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CULTIVAR RELEASE

BRS 500 B2RF: transgenic cotton cultivar expressing Cry1Ac, Cry2Ab, and CP4-EPSPS with multiple disease resistance

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Abstract: *BRS 500 B2RF is a high-yielding cotton cultivar with transgenic resistance to lepidopteran pests and tolerance to the herbicide glyphosate. Additionally, BRS 500 B2RF has resistance to Ramularia leaf spot and partial resistance to root-knot nematode. It represents an important contribution to sustainable cotton production in Brazil.*

Keywords*:* Fiber yield*, Gossypium hirsutum, Mycosphaerella areola, Meloidogyne incognita.*

INTRODUCTION

Cotton (*Gossypium hirsutum* L.) is the most important natural textile fiber in the world. It is cultivated mainly to obtain fibers, although it also stands out as a source of oil and food products. In Brazil, since the early 1990s, the main cotton-growing area is located in the Brazilian tropical savannah (Morello et al. 2020). Due to a well-defined rain season, most cotton fields are rainfed and the lint yield is often very high, an average of 1672.94 kg ha^{-1} in the 2018-2019 growing season (ABRAPA 2020). In 2019, Brazil was the second largest cotton fiber exporter and fourth largest cotton fiber producer in the world (OECD-FAO 2020). However, the production system is based on extensive areas requiring several pesticide applications to achieve high levels of fiber production. In this scenario, Ramularia leaf spot disease (RLS), caused by *Ramulariopsis pseudoglycines* (teleomorph: *Mycosphaerella areola*), became epidemic, with considerable economic impact (Silva et al. 2019a). Additionally, the root-knot nematode (RKN), *Meloidogyne incognita*, is an emerging threat in cotton-growing regions (Belot et al. 2020).

Recently, sources of resistance to RLS have been identified and efforts have been made to develop cultivars tolerant or resistant to RLS (Silva et al. 2019a, Suassuna et al. 2020). Sources of resistance to RKN are available (Lopes et al. 2020), and the stocks most used for resistance are sourced from Auburn 623 RNR (Shepherd 1974). The high resistance in Auburn 623 RNR has been transferred to breeding lines (M-lines). Genetic resistance in M-lines is under

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oligogenic inheritance, determined by two QTLs located on chromosomes 11 and 14. The QTL (*qMi-C11*), located on chromosome 11, has a dominant effect on gall formation, whereas the QTL (*qMi-C14*), located on chromosome 14, has a partial dominant effect and is associated with reduced egg production (Silva et al. 2019b). Resistance (near-immunity) to the RKN is conferred by an epistatic interaction between the two QTLs (Gutiérrez et al. 2010, Lopes et al. 2020).

A cotton-breeding program was established by Embrapa in the 1980s to develop cultivars adapted to the *cerrado* (Brazilian tropical savanna) environment focusing on high-quality fiber (Morello et al. 2020) or enhanced disease resistance (Suassuna et al. 2020). Here, we report the development of the transgenic cotton cultivar BRS 500 B2RF, with resistance to RLS and partial resistance to RKN, which represents an important genetic contribution to sustainable cotton production in tropical environments.

BREEDING METHODS

The cultivar BRS 500 B2RF was developed through crossing the conventional cultivar BRS 372 (Suassuna et al. 2020) with the transgenic cultivar BRS 432 B2RF (Suassuna et al. 2018). BRS 372 has high fiber yield with good fiber quality and resistance to RLS (Suassuna et al. 2020). In addition, the BRS 372 plants used in the crosses express the gene *qMi-C11* in chromosome 11, the main RKN resistance gene, identified by the molecular markers i16205Gh and i39716Gh. BRS 432 B2RF is a transgenic cultivar that carries insect resistance and herbicide tolerance through the Bollgard II® (B2) and Roundup Ready Flex® (RF) events (MON 15985 and MON 88913, respectively), though it is susceptible to RLS. BRS 500 B2RF was derived from a cross of the cultivars BRS 372 and BRS 432 B2RF, followed by a selfing generation, to produce the F_2 population. Hybridizations and self-fertilizations were performed in Santo Antônio de Goiás, GO, in 2014. In 2015, plants from the F_2 segregating population, homozygous for the transgenic B2 and RF commercial traits, were selected using TaqMan™ SNP genotyping assays in a Real-Time PCR System (Applied Biosystems, USA). Moreover, marker-assisted selection was used to confirm resistance to cotton blue disease (CBD), caused by *Cotton Leafroll Dwarf Virus* (CLRDV); bacterial blight (BB), caused by *Xanthomonas citri* subsp. *malvacearum*; RKN (*qMi-C11*); and RLS. After the genotyping process, the plant CNPA 2015-126 was selected.

