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ORIGINAL ARTICLE

Resistance to Asian soybean rust and yield of new soybean cultivars, JFNC 1 and JFNC 2, harboring three resistance genes

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Abstract
Asian soybean rust (ASR) caused by *Phakopsora pachyrhizi* is one of the most devastating diseases affecting soybean production. Recently, two new soybean cultivars, JFNC 1 and JFNC 2, harboring three ASR-resistance genes, *Rpp2*, *Rpp4*, and *Rpp5*, developed by line breeding and marker-assisted selection have been released in Paraguay. Furthermore, the cultivars JNFC 1 and JNFC 2 were evaluated by comparing them with their recurrent parents Aurora and YG 203, respectively, for disease severity and yield in the plots sprayed and unsprayed with fungicides during 2017/2018 and 2018/2019 in Paraguay. Disease parameters including infection index, number of uredinia per lesion, frequencies of lesions with uredinia, and sporulation level caused by ASR infection were also compared between the resistant cultivars and the recurrent parents in the laboratory. Disease severity was low in JFNC 1 and JFNC 2, irrespective of fungicide treatment; however, the disease severity in Aurora and YG 203 decreased considerably in plots sprayed with fungicides than those in the unsprayed plots. JFNC 1 and JFNC 2 were highly resistant to all disease parameters than Aurora and YG 203. Yield loss in unsprayed plots was in the range of 10–23% and 37–49% for JFNC 1 and Aurora, respectively, while it was 9–12% and 16–20% for JFNC 2 and YG 203, respectively. Yield loss in unsprayed plots was lower in JFNC 1 than that in Aurora. YG 203 may exhibit tolerance, considering its high disease severity and minimal yield loss in the unsprayed plots. JFNC 1 and JFNC 2 resisted ASR as observed from the values of the disease parameters. These results indicate that JFNC 1 and JFNC 2 were more resistant to ASR than their recurrent parents. Therefore, pyramiding the three *Rpp* genes into susceptible cultivars contributes to enhanced ASR resistance.

Keywords Gene pyramiding · Line breeding · Marker-assisted selection · *Phakopsora pachyrhizi* · *Rpp* genes

Introduction

Soybean [*Glycine max* (L.) Merr.], as a source of protein and oil, is a staple food crop for humans and a major feed crop in livestock farming. More than 80% of soybean is produced in the Americas, including the USA, Brazil, Argentina, Paraguay, and Canada (FAOSTAT 2021). Stable soybean production is vital for global food security. Asian soybean rust (ASR) caused by *Phakopsora pachyrhizi* Syd. & P. Syd. is one of the most serious biotic threats affecting soybean producers in South America. It was first reported in 2001 in the American continent, Paraguay and Brazil (Yorinori et al. 2005), spreading within a few years to most American countries, from Argentina to Canada.

The soybean-free period is one of the cultural control measures that has been adopted in Brazil and Paraguay. It reduces the amount of spores between crop seasons, thus retarding the onset of the disease in the regular sowing. However, it is difficult to eliminate all soybean plants, including volunteer soybeans and other hosts, in soybean production regions. A major control method for ASR is fungicide treatment. In Paraguay, fungicides were applied two to six times per crop season depending on the sowing date (Maldonado et al. 2019), and its use has increased the production cost of soybeans (Ishiwata and Furuya 2020). In Brazil, repeated application of a fungicide reduced its efficacy over time (Dalla Lana et al. 2018; Godoy et al. 2016). Recently, isolates with multiple resistance to demethylation

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inhibitors (DMIs), quinone-outside inhibitors (QoIs), and succinate dehydrogenase inhibitors (SDHIs) have been collected and reported in Brazil (Müller et al. 2021).

Another control measure is the use of resistant cultivars; eight loci resistant to ASR (*Rpp1* to *Rpp7*, including *Rpp1-b*) have been reported (Childs et al. 2018). The breeding of resistant cultivars has been performed in several countries. Pyramiding resistance genes is a promising strategy for developing resistant cultivars (Mundt 2014). JFNC 1 and JFNC 2 were developed by incorporating the *Rpp2*, *Rpp4*, and *Rpp5* genes of No6-12–1 (Yamanaka et al. 2013) into susceptible Paraguayan cultivars, Aurora and YG 203, through line breeding. Although the ASR resistance presented by No6-12–1 in a field was reported in Brazil, no yield was observed (Kato and Soares 2021). In this study, we evaluated the ASR resistance and yield of JFNC 1 or JFNC 2 by comparing them with their recurrent parents in a field in Paraguay to confirm the effect of gene pyramiding. Disease parameters of the cultivars were also compared in the laboratory. This study makes a novel contribution to the literature by demonstrating resistance of soybean cultivars pyramided with three ASR resistance genes.

