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SHORT COMMUNICATION

Mixture of *Colletotrichum lindemuthianum* races in anthracnose resistance screening and its implication for common bean breeding

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Abstract

Evaluations by artificial inoculation of different races of *Colletotrichum lindemuthianum* can be unfeasible in a breeding program aiming at obtaining cultivars with durable resistance to anthracnose in common bean, since many physiological races have already been found. An alternative would be the use of mixtures of different races. However, information is scarce about the efficiency of this method to select resistant plants and to evaluate the virulence of the pathogen. Thus, the objective of this work was to demonstrate the feasibility of using mixtures of *C. lindemuthianum* races in breeding programs aiming at the development of common bean cultivars with resistance to anthracnose. Two experiments were carried out, in which races 65, 73 and 81 of *C. lindemuthianum* were inoculated individually and in a mixture of the three races (M1), and also mixtures of first (M2 and M3) and second (M4, M5, M6 and M7) generations, for cultivars Pérola and BRSMG Majestoso. The use of mixtures of races of *C. lindemuthianum* was efficient in evaluating anthracnose severity and could be recommended as an alternative method to save costs and time.

Keywords *Phaseolus vulgaris* · Artificial inoculation · Breeding · Common bean · Genetic resistance

Common bean (*Phaseolus vulgaris* L.) is one of the main components of the Brazilian population diet. Its grains are excellent sources of protein, calories and minerals. Occurrence of diseases can cause significant damage to the crop, with reduction in grain yield and quality. Anthracnose, caused by the fungus *Colletotrichum lindemuthianum* (Sacc. & Magnus), can cause losses of up to 100% (Paula-Júnior et al. 2015).

Anthracnose management using resistant cultivars is a strategy that minimizes production costs and reduces damage to the environment. However, the large pathogenic variability of *C. lindemuthianum* limits disease control and development of new cultivars with durable resistance (Pinto et al. 2012). More than 100 races of *C. lindemuthianum* have been found in the world, and races 65, 73, 81 and 89 are the most frequent in Brazil (Silva et al. 2007; Pereira et al. 2010; Pinto et al. 2012). In addition, pathogenic variability has been detected within races 65 and 81 in Brazil (Ishikawa et al. 2011, 2013; Costa et al. 2017).

Common bean resistance to anthracnose is predominantly vertical, and a gene-a-gene model has been verified in the *C. lindemuthianum-P. vulgaris* pathosystem. Twenty resistant genes to anthracnose have been reported in common bean (Ferreira et al. 2013; Zuiderveen et al. 2016). In addition, 12 independent genes have been identified that confer resistance to six different isolates belonging to race 65 of *C. lindemuthianum* (Costa et al. 2017). Despite the existence of vertical resistance, several genes already identified display a polygenic inheritance of this trait (Costa et al. 2017). Therefore, cultivars with durable resistance should present resistance to the most frequent *C. lindemuthianum* races in different common bean crop regions.

The reaction of common bean lines and progenies to *C. lindemuthianum* has been assessed using artificial inoculation of isolates of the most frequent races (Barcelos et al. 2013; Costa et al. 2017). However, when many races are used, a large amount of seeds is necessary, hindering the evaluation of progenies in a breeding program. A strategy could be to use mixtures of different isolates or races. However, literature information about the efficiency of this method for evaluation of anthracnose resistance in common bean is scarce (Chilipa et al. 2016), and there is no information about pathogen virulence when mixtures of isolates are used.

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In the present study, anthracnose severity on common bean was evaluated after inoculation of *C. lindemuthianum* races 65, 73 and 81 individually and also using mixtures of isolates. Implications for common bean breeding to obtain cultivars with resistance to anthracnose are discussed.

