance reactions (IM, RB1, RB2, RB3, and TAN), and only populations of the best combinations of Rpp-pyramided genes (showing IM and RB1 resistance reactions), and with enough seed to perform field trials, with replicates, were selected to be further evaluated in this study. Sporulation levels, number of uredia per lesion, frequency of lesions with uredia, number of lesions, in addition to photographs of lesions of each line, resistance source and resistance control (PI), and susceptible and resistant cultivars were performed in the present study. Subsequently, these were classified according to Yamanaka et al. (2020). Therefore, more generous information on combinations of Rpp-pyramided genes and resistance sources and resistance control is described in the present study.

In the present study, Rpp-pyramided lines 52117-21 (Rpp2+Rpp1-b), 52117-57 (Rpp2+Rpp1-b), 52117-59 (Rpp2+Rpp1-b)+52117-60 (Rpp2+Rpp4) showed high resistance levels compared to resistant control, carrying a single Rpp gene such as PI 200487 (Rpp5), PI 200492 (Rpp1), and PI 506764 (Rpp3+Rpp5), and resistance source PI 230970 (Rpp2), PI 459025A (Rpp4). Similar results were reported by Yamanaka et al. (2015), who showed higher resistance in pyramided lines No6-12-B (Rpp4+Rpp5), Oy49-4 (Rpp2+Rpp3+Rpp4), and No6-12-1 (Rpp2+Rpp4+Rpp5) than in the resistant sources PI 230970 (Rpp2), PI 506764 (Rpp3+Rpp5), PI 459025 (Rpp4), and PI 200487 (Rpp5). Lemos et al. (2011) also reported successful
results for Rpp-pyramided lines. These authors obtained higher resistance levels to lines carrying Rpp2 + Rpp4 + Rpp5 than their parents carrying a single Rpp gene (PI 230970, PI459025, and PI 200487). The line 52117-54 (Rpp2 + Rpp1-b), showed high resistance levels compared to resistant source PI 230970 (Rpp2), but showed resistance level smaller than PI 594723, the resistance source of Rpp1-b. The line 52117-63 (Rpp2 + Rpp4) showed similar resistance levels than resistance source PI 230970 (SR). Similar results were reported by Yamanaka and Hossain (2019) when line No12-1A carrying Rpp2 + Rpp5 showed resistance levels less than source resistance PI 200487 (Rpp5). The authors suggested that genetic factors important to resistance to ASR were lost with the pyramiding.

The Number of uredinia per lesion (NoU) and frequency of lesions with uredinia (%LU) are reliable parameters for determining resistance to ASR, classifying resistant and susceptible genotypes (Kashiwa et al. 2020). In our studies, Rpp-pyramided lines showed less %LU and NoU than those of their parents and resistance control. Similar results have been reported in several studies, with different gene pyramiding combinations (Lemos et al. 2011; Yamanaka et al. 2013, 2015; Yamanaka and Hossain 2019). In addition, the lines 52117-21, 52117-57, 52116-74 and 52117-60 showed no sporulation (Fig. 1, Table 5). These results corroborate those of Yamanaka et al. (2013) and Yamanaka and Hossain (2019), who observed the absence of uredinia formation and sporulation in lines with pyramided Rpp genes.

Gene pyramiding directly affects the formation of the pathogen’s reproductive structures, preventing and/or reducing uredinia formation and sporulation. Uredinias are responsible for the release of spores, which spread the fungus (Kashiwa et al. 2020). With the reduction of these structures, damage to the leaf area is minimized, maintaining a larger photosynthetically active area, and improving light interception, generating a greater accumulation of photoassimilates, resulting in higher yields (Godoy et al. 2016).

Among the Rpp-pyramided combinations, lines carrying Rpp2 + Rpp1-b showed immunity and high resistance phenotypic reactions (IM, HR, and R respectively), and lines carrying Rpp2 + Rpp4 presented HR and SR levels (Fig. 2). The level of resistance of the genotype is influenced by the number of genes and the combination of genes in the plant (Nelson et al. 2018). Within the same combination, with the same genetic basis, it was possible to observe differences between the levels of resistance of the sister lines (showed in Rpp2 + Rpp1-b and Rpp2 + Rpp4, for example). Small allelic differences in resistance genes, as well as different interactions between Rpp-pyramided genes, unknown genetic factors (in addition to Rpp genes), and the interaction by Rpp genes with plant genetic basis, besides environmental effects can influence resistance level (Yamanaka et al. 2015; Nelson et al. 2018; Kashiwa et al. 2020). All these factors may be contributing to different phenotypes in genotypes with the same genetic basis. Therefore, within the same combination of Rpp-pyramided genes, using the same parents, it is possible to observe differences in the phenotypic reactions to ASR as is between lines 52117-60 and 52117-63.

Among the resistance sources and resistance control evaluated in this study, the PI carrying Rpp1-b (PI 594538A, and PI 594723), and resistance control PI 587880A, and the resistant commercial cultivar BRS531 showed higher resistance levels (HR and R classification) (Fig. 1, Table 5). Genotypes carrying Rpp1-b have been reported to show high levels of resistance, especially against South American P. pachyrhizi isolates (Akamatsu et al. 2017; Hossain et al. 2015; Yamanaka et al. 2016) and African isolates (Murithi et al. 2021), corroborating the results obtained in this study. Thus, using the Rpp1-b gene as a resistance source can be a promising strategy for breeding programs with higher levels of resistance, particularly against highly aggressive isolates of ASR in South America (Hossain et al. 2015). However, it is worth noting that no isolated strategy can maintain the sustainability of the culture (Chander et al. 2019).

Thus, the genetic resistance promoted by simple or pyramided genes needs to be used strategically, maintaining an integrated long-term management to increase its durability and efficiency (Chander et al. 2019). The use of fungicides, for example, associated with the use of resistant cultivars, can help reduce inoculum, as well as reduce selection pressure applied by P. pachyrhizi on resistance genes (Godoy et al. 2016). Likewise, the use of resistant cultivars helps to reduce the selection pressure on fungicides by reducing the number of applications during the crop cycle.
Kato et al. (2022), for example, evaluated two soybean cultivars carrying three pyramided genes for resistance to ASR under field conditions, with and without fungicide application. The authors observed higher levels of resistance of cultivars with pyramided genes compared to susceptible parents, regardless of fungicide application. In other words, the association of the two control methods further increases the field resistance against ASR. In this way, using different management methods in an integrated way, in addition to increasing resistance levels, can help prevent fungicide resistance, in addition, increase the durability of genetic resistance.

In conclusion, Rpp-pyramided lines showed higher resistance levels to ASR, with significant reductions in SL, NoU, and %LU. The line 52117-57 carrying Rpp2+Rpp1-b showed phenotypic reaction of immunity under field conditions, and all evaluated Rpp-pyramided lines were classified as HR and R. Only the line 52117-63 showed resistance level SR, close to susceptibility. Furthermore, the different phenotypic reactions to ASR observed in sister lines highlighted the difference between genetic bases and phenotypic reactions.

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Author’s contributions All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by MCP, RATF, CPM, ORC, FCQGB. The first draft of the manuscript was written by MCP. LAM, DM, GM and SLBJ and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Data availability The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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