Material and methods

Plant materials

Both JFNC 1 and JFNC 2, which are gene-pyramided cultivars harboring three ASR resistance genes, *Rpp2*, *Rpp4*, and *Rpp5*, were developed by Fundación Nikkei-Cetapar and the Japan International Research Center for Agricultural Sciences and registered in Paraguay in 2018. JFNC 1 was bred by back-crossing a genotype No6-12–1 (*Rpp*, *Rpp4*, and *Rpp5* donor) with Aurora (susceptible recurrent parent); the development of the resistant donor line No6-12–1 has been reported in previous studies (Yamanaka et al. 2013). JFNC 2 was bred by back-crossing No6-12–1 with YG 203 (susceptible recurrent parent, alternative name: YG00/26–19). Certification numbers for JFNC 1 and JFNC 2 in Departamento de Protección y Uso de Variedades, Paraguay, in 2021 are 955 and 1010, respectively.

Cultivation information

The experiments were conducted during the 2017–2018 crop season and the 2018–2019 season at the Capitán Miranda Investigation Center, Paraguayan Institute of Agriculture Technology, Capitán Miranda, Itapúa, Paraguay (27°12′ S; 55°48′ W). The experimental design was a randomized block design with three replicates. In the first experiment conducted on November 14, 2017, the seeds of the cultivars were sown at a density of 10 seeds per meter. Each plot had four rows of 5 m long with 0.45-m row spacing. Fungicides were applied three times (200 g/ha of 30% azoxystrobin + 15% benzovindiflupyr on January 30, 2018; 300 g/ha of 10% picoxystrobin + 20% cyproconazole on February 12, 2018; 200 g/ha of 30% azoxystrobin + 15% benzovindiflupyr on March 2, 2018). In the second experiment, the seeds were sown on October 30, 2018. Fungicides (200 g/ha of 30% azoxystrobin + 15% benzovindiflupyr) were applied on February 6 and 26, 2019.

Disease severity and yield assessment

Disease severity was rated on March 2, 2018, at the growth stage of R5 for Aurora and JFNC 1 and R6 for YG 203 and JFNC 2 (first experiment) and at the growth stage of R5 for all the cultivars on February 28, 2019 (second experiment). Ten leaflets of each cultivar were collected from the mid-height of the plants, and the disease severity was rated as diseased leaf area using a diagram illustrating ASR disease severity (Godoy et al. 2006; Yamanaka et al. 2021). For yield assessment, plants were harvested from the two rows at the center eliminating the 0.5-m border at both ends (4 m × 0.9 m) to avoid a border effect. After harvest, the plants were air-dried, and the grain yield and moisture were measured using an electronic balance and a grain moisture meter, respectively. The grain yield was adjusted to 13% moisture. Data for days to maturation, plant height, and 100-seed weight were obtained along with yield as reference data (Supplemental Table 1).

Disease parameter evaluation

Disease parameters, such as infection index, the number of uredinia per lesion, frequency of lesions with uredinia, and sporulation level were evaluated on the four soybean cultivars and No6-12–1 line in the laboratory using the Brazilian ASR population BRP-2. The BRP-2 population was obtained from Embrapa Soybean, Londrina, Parana, and was not an isolate derived from a single uredinium. BRP-2 was imported to Japan (import permit # 20Y157), stored in a deep freezer in JIRCAS, and used in disease parameter studies (Yamanaka et al. 2010, 2011, 2013). The urediniospores on detached leaflets of a susceptible cultivar 'BRS 184' were multiplied and cultured in an incubator. Urediniospores were collected using a paintbrush and suspended in 0.04% Tween 20 and adjusted to approximately 5.2×10^4/mL. Healthy first trifoliate leaflets were inoculated by uniformly spreading the suspension with a paintbrush, which were incubated at 24 °C with a photoperiod of 14-h light and 10-h dark. Urediniospore germination was evaluated one day after loading on 0.6% agar at 24 °C, and the percent germination was 95.3%. Infection index, number of uredinia per lesion, frequency of lesions with uredinia, and sporulation level were evaluated

14 days after inoculation. Infection index was obtained by visually counting the number of lesions and measuring the leaflet areas using a leaf area meter (AM200, ADC Bioscientific Ltd., Hoddesdon). Infection index was calculated by dividing the number of lesions by the product of the average leaflet area and the number of germinated urediniospores in 1 mL of spore suspension. Number of uredinia per lesion, frequency of lesions with uredinia, and sporulation level were evaluated according to the laboratory manual (Yamanaka et al. 2021). The average and standard error of the values were expressed by combining the scores obtained from 90 leaflets.