In the 2015/2016 growing season, the progeny CNPA 2015-126 B2RF (F_3) was evaluated in a field progeny trial in Santo Antônio de Goías, GO, and selected based on fiber yield, fiber properties (length, strength, and micronaire index), disease resistance (BB, CBD, and RLS), plant height, resistance to lodging, stormproofness (lock tenacity), and early maturity. Simultaneously, the progeny was grown in a controlled environment to produce seeds from self-pollination. In the 2016/2017 growing season, the preliminary line CNPA 15-126 B2RF (F_4) was evaluated in three independent trials across the states of Bahia and Goiás, when was selected as an elite line. In the 2017/2018, 2018, 2018/2019, and 2019 growing seasons, CNPA 15-126 B2RF (F_5) was evaluated in twenty-seven VCU trials (Table 3).

TRAITS AND PERFORMANCE

BRS 500 B2RF is a medium/tall cultivar with a cylindrical morphology. Plant height ranges from 135 to 145 cm when 200 to 250 grams of mepiquat chloride are applied in fractions over the season. BRS 500 B2RF has midseason to late maturity, and fruit branches usually have fewer than 3 bolls. Trichomes are found on the main stem and on the leaves as well. Leaves have nectaries and gossypol glands and are normal shaped. Bract teeth numbers range from 7 to 12. Fruiting branches usually have one or two bolls, providing the cylindrical plant morphology. Anthers, flower petals and pollen are cream in color. Mature bolls are elliptical in form, predominantly with four locules. Both the lint and the fuzz fibers are white. Completely open bolls are suitable for mechanical harvesting, even though they are not stormproof.

When grown at 830 meters asl (Table 1), the first flowers appear at about 55 to 60 days after emergence (DAE), and the first bolls open at about 110 to 120 DAE. Under these environmental conditions and use of chemical defoliants (Thidiazuron + Diuron) and harvest aid (Ethephon + Cyclanilide) to stimulate opening of physiologically mature green cotton bolls, BRS 500 B2RF was harvested at 175 DAE.

The cultivar BRS 500 B2RF is resistant to BB, CBD, RLS, and RKN (Table 1). Molecular markers were used to make inferences regarding genetic resistance to RLS, RKN, BB, and CBD. The SSR molecular marker CIR 246, linked to the B_{12} gene of resistance to bacterial blight, was used to check resistance to bacterial blight. The SSR DC20027, linked to the *Cdb* resistance gene, was used to check resistance to typical CBD. Additionally, field or greenhouse experiments were

Table 1. Fiber quality and agronomic traits of BRS 500 B2RF and check (BRS 432 B2RF)

Traits	BRS 500 B2RF	BRS 432 B2RF
Fiber percentage (%) [a]	40.3	40.0
Boll weight (g) [a]	5.6	5.3
Appearance of the first flower (days)[*]	50-55	50-55
First boll (days)[*]	110-120	110-120
Fiber length (mm) [a]	30.4	30.9
Uniformity index (%) [a]	84.0	84.6
Fiber strength (gf tex^{-1}) [a]	30.8	30.7
Micronaire index[a]	4.6	4.5
Fiber elongation (%) [a]	6.7	6.4
Fiber reflectance (%) [a]	78.8	79.8
Yellowness [a]	9.2	8.9
SFI index (%) [a]	7.5	7.1
Ramularia leaf spot [b]	1.1[¥]	2.9[¥]
Root-knot nematode [c]	0.2	10.1[**]

[*] Data recorded in Santo Antônio de Goiás (16°28'00" S, 49°17'00" W, alt 830 m asl), GO, Brazil.
[a] Means from twenty field trials conducted in the 2017/2018, 2018, 2018/2019, and 2019 growing seasons; [b] Percentage of plants with typical CBD symptoms; [b] Ordinal scale of severity (1 = completely resistant to 5 = highly susceptible); [c] Reproduction factor of root-knot nematodes (*Meloydogine incognita* - race 3) after inoculation of 9,798 eggs. [**] Cultivar FM 966 was used as a susceptible check. [¥] Means from four field trials conducted in Luís Eduardo Magalhães, BA, and São Desidério, BA, Brazil, in the 2017/2018 and 2018/2019 growing seasons.