Three isolates of *C. lindemuthianum* were used, which were previously identified as race 65 (differential cultivars Michelite and Mexico 222 are susceptible), race 73 (differential cultivars Michelite, Cornell 49.242 and Mexico 222 are susceptible) and race 81 (differential cultivars Michelite, Widusa and Mexico 222 are susceptible). In addition, inoculum obtained from the mixture of the three races (M1) and from mixtures (M2, M3, M4, M5, M6 and M7) obtained after inoculation on cultivars Pérola (susceptible to the three races) and BRSMG Majestoso (resistant to the races 73 and 81) was used.

Each isolate was grown in sterile pods partially immersed in agar-water medium in test tubes, and incubated for 15 days at 22 °C in a BOD in the dark. A conidial suspension was prepared as described by Pinto et al. (2012) and the concentration was adjusted to 1.2×10^6 conidia/mL. A conidial suspension of the mixture of the three races (M1) was obtained by equally mixing the suspensions at the adjusted concentration of conidia of each race.

Inoculations of each isolate individually and of the mixtures were carried out as described by Pinto et al. (2012). Inoculated common bean seedlings were incubated for 48 h at 100% relative humidity with a photoperiod of 12 h and temperature of approximately 20 °C. After incubation, seedlings were transferred to a greenhouse with a temperature of approximately 24 °C and 95% relative humidity.

Two experiments were conducted in a completely randomized design with subdivided plots, with two replicates. A plot consisted of 36 plants. A previous test was carried out by inoculating each race individually and also M1 on plants of the cultivars Pérola and BRSMG Majestoso, as described above. Isolations of the pathogen from lesions on leaves and stems were carried out in order to obtain mixtures of first generation isolates. The isolate obtained from the cultivar Pérola was denominated M2 and the one obtained from cultivar BRSMG Majestoso was denominated M3.

Mixtures of second generation isolates were obtained by isolation from lesions caused by M2 and M3 inoculated on leaves and stems of cultivars Pérola and BRSMG Majestoso. Thus, isolates obtained from the inoculation of M2 on Pérola and BRSMG Majestoso were named M4 and M5, respectively. Likewise, isolates obtained from the inoculation of M3 on Pérola and BRSMG Majestoso were named M6 and M7, respectively.

In the second experiment, inoculations of each isolate and each mixture (M1, M2, M3, M4, M5, M6 and M7) were carried out on plants of the cultivars Pérola and BRSMG Majestoso. In all experiments anthracnose severity was evaluated at three to 10 days after inoculation (dai) using the scale proposed by Schoonhoven and Pastor-Corrales (1987).

The area under the disease progress curve (AUDPC) was estimated using severity mean scores of expression adopted by Shaner and Finney (1977), according to the following equation:

$$AUDPC = \sum_{k=1}^{n} \left[\frac{(Y_{k+1} + Y_k)}{2} x (T_{k+1} - T_k) \right]$$

where Y_k e Y_{k+1} are disease severity at evaluation times k and k + 1, respectively, and T_k e T_{k+1} are the time of evaluation k and k + 1, respectively, in number of dai.

AUDPC and anthracnose severity at 10 dai data were subjected to analysis of variance (ANOVA), and means were compared by the Scott-Knott test ($p = 0.05$) using software R (R Core Team 2015).

All sources of variation were significant in both experiments, except for the interaction isolate/mixture x cultivars in experiment 1. Isolates/mixtures that caused susceptibility reaction in 'Pérola' displayed scores above 3.0 from the fifth and sixth days after inoculation in experiments 1 and 2, respectively (Figs. 1 and 2). The cultivar BRSMG Majestoso was resistant to all isolates/mixtures, except for race 65 and mixture M1, in which cases it displayed scores above 3.0 from the fifth day on (Fig. 1d-f).

Mean AUDPC was higher in experiment 1 than in experiment 2 (Table 1). Anthracnose severity progress was faster and more intense in experiment 1 for isolates/mixtures of *C. lindemuthianum* that were evaluated in both experiments. It is possible that environmental conditions were more favorable to the disease during experiment 1. In spite of humidity and temperature control, outside temperatures above 30 °C can make it difficult to keep conditions between 15 and 25 °C (most favorable to disease development) inside the greenhouse (Paula-Júnior et al. 2015).