Statistical analysis

Disease severity (%) was analyzed after logit transformation: log (((disease severity + 0.05)/100)/(1 − (disease severity + 0.05)/100)). To avoid infinitive values by logit transformation, half of the minimum rating unit (0.05%) was added to the disease severity. The transformed disease severity was analyzed by analysis of variance (ANOVA) and compared among treatments using Tukey's honest significant difference (HSD) test. Yield was analyzed by ANOVA and compared using Tukey's HSD test. The statistical analysis package JMP software version 11 (SAS Co., Ltd. California) was used for the analyses.

Results

Disease severity

Comparing Aurora (susceptible recurrent cultivar) and JFNC 1 (resistant cultivar) produced by pyramiding *Rpp2*, *Rpp4*, and *Rpp5* genes into Aurora, disease severity was the highest in Aurora in the unsprayed plots, followed by Aurora in plots sprayed with fungicides, and was the lowest in JFNC 1 in unsprayed and sprayed plots in the two seasons (Fig. 1). A similar trend was also observed in YG 203 (susceptible recurrent cultivar) and JFNC 2 (resistant cultivar) produced by pyramiding the three *Rpp* genes into YG 203 (Fig. 1). The disease severity on JFNC 1 and JFNC 2 in the unsprayed plots was lower than that of their recurrent parents in the sprayed plots.

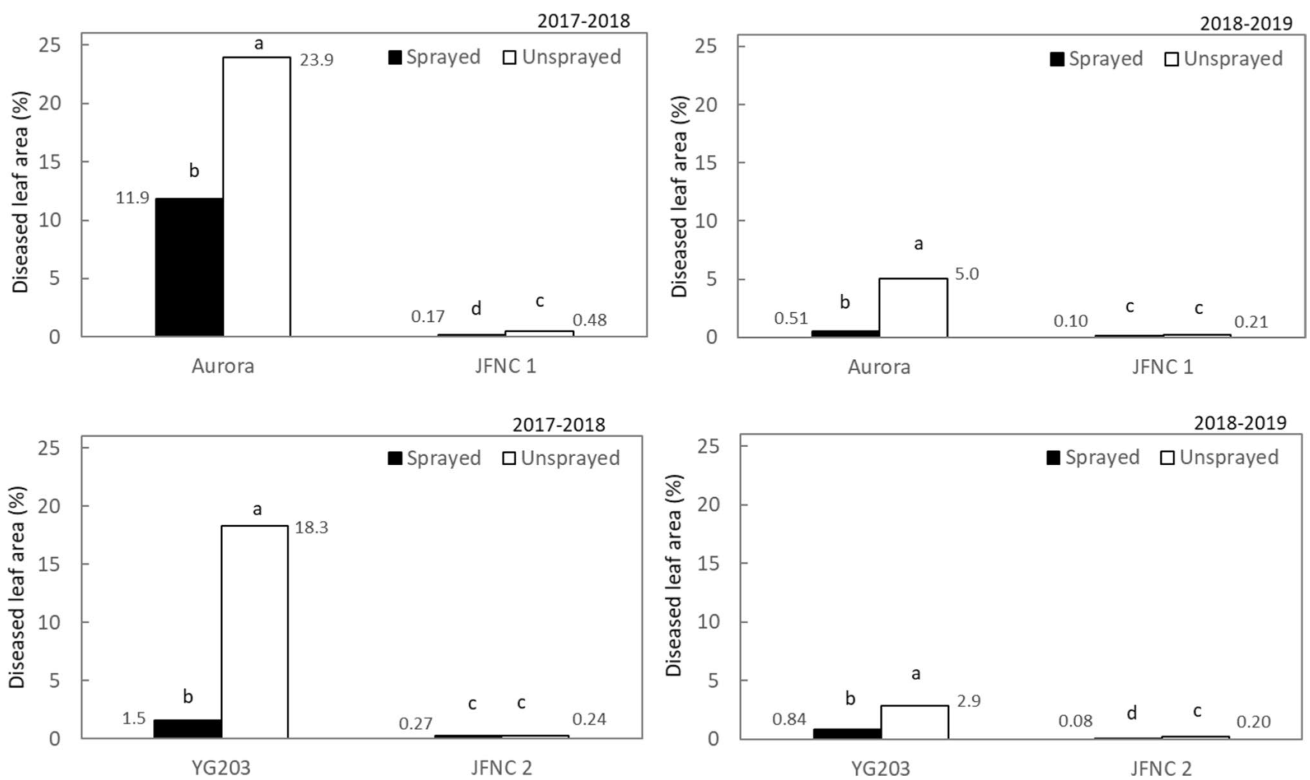

Fig. 1 Disease severity of Asian soybean rust on Aurora, JFNC 1, YG 203, and JFNC 2 in plots sprayed and unsprayed with fungicides during the 2017–2018 and 2018–2019 seasons assessed at R5–R6 stage. Disease severity is expressed as diseased leaf area (%). Letters at the top of bars correspond to a Tukey's HSD test with $P < 0.05$ significance