carried out to confirm the genotyping data. RKN was tested under greenhouse conditions to measure the reproduction factor (RF), following the methodology used by Lopes et al. (2020). Briefly, eight individual plants of BRS 500 B2RF, M-315 (resistant check), and FM 966 (susceptible check) were grown in pots (20 x 15 cm) filled with autoclaved soil under greenhouse conditions. Twenty-five days after seedling emergence, pots were inoculated with 9,798 eggs of *M. incognita* by pipetting a nematode suspension around the stem base. Four months after inoculation, the root systems were rinsed under tap water and weighed. Eggs were extracted from roots, and resistance to RKN was measured based on the reproduction factor (RF), calculated as RF = FP/IP, where FP = final nematode population and IP = initial nematode population (IP = 9,789). RF in BRS 500 B2RF ranged from 0.05 to 0.26 (average of 0.2), lower than the 10.1 estimate in FM 966 and higher than the 0.025 estimate in M-315. The experiment was conducted twice. The partial resistance found in BRS 500 B2RF is associated with the SNP molecular marker i16205Gh linked to the resistance gene *qMi-C11* in chromosome 11.

Resistance to RLS was determined in two independent field assays in the 2017/2018 growing season and replicated in the 2018/2019 season in the municipalities of Luís Eduardo Magalhães and São Desidério in Bahia State (Table 1). Additionally, RLS evaluations were performed in the 2018/2019 season in Campo Verde, MT; Santo Antônio de Goiás, GO; and Santa Filomena, PI, with mean severity scores of 1.0, 1.0, and 1.3 for BRS 500 B2RF and 2.9, 2.7, and 2.3 for BRS 432 B2RF, respectively, showing apparently stable resistance against pathogen populations at the test sites. Resistance to RLS was confirmed by using SNP molecular markers linked to resistance genes in chromosomes 1 and 4. In addition, RLS severity was measured in the cotton cultivars BRS 436 B2RF (susceptible) and BRS 500 B2RF, either untreated or sprayed 2, 4, 6, 8, or 10 times with fungicide throughout the growing season. Disease severity was evaluated 135 days after emergence using a disease grading scale developed by Aquino et al. (2008). Severity in the susceptible check ranged from 4.9% to 29.3%, depending on the number of fungicide spray applications. The number of fungicide applications tested did not affect the severity of RLS in the cultivar BRS 500 B2RF, with severity ranging from 0.1% to 0.7%. Although the resistance level of BRS 500 B2RF to RLS is high, two applications of fungicides during the season may be necessary to control secondary leaf diseases, such as target spot (*Corynespora cassiicola*), as well as to prevent or delay the emergence of putative resistance-breaking *R. pseudoglycines* isolates.

In field evaluations, there was no occurrence of symptoms typical of CBD or BB in two independent assays conducted in Santo Antônio de Goiás and Luís Eduardo Magalhães. Paired *t*-test comparisons were performed both in agronomic traits: cottonseed yield - CY, lint percentage - LP, and lint yield - LY and in fiber quality traits: fiber length - FL and fiber strength - FS between BRS 500 B2RF and BRS 432 B2RF (Table 2). Fiber was significantly shorter in BRS 500 B2RF (30.4

mm) than in BRS 432 B2RF (30.9 mm). FS did not differ between cultivars, 30.8 and 30.7 gf tex^{-1} for BRS 500 B2RF and BRS 432 B2RF, respectively. There was significant difference in CY between the cultivars BRS 500 B2RF (5.667 kg ha^{-1}) and BRS 432 B2RF (5.187 kg ha^{-1}). In addition, LY was higher for BRS 500 B2RF (2.579 kg ha^{-1}) than for BRS 432 B2RF (2.383 kg ha^{-1}). The LP was significantly higher in BRS 500 B2RF than in BRS 432 B2RF (Table 2).

Averaged across the 27 field trials in the 2017/2018, 2018, 2018/2019, and 2019 growing seasons (Table 3), BRS 500 B2RF had 11.4% more cotton seed yield and 9.5% more cotton lint yield than the control (BRS 432 B2RF). BRS 500

Table 2. Comparison (*t*-test) of BRS 500 B2RF and BRS 432 B2RF cultivars for cottonseed yield (CY), lint yield (LY), lint percentage (LP), fiber length (Len), and fiber strength (Str) in 27 locations during the 2017/2018 and 2018/2019 growing seasons

	CY (kg ha^{-1})	**LY** (kg ha^{-1})	**LP** (%)	**Len** (mm)	**Str** (gf tex^{-1})
Error	468777	95620	1.05	0.59	2.31
df	1377	1020	1020	1071	1071
N	108	80	80	84	84
BRS 500 B2RF	5666.5	2578.8	40.3	30.4	30.8
BRS 432 B2RF	5186.9	2383.4	40.0	30.9	30.7
t-value	5.15**	4.00**	2.33*	-4.22**	0.49

Significance: * Significant at 5%; ** Significant at 1%.