In experiment 1, AUDPC means for both cultivars were grouped in two classes by the Scott-Knott test, and were higher for the isolate of race 65 and mixture M1 in both cultivars (Table 1). However, in experiment 2, the mixtures M3, M4 and M5 presented higher AUDPC estimates for Pérola. Race 81 presents an additional virulence factor compared to race 65. However, isolates of these races presented the same virulence in experiment 2. Race 65, the simplest race evaluated, and the M1 mixture were the most virulent in experiment 1. These results corroborate with those from other authors, who have found that series of international differential cultivars are not suitable to discriminate the pathogenic variability of Brazilian isolates of *C. lindemuthianum* (Ishikawa et al. 2011; Carbonell et al. 2012;

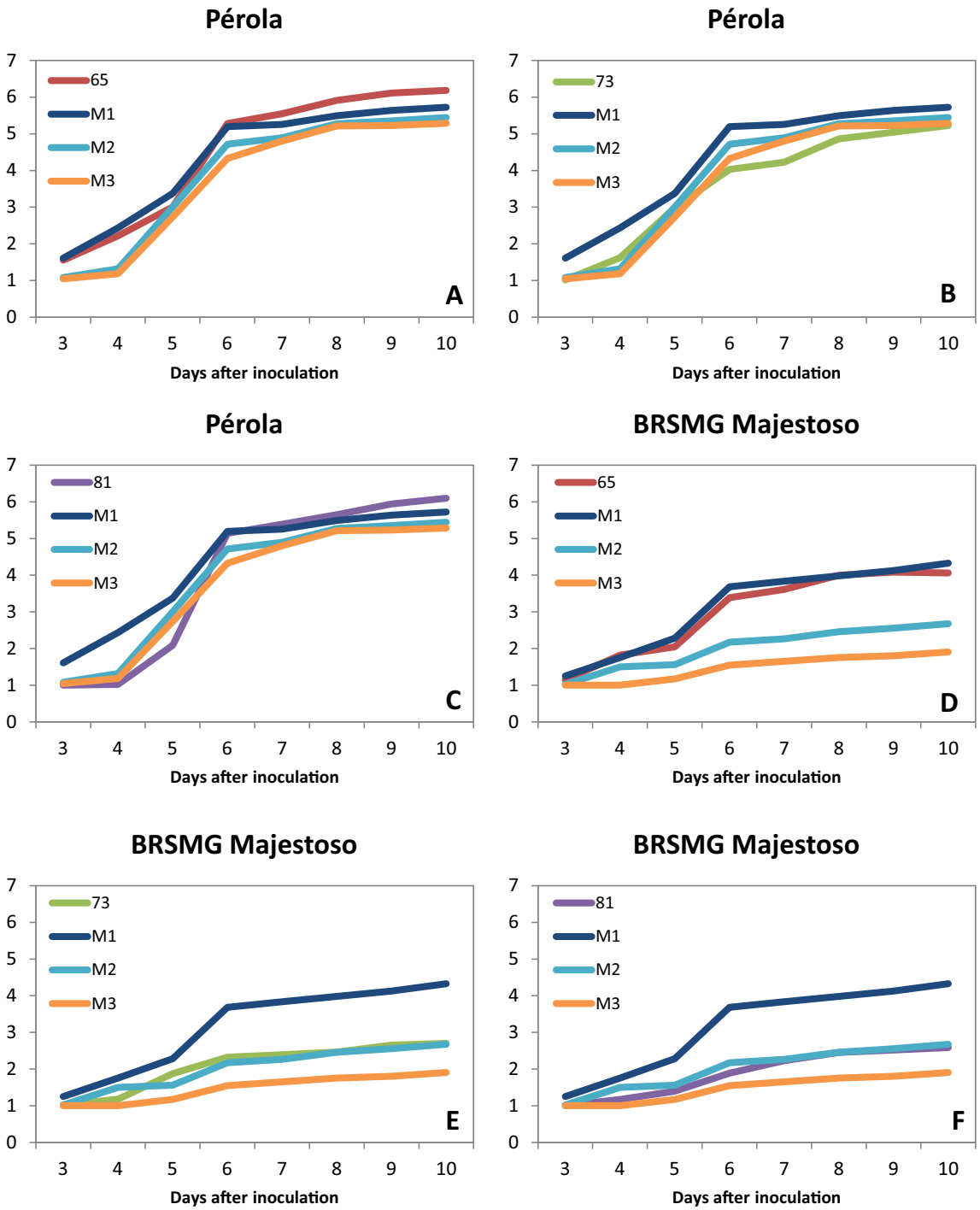

Fig. 1 Anthracnose severity progress between 3 and 10 days after inoculation (dai) of *Colletotrichum lindemuthianum* on plants of cultivars Pérola and BRS Majestoso, comparing individual inoculations of races with inoculations of mixtures of races (M1, M2 and M3)

Costa et al. 2017). Considering virulence factors, M1 has factors 2^0 and 2^6 in higher proportion since all races evaluated present these factors. Therefore, virulence factors 2^3 and 2^4, present in isolates of races 73 and 81, respectively, are in a lower proportion in the mixture. This fact may justify the similar behavior of M1 and of the isolate of race 65.