Resistance characteristics evaluated in the laboratory

The values of the evaluated disease parameters, such as infection index, number of uredinia per lesion, frequency of lesions with uredinia, and sporulation level, were high in Aurora and YG 203 (Table 1 and Supplemental Figs. 2 and 3). In contrast, JFNC 1 and JFNC 2 presented low values and were highly resistant. The infection index values for JFNC 1 and JFNC 2 were 19-fold and sevenfold lower than those for Aurora and YG 203, respectively. BRP-2 produced approximately 25-fold less number of uredinia per lesion on JFNC 1 and JFNC 2 than that on Aurora and YG 203 (Table 1). Values of frequency of lesions with uredinias for JFNC 1 and JFNC 2 were approximately tenfold less than those for Aurora and YG 203 (Table 1). The average scores of sporulation level for JFNC 1 and JFNC 2 were less than one, whereas those for Aurora and YG 203 were equal to or close to the highest score of three (Table 1). The values of the disease parameters of No6-12–1, the donor parent of *Rpp2*, *Rpp4*, and *Rpp5*, were similar to those of JFNC 1 and JFNC 2, although the values for No6-12–1 were slightly lower.

Yield

The yields of cultivars with and without fungicide treatments are shown in Tables 2 and 3. The yield recovered after fungicide treatments varies with season and cultivar. The yield reduction for Aurora was larger than that of JFNC 1 in the unsprayed plots. The ratio of yield in unsprayed plots to that in sprayed plots was 0.51–0.63 in Aurora and

Table 1 Infection index (IFI), the number of uredinia per lesion (NoU), frequency of lesion with uredinia (%LU), and sporulation level (SL) of new soybean cultivars JFNC1 and JFNC 2 and their recurrent and the donor parents.[1] by the Brazilian rust population BRP-2 infection under the leaf-culture experiment

Cultivar/line [2]	Resistance genes	IFI [3]	NoU [4]	%LU [5]	SL [6]
JFNC 1	*Rpp2*, *Rpp4*, *Rpp5*	0.24 ± 0.04	0.17 ± 0.07	8.9	0.17 ± 0.06
Aurora	– (susceptible)	4.70 ± 0.33	4.64 ± 0.18	100.0	3.00 ± 0.00
JFNC 2	*Rpp2*, *Rpp4*, *Rpp5*	0.40 ± 0.05	0.17 ± 0.05	12.2	0.23 ± 0.07
YG 203	– (susceptible)	2.94 ± 0.72	4.04 ± 0.17	100.0	2.98 ± 0.02
No6-12–1	*Rpp2*, *Rpp4*, *Rpp5*	0.19 ± 0.04	0.03 ± 0.02	2.2	0.00 ± 0.03

[1]Values are presented by the average ± standard error ($n=9$ for IFI and $n=90$ for NoU and SL)

[2]The donor parent of the resistance genes and the recurrent parent of JFNC 1 are No6-12–1 and Aurora, respectively. The donor parent of the resistance genes and the recurrent parent of JFNC 2 are No6-12–1 and YG 203, respectively

[3]IFI indicates the number of lesions per 1 cm^2 by germinated urediniospores in 1 mL of spore suspension

[4]NoU indicates the number of uredinia per lesion

[5]%LU indicates percent lesions producing uredinia

[6]SL sporulation score; 0, none; 1, few; 2, medium; 3, abundant

Table 2 Yield of soybean cultivars, Aurora and JFNC 1, in fungicide treated and untreated plots during the 2017–2018 and 2018–2019 seasons

Cultivar [1]	Fungicide	2017–2018			2018–2019		
		Yield (kg/ha)[2]		Ratio[3]	Yield (kg/ha)[2]		Ratio[3]
Aurora	Sprayed	2330	a	0.51	2410	a	0.63
	Unsprayed	1181	b		1516	a	
JFNC 1	Sprayed	2713	a	0.77	2871	a	0.90
	Unsprayed	2096	a		2586	a	
Factor	Degree of freedom	*F*-value	*P*-value		*F*-value	*P*-value	
Cultivar	1	17.3797	0.0059		6.5198	0.0433	
Fungicide	1	32.1959	0.0013		3.8653	0.0969	
Cultivar × fungicide	1	2.9195	0.1384		1.0324	0.3488	
Block	2	0.5189	0.6197		0.3463	0.7206	

[1]JFNC 1 was developed with line breeding of a donor parent No6-12-1 harboring resistance genes, *Rpp2*, *Rpp4*, and *Rpp5*, and a recurrent susceptible parent Aurora

[2]Different letters after the yield values indicate that the yield significantly differed within a column