Table 3. BRS 500 B2RF and BRS 432 B2RF means of cottonseed yield (CY) and lint yield (LY) in twenty-seven trials carried out in the 2017/2018, 2018, 2018/2019, and 2019 growing seasons

Counties/Brazilian State	**Seasons**	**BRS 500 B2RF**		**BRS 432 B2RF**		**PCC¥**		**CV***
		CY (kg ha^{-1})	**LY** (kg ha^{-1})	**CY** (kg ha^{-1})	**LY** (kg ha^{-1})	**CY**	**LY**	
Luís Eduardo Magalhães - BA	2017/18	8.420	3.500	7.734	3.165	108.9	110.6	8.4
Riachão das Neves - BA	2017/18	8.858	3.803	6.908	2.910	128.2	130.7	8.0
São Desidério - BA	2017/18	8.117	3.431	6.662	2.732	121.8	125.6	13.0
Uruçuí - PI	2017/18	5.624	2.258	5.004	2.003	112.4	112.7	13.3
Barbalha - CE	2018	6.762	2.852	5.969	2.505	113.3	113.8	22.9
Santo Antônio de Goiás - GO	2017/18	6.132	2.429	5.190	2.048	118.2	118.6	20.4
Cristalina - GO	2017/18	5.321	2.148	3.873	1.496	137.4	143.6	13.5
Sorriso - MT	2018**	5.960	2.225	6.705	2.522	88.9	88.2	11.3
Campo Verde - MT	2017/18**	6.186	2.466	5.120	1.935	120.8	127.4	9.9
Sapezal - MT	2018**	7.026	2.594	6.086	2.174	115.5	119.3	10.8
Chapadão do Sul - MS	2018**	5.423	-	4.874	-	111.3	-	10.6
Cambará - PR	2017/18	1.998	-	1.851	-	107.9	-	22.3
Palmas -TO	2018	6.185	-	4.409	-	140.3	-	12.2
Boa Vista - RR	2018	2.378	-	1.722	-	138.1	-	8.3
Luís Eduardo Magalhães - BA	2018/19	7.016	2.742	6.816	2.705	102.9	101.4	6.9
Riachão das Neves - BA	2018/19	6.951	2.781	6.537	2.577	106.3	107.9	11.9
São Desidério - BA	2018/19	8.400	3.392	9.501	3.885	88.4	87.3	7.9
Uruçuí - PI	2018/19	5.387	2.181	5.234	2.076	102.9	105.1	13.5
Barbalha - CE	2019	4.307	1.808	4.370	1.803	98.6	100.2	18.1
Santo Antônio de Goiás - GO	2018/19	5.346	2.123	5.184	2.069	103.1	102.6	9.9
Sorriso - MT	2019**	5.279	2.199	6.048	2.559	87.3	85.9	11.2
Campo Verde - MT	2018/19**	5.843	2.442	5.793	2.436	100.9	100.2	7.7
Sapezal - MT	2019**	6.696	2.696	6.485	2.651	103.3	101.7	11.3
Chapadão do Sul - MS	2019**	3.956	1.512	3.747	1.421	105.6	106.4	12.1
Cambará - PR	2018/19	2.378	-	1.853	-	128.3	-	24.1
Palmas - TO	2019	4.782	-	4.176	-	114.5	-	11.4
Boa Vista - RR	2019	2.273	-	2.201	-	103.3	-	12.8
Mean		5.667	2.579	5.187	2.383	111.4	109.5	

¥ Percentage in comparison to the mean of BRS 432 B2RF; * Coefficient of variation (%) for CY; ** Second growing season

B2RF has desirable fiber quality for a medium staple upland cotton: fiber length (UHML), 30.4 mm; micronaire index, 4.6; fiber strength, 30.8 gf tex^{-1}; reflectance, 78.8%; yellowness (+b), 9.2; and short fiber index, 7.5 (Table 3).

AVAILABILITY

Foundation seeds of BRS 500 B2RF (registration number 44648 and protection certificate number 20200117) are produced by Embrapa. Commercial seeds can be purchased from IST Cotton Brasil. Inquiries regarding availability of seed for research purposes or for commercial use should be directed to the corresponding authors.

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