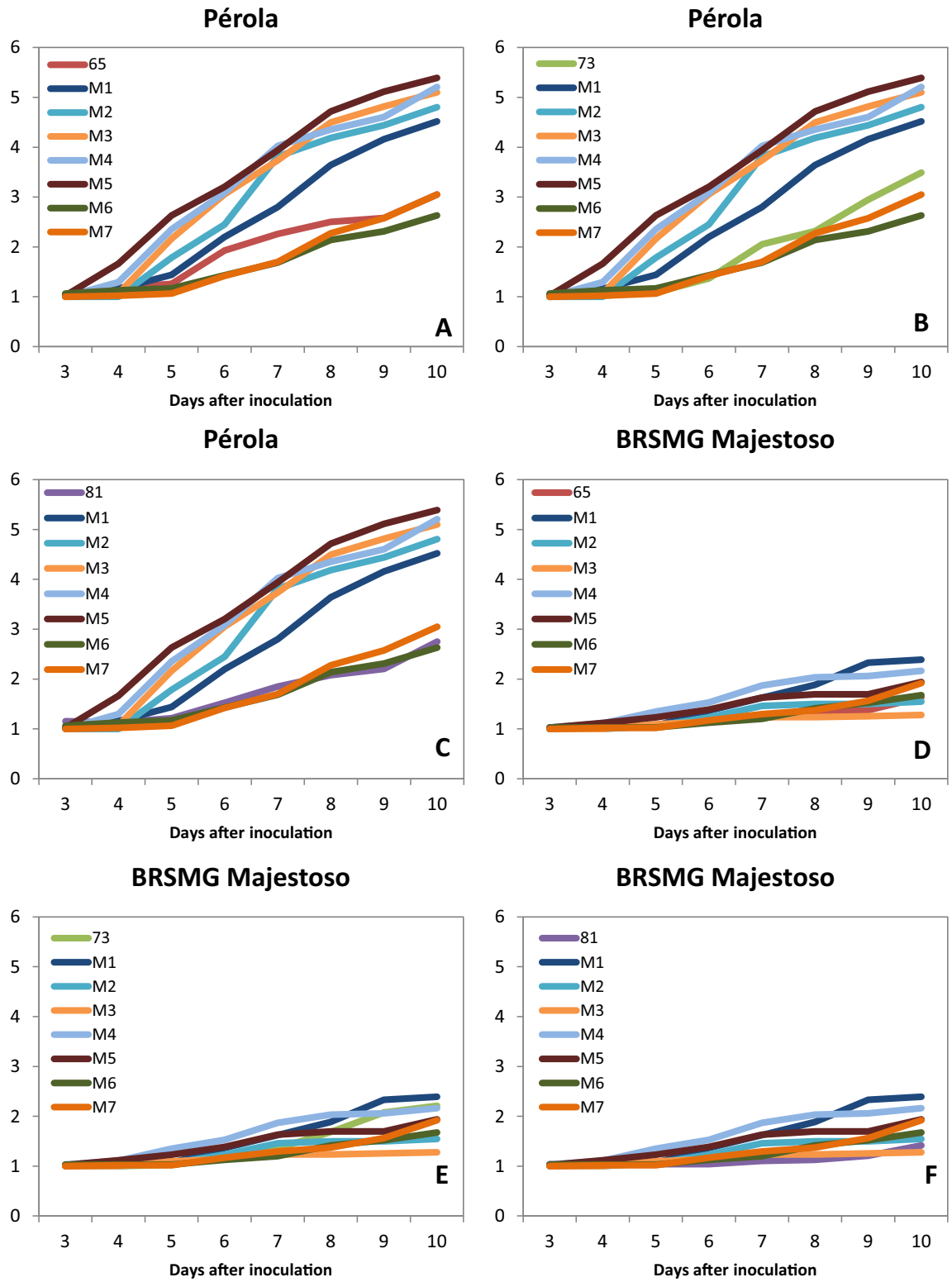

Fig. 2 Anthracnose severity progress between 3 and 10 days after inoculation (dai) of *Colletotrichum lindemuthianum* on plants of cultivars Pérola and BRS Majestoso, comparing individual inoculations of races with inoculations of mixtures of races (M1, M2, M3, M4, M5, M6 and M7)

Table 1 Area under the disease progress curve (AUDPC) for common bean cultivars Pérola and BRSMG Majestoso 10 days after inoculation (dai) with different isolates and mixtures of *Colletotrichum lindemuthianum*

Race/Mixture*	Experiment 1		Experiment 2	
	Pérola	BRSMG Majestoso	Pérola	BRSMG Majestoso
65	32.0 A[#]	21.6 A	13.8 C	8.5 A
73	25.9 B	14.8 B	13.0 C	9.9 A
81	28.8 B	13.5 B	12.0 C	7.8 A
M1	31.1 A	22.5 A	18.2 B	11.0 A
M2	27.8 B	14.4 B	20.6 B	9.0 A
M3	26.7 B	10.4 B	22.4 A	8.1 A
M4	–	–	22.8 A	11.6 A
M5	–	–	24.5 A	10.2 A
M6	–	–	11.7 C	8.7 A
M7	–	–	12.1 C	8.9 A

*Mixtures: M1: mixture of races 65, 73 and 81; M2 and M3: isolates obtained from inoculation of M1 on Pérola and BRSMG Majestoso, respectively; M4 and M5: isolates obtained from inoculation of M2 on Pérola and BRSMG Majestoso, respectively; M6 and M7: isolates obtained from inoculation of M3 on Pérola and BRSMG Majestoso, respectively

[#] Means followed by same letter in the same column belong to same group according to the Scott-Knott test at 5% probability level

The greater magnitude of AUDPC and also of anthracnose severity estimates for Pérola using M1, M2, M3, M4 and M5 reveals an increase in race virulence for mixed isolates and also for new generations of isolations, except for M6 and M7. For M1 (mixture of the three races), although the concentration of each race was reduced by 1/3 (4×10^5 conidia/mL), the virulence of the mixture was greater or similar to the other isolates/mixtures. It has been shown that a concentration of 10^5 conidia/mL provides similar results to those obtained by using the usual recommended concentration of 1.2×10^6 for assessing anthracnose severity on common bean (Davide and Souza 2009). The higher virulence of M1 may be due to a synergistic effect among the three isolates, as already verified in this pathosystem by Chilipa et al. (2016). Another probable explanation would be the occurrence of heterosis, because alleles of different races are contributing to the virulence in the mixture.