[3]Ratio = unsprayed/sprayed treatments of fungicides

Table 3 Yield of soybean cultivars, YG 203 and JFNC 2, in fungicide treated and untreated plots in the 2017–2018 and 2018–2019 seasons

Cultivar [1]	Fungicide	2017–2018			2018–2019		
		Yield (kg/ha)[2]		Ratio[3]	Yield (kg/ha)[2]		Ratio[3]
YG 203	Sprayed	2196	bc	0.84	2811	a	0.80
	Unsprayed	1834	c		2238	c	
JFNC 2	Sprayed	3459	a	0.88	2715	ab	0.91
	Unsprayed	3059	ab		2461	bc	
Factor	Degree of freedom	*F*-value	*P*-value		*F*-value	*P*-value	
Cultivar	1	27.5195	0.0019		0.8242	0.3990	
Fungicide	1	2.5843	0.1591		34.7031	0.0011	
Cultivar × fungicide	1	0.0068	0.9371		5.1485	0.0638	
Block	2	2.0159	0.2140		5.8495	0.0390	

[1]JFNC 2 was developed with line breeding of a donor parent No6-12–1 harboring resistance genes, *Rpp2*, *Rpp4*, and *Rpp5*, and a recurrent susceptible parent YG 203

[2]Different letters after the yield values indicate that the yield differed significantly within a column

[3]Ratio = unsprayed/sprayed treatments of fungicides

0.77–0.90 in JFNC 1. ANOVA revealed that the yield was significantly affected by the cultivar type, that is, Aurora and JFNC 1, and was significantly affected by fungicides during the 2017–2018 season. Yield reduction of YG 203 in the unsprayed plots was as large as that of JFNC 2. The ratio of yield in unsprayed plots to that in sprayed plots was 0.80–0.84 for YG 203 and 0.88–0.91 for JFNC 2. ANOVA revealed that the yield of YG 203 and JFNC 2 was significantly affected by the cultivar type during the 2017–2018 season and was significantly affected by fungicides during the 2018–2019 season. Interactions between cultivars and fungicides were not significant.

Discussion

JFNC 1 and JFNC 2 were developed in a line breeding program between susceptible recurrent parents (Aurora and YG 203) and a resistant donor parent (No6-12–1 experimental line). Although both JFNC 1 and JFNC 2 harbor ASR-resistance genes, *Rpp2*, *Rpp4*, and *Rpp5* derived from No6-12–1, theoretically, the genome of JFNC cultivars is 98.4% identical to those of the original cultivars, because they belong to BC_5F_2 lines. Thus, they can be considered essentially derived cultivars of the recurrent parents. The combination of these three resistance genes was effective against ASR strains in Latin American countries such as Argentina, Brazil, Mexico, Paraguay, and Uruguay (Akamatsu et al. 2013, 2017; García-Rodríguez et al. 2022; Lemos et al. 2011; Stewart et al. 2019; Yamanaka et al. 2013). JFNC 1 and JFNC 2 are derived from Latin American cultivars by combining resistance genes effective in Latin America; hence, they are promising cultivars for reducing the damage caused by ASR.

The disease severity of JFNC 1 was considerably lower than that of Aurora, irrespective of fungicide treatment; even in the plots sprayed with fungicides, Aurora displayed a higher diseased severity than JFNC 1 cultivated in the unsprayed plots (Fig. 1). The difference was observed in the 2017—2018 season with severe epidemics of ASR and in 2018–2019 season with epidemics. Values of infection index, number of uredinia per lesion, frequency of lesions with uredinia, and sporulation level in JFNC 1 evaluated using a Brazilian rust population in the laboratory were lower than those in Aurora (Table 1). Infection index is related to lesion production, whereas number of uredinia per lesion, frequency of lesions with uredinia, and sporulation level relate to urediniospore production. The low values of the disease parameters in JFNC 1 decreased its disease severity in the field. A similar trend was also observed for JFNC 2 and YG 203 (Table 1). Values of the disease parameters in JFNC 1 and JFNC 2 were similar or slightly higher than those of No6-12–1 (Table 1), suggesting that the resistant donor line No6-12–1 may contain minor resistance genes, in addition to the three major *Rpp* genes, such as *Rpp2*, *Rpp4*, and *Rpp5*, incorporated into JFNC cultivars. However, the high resistance of JFNC cultivars has been observed by assessing their two independent genetic backgrounds, Aurora and YG 203; their high resistance is considered to be derived from the combination of three *Rpp* genes. Inheritance of high resistance from No6-12–1 was also observed in the early generation of JFNC 1 in a previous study (Yamanaka et al. 2013). The low disease severity, number of uredinia per lesion, and sporulation level of No6-12–1 have also been observed in the Brazilian fields (Kato and Soares 2021). The strong resistance offered by the combined three *Rpp* genes observed in this study supports the results of these previous studies.