The behavior of isolates and mixtures was similar at 3 and 4 dai. After that, differences in anthracnose severity were observed (Fig. 1). This can be confirmed by the estimates of the variation limits, which were of greater magnitude after 4 dai (Table 2). However, mean scores higher than 4.0 were observed at 6 and 10 dai in experiments 1 and in 2, respectively.

Isolates of races 65 and 81 and mixtures M1, M2 and M3 presented similar virulence for cultivar Pérola, as well as similar anthracnose intensity in experiment 1 (Fig. 1a-c). Most mixtures were more virulent on cultivar Pérola in experiment 2 (Fig. 2a-c). Mixtures were more virulent than isolates of races 65, 73 and 81 in both experiments on cultivar BRSMG Majestoso (Figs. 1d-f and 2f). This cultivar was evaluated as resistant to all isolates and mixtures, as scores were lower than 3.0 in experiment 2.

Disease severity scores at 10 dai were used to carry out ANOVA in experiment 2. All sources of variation were significant, including the interaction that was partitioned; there were significant difference for isolates/mixtures for Pérola. The means test grouped M1, M2, M3, M4 and M5 mixtures that caused higher mean scores and therefore were more virulent than individual isolates and mixtures M6 and M7 (Table 1).

The lower magnitude of AUDPC and of anthracnose severity obtained upon inoculation of M6 and M7 (Table 1) reveals that there was a reduction in virulence using new generations of M1 initially inoculated on BRSMG Majestoso. This reduction may be related to the stabilizing selection action that is effective against races with unnecessary virulence alleles in a pathogen population (Vanderplank 1968), favoring 1 (2^0) and 64 (2^5) virulence factors, which were more frequent in M1, in comparison to virulence factors 8 and 16 of races 73 and 81, respectively. Casela et al. (2001) observed differences in competitive capacity of *C. sublineolum* races after five and six inoculations of mixture generations in sorghum cultivars, indicating a possible action of stabilizing selection against races of distinct virulence in the pathogen population. Our results were obtained with only two generations, but there are no reports of occurrence of conidial anastomosis tubes (CATs) recombinants for *C. sublineolum*, a mechanism that may be related to the asexual recombination of *C. lindemuthianum* (Ishikawa et al. 2012; Pinto et al. 2012; Mota et al. 2015).

The inoculation of mixtures resulted in similar or greater virulence compared to the inoculation of individual isolates. In addition, mixtures of isolates of *C. lindemuthianum* can be more efficient in selecting common bean progenies with different levels of resistance, as they have a larger number of virulence alleles. We recommend the use of mixtures of races at each inoculation. On the other hand, we do not recommend the use of mixtures obtained from new isolations done in

Table 2 Mean anthracnose severity scores (on a scale of 1 to 9) and variation limits from 3 to 10 days after inoculation (dai) on cultivars Pérola and BRSMG Majestoso

Cultivar	3 dai	4 dai	5 dai	6 dai	7 dai	8 dai	9 dai	10 dai
Experiment 1								
Pérola	1.22 (1.00–1.61)	1.63 (1.02–2.44)	2.87 (2.09–3.38)	4.78 (4.03–5.28)	5.02 (4.23–5.55)	5.40 (4.87–5.91)	5.55 (5.05–6.12)	5.66 (5.23–6.19)
BRSMG Majestoso	1.07 (1.00–1.25)	1.40 (1.00–1.82)	1.72 (1.17–2.29)	2.50 (1.55–3.69)	2.66 (1.65–3.84)	2.85 (1.76–4.00)	2.96 (1.80–4.13)	3.04 (1.91–4.33)
Experiment 2								
Pérola	1.03 (1.00–1.16)	1.17 (1.00–1.67)	1.62 (1.07–2.63)	2.17 (1.37–3.21)	2.79 (1.69–4.03)	3.27 (2.08–4.72)	3.58 (2.21–5.12)	4.00 (2.63–5.39)
BRSMG Majestoso	1.01 (1.00–1.04)	1.04 (1.00–1.12)	1.10 (1.02–1.35)	1.23 (1.04–1.53)	1.40 (1.11–1.87)	1.53 (1.13–2.04)	1.66 (1.21–2.33)	1.82 (1.28–2.39)