Fungicide treatment reduced disease severity to less than 2%, except in Aurora during the 2017–2018 season, suggesting that the fungicides used in this study could effectively control ASR. The low effectiveness of sprayed plots for Aurora during 2107–2018 is likely due to the conducive weather. Precipitation reached 380 mm during mid- and late January, 2018, and 18 days of rain was observed from mid-January to the end of February, 2019 (Supplemental Fig. 1). Fungicide residues decreased with time after application. Since rust urediniospores can infect soybean multiple times in a season favoring ASR epidemics, ASR pathogens can successfully infect soybean and produce lesions. If the weather is not conducive to ASR, fungicide applications can effectively reduce disease severity, even in Aurora. Fungicide application effectively reduced the disease severity of another susceptible cultivar, YG 203. This suggests that YG 203 can defend against pathogen attack if the aggressiveness of the pathogen is slightly weakened using fungicides.

Fungicide treatment generally increases the yield via disease control. When the yield loss of resistant cultivars is smaller than those of susceptible cultivars under untreated conditions, the effect of fungicide on yield recovery is expected to be larger in susceptible cultivars. Interactions between cultivars and fungicides for yield were expected; however, their interaction was not significant, although the amount of yield loss in unsprayed plots was larger in Aurora in this study. Since the yield is affected by several factors, such as diseases (except for ASR), pests, and cultural conditions, the effect of fungicides might have been reduced. In this experiment, fungicide treatments recovered yield in Aurora in the 2017–2018 season and in YG 203 in the 2018–2019 season (Tables 2 and 3 and Fig. 2). There was a tendency of yield recovery by fungicide treatments in Aurora in the 2018–2019 season although it was not significant. These recoveries are probably due to reduction of ASR by the fungicide treatment. Yield was higher in the sprayed plots although some were not significant. Foliar and stem diseases such as ASR, Cercospora leaf blight, anthracnose, brown spot, seed diseases, and pod and stem blight are reported as diseases causing yield reduction in Paraguay (Wrather et al. 2010). Cercospora leaf blight, brown spot, and pod and stem blight other than ASR are labeled as target diseases of soybean on the fungicides used in this experiments. Actually Cercospora leaf blight was observed in the experiments. The fungicides may recover the yield through the control of these foliar diseases although the severity of the diseases was not recorded. Even though disease severity on unsprayed YG203 during the 2018–2019 season was less than 5%, yield was significantly lower than sprayed YG 203 (Table 3 and Fig. 2). The yield difference may be explained by the same reason.

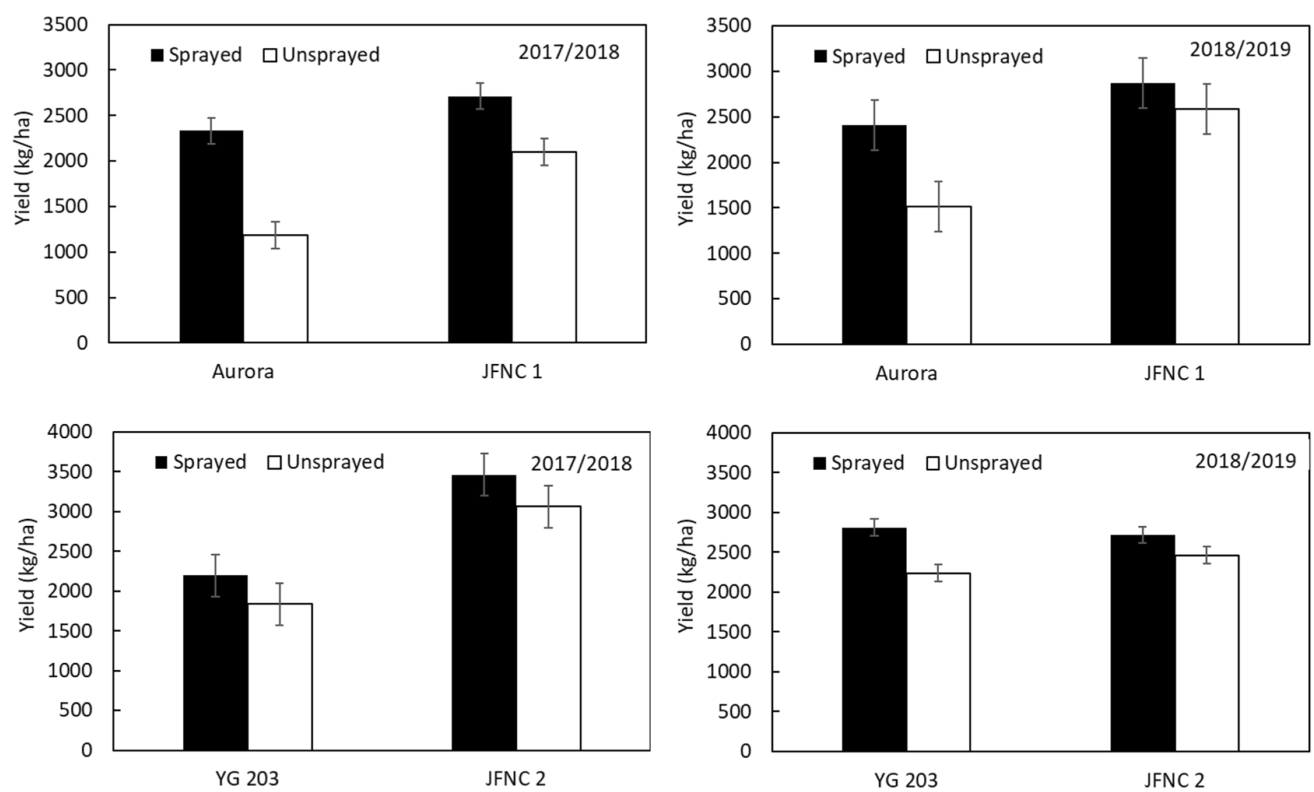

Fig. 2 Yield of soybean cultivars of Aurora, JFNC 1, YG 203, and JFNC 2 in fungicide treated and untreated plots in the 2017–2018 and 2018–2019 seasons. Vertical bars indicate standard error ($n=3$). Aurora and YG 203 are recurrent parents of JFNC 1 and JFNC 2, respectively

YG 203 exhibited disease severity values similar to those of Aurora in the unsprayed plots. However, the percent yield loss of YG 203 was smaller than that of Aurora. These results suggested that YG 203 is more tolerant to ASR than Aurora. The contribution of fungicide spray to yield recovery is expected to be minimal. The yield loss of JFNC 2 in the unsprayed plots was approximately 10%, indicating that JFNC 2 cultivation can save fungicide cost without a large reduction in profit from marketing soybean products. The yield of JFNC 2 was higher during the 2017–2018 season than that of YG 203, which may be due to line selection for better traits in the BC_5F_2 generation. This suggests that cultivars with high yield and ASR resistance by gene pyramiding can be selected.

Although yield reduction in unsprayed JFNC cultivars compared to sprayed their recurrent parents was observed in some cases, unsprayed JFNC varieties generally showed similar yield levels to sprayed their recurrent parents and JFNC cultivars (Supplemental Fig. 4). Cultivars that are resistant to diseases are expected to reduce fungicide costs. In Paraguay, farmers apply fungicides three or four times to soybeans sown in October and November (Maldonado et al. 2019). In the Pirapó cooperative, Itapúa, Paraguay, which is a major soybean-producing area, fungicide cost in 2018 was 146.0 USD and 55.8 USD for conventional cultivars and ASR-resistant cultivars, respectively (Ishikawa-Ishiwata and Furuya 2021). They estimated that fungicide application cost of 252.6 million USD can be saved by disseminating an ASR-resistant cultivar to 75% of the soybean-production area in Paraguay under the assumption that the fungicides are ineffective and the yield of resistant cultivars is the same as that of the conventional cultivars (Ishikawa-Ishiwata and Furuya 2021). The yield of unsprayed JFNC 1 was 0.90 and 1.07 times that of the sprayed Aurora during the 2017–2018 and 2018–2019 seasons, respectively. In case fungicides become ineffective, a reduction in fungicide cost similar to that estimated by Ishikawa-Ishiwata and Furuya (2021) can be adopted for JFNC 1 cultivation. The yield of unsprayed JFNC 2 was 1.39 times that of sprayed YG 203 during the 2017–2018 season. A similar reduction in fungicide costs is also expected for JFNC 2 in the season. However, yield of unsprayed JFNC 2 was significantly lower than that of sprayed YG 203 in the 2018–2019 season. Cercospora leaf blight was observed in JFNC 2 and YG 203. The disease may have caused the yield reduction.

Higher yield of JFNC 2 compared to YG 203 was observed in the 2017–2018 season but not in the 2018–2019 season. Small differences in the genetic background between the cultivars may have affected the yield under the 2017–2018 environment. However, it is unknown which combination of genetic traits and environmental factors affected.

The efficacy of fungicides has been shown to reduce over time in Brazil (Dalla Lana et al. 2018; Godoy et al. 2016), which has increased the importance of resistance genes in controlling ASR. Ishiwata and Furuya (2020) observed that adopting ASR-resistant cultivars reduced the estimated cost of controlling ASR by half in Brazil. Fungicide treatment was still effective against Aurora and YG 203 in our experiments. A considerable decrease in its efficacy would further reduce the yield of Aurora and YG 203, and the difference in yield between the unsprayed pyramided cultivars and their sprayed recurrent parents would be smaller or even reverse. In the present study, only site-specific fungicides were used. A mixture of a site-specific and multisite fungicide, such as prothioconazole and mancozeb, can enhance the control efficacy compared to only the site-specific fungicide (Reis et al. 2020). Such fungicide combinations can recover the yield of susceptible cultivars.

Although the values of disease severity, irrespective of fungicide application, were as low as 1% in JFNC 1 and JFNC 2, fungicide application reduced yield loss by 9–23%. Using the Brazilian data from 2004 to 2013, Dalla Lana et al. (2015) reported that the yield decreased by 0.6% when ASR-mediated disease severity at the full-seed growth stage increased by 1%. Fungicide application may have recovered the yield loss caused by other foliar diseases, resulting in the reduced yield loss observed in this experiment. In Paraguay, purple seed stain and Cercospora leaf blight, anthracnose, brown spot, seed diseases, and pod and stem blight are important foliar diseases causing yield reduction after ASR (Wrather et al. 2010). Cercospora leaf blight was actually observed on YG 203 and JFNC 2 as mentioned above. The cultivars were likely more susceptible than Aurora and JFNC 1. In Argentina, *Cercospora* species causing Cercospora leaf blight and purple seed stain of soybean were resistant or insensitive to quinone outside inhibitors and succinate dehydrogenase inhibitors (Sautua et al. 2020), which one of fungicides used in this experiment belongs to. Resistance of the pathogens to fungicides in Paraguay is unknown. The main significance of resistant cultivars is a reduction in the fungicide treatment costs. When the fungicide resistance become a problem in soybean production, resistant cultivars will be more required. To enhance the importance of the resistant cultivars, incorporating resistance genes to other foliar diseases in the cultivars is desirable.

A large pathogenic diversity exists in *P. pachyrhizi* worldwide (Chander et al. 2019). Several types of pathogenic diversity have been observed in Argentina, Brazil, and Paraguay (Akamatsu et al. 2013, 2017; Darben et al. 2020). Lemos et al. (2011) observed the effectiveness of the combined *Rpp2*, *Rpp4*, and *Rpp5* resistance genes using the *P. pachyrhizi* population collected in Brazil (Lemos et al. 2011). Darben et al. (2020) also reported that these three genes were effective against Brazilian isolates. JFNC 1 and

JFNC 2 are promising resistant cultivars in South America. Gene pyramiding provides increased resistance to plant diseases (Mundt 2014). Several reports have indicated that pyramiding *Rpp* genes are effective against ASR. Pyramiding of *Rpp4* + *Rpp5*, *Rpp2* + *Rpp3* + *Rpp4*, and *Rpp2* + *Rpp4* + *Rpp5* demonstrated higher resistance than that of the original resistance source carrying single *Rpp* genes against Brazilian rust populations (Yamanaka et al. 2015). Maphosa et al. (2012) observed that pairwise pyramiding of *Rpp2*, *Rpp3*, and *Rpp4* enhanced resistance in fields in Uganda. Combinations of *Rpp3* + *Rpp4* and *Rpp3* + *Rpp4* + *Rpp5* conferred soybean with increased ASR resistance, and the combination of *Rpp2* + *Rpp4* + *Rpp5* conferred immunity to Bangladeshi ASR isolates (Yamanaka and Hossain 2019). In contrast, certain combinations such as *Rpp1* + *Rpp2*, *Rpp2* + *Rpp4*, *Rpp2* + *Rpp5*, and *Rpp1* + *Rpp2* + *Rpp4* showed no effective resistance against Brazilian rust isolates (Yamanaka et al 2015). Therefore, confirming the combination of effective *Rpp* genes in a region is of utmost necessity. In many cases, plant pathogens can overcome resistance genes; hence, it is also necessary to monitor the efficacy of the resistance in JFNC 1 and JFNC 2 over time.

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Author contribution All authors have contributed to the conception and design of the study. NY contributed to the development of new cultivars, JFNC 1 and JFNC 2, and the *Rpp*-pyramided line: No6-12–1. AM and NY conducted the field and laboratory experiments, respectively. MK analyzed the data. MK and NY prepared the manuscript. All authors have read and approved the final version of the manuscript.

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Data availability The data generated or analyzed in this study are available as supplementary materials or will be supplied by the corresponding author upon reasonable request.

Declarations

Ethics approval No studies involving human participants or animals performed by any of the authors are described in this article.

Conflict of interest New soybean cultivars, "JFNC 1" and "JFNC 2," resistant to Asian soybean rust used in this study were developed by "Fundación Nikkei CETAPAR" and "Japan International research Center for Agricultural Sciences (JIRCAS)." These two organizations share breeder's rights for the two cultivars.

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