plants inoculated with mixtures, due the action of stabilizing selection on the pathogen population.

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References

Barcelos QL, Souza EA, Pádua PF, Ishikawa FH, Costa LC, Pereira R, Abreu AFB, Ramalho MAP (2013) Investigation of sources of resistance to anthracnose disease in a *Phaseolus vulgaris* germplasm collection in Brazil. Annual Report of the Bean Improvement Cooperative 56:37–38

Carbonell SAM, Chiorato AF, Ito MF, Wendland A, Del Peloso MJ, Melo LC, Souza EA, Ramalho MAP, Carneiro JES, Moda-Cirino V, Fonseca Júnior NS, Barcelos QL, Costa LC, Celin EF (2012) Antracnose. In: Paula-Júnior TJ, Wendland A (eds) Melhoramento genético do feijoeiro-comum e prevenção de doenças. Epamig, Viçosa, pp 15–39

Casela CR, Ferreira AS, Santos FG (2001) Differences in competitive ability among races of *Colletotrichum graminicola* in mixtures. Fitopatologia Brasileira 26:217–220

Chilipa LNK, Lungu DM, Tembo L (2016) Multiple race inoculation as an option in breeding for resistance to *C. lindemuthianum* in common beans. Journal of Agriculture and Crops 2:45–50

Costa LC, Nalin RS, Ramalho MAP, Souza EA (2017) Are duplicated genes responsible for anthracnose resistance in common bean? PLoS ONE 12:e0173789

Davide LMC, Souza EA (2009) Pathogenic variability within race 65 of *Colletotrichum lindemuthianum* and its implications for common bean breeding. Crop Breeding and Applied Biotechnology 9:23–30

Ferreira JJ, Campa A, Kelly JD (2013) Organization of genes conferring resistance to anthracnose in common bean. In: Varshney RK, Tuberosa R (eds) Translational genomics for crop breeding. John Wiley & Sons, Ames, pp 151–176

Ishikawa FH, Ramalho MPA, Souza EA (2011) Common bean lines as potential differential cultivars for race 65 of *Colletotrichum lindemuthianum*. Journal of Plant Pathology 93:461–464

Ishikawa FH, Souza EA, Shoji J, Connolly L, Freitag M, Read ND, Roca MG (2012) Heterokaryon incompatibility is suppressed following conidial anastomosis tube fusion in a fungal plant pathogen. PLoS ONE 7:e31175

Ishikawa FH, Souza EA, Read ND, Roca MG (2013) *Colletotrichum lindemuthianum* exhibits different patterns of nuclear division at different stages in its vegetative life cycle. Mycologia 105:795–801

Mota SF, Barcelos QL, Dias MA, Souza EA (2015) Variability of *Colletotrichum* spp in common bean. Genetics and Molecular Research 15:1/15027176

Paula-Júnior TJ, Vieira RF, Teixeira H, Lobo Junior M, Wendland A (2015) Doenças do feijoeiro: estratégias integradas de manejo. In: Carneiro JE, Paula Júnior TJ, Borém A (eds) Feijão: do plantio à colheita. Editora UFV, Viçosa, pp 270–299

Pereira R, Ishikawa FH, Pinto JMA, Souza EA (2010) Occurence of anthracnose in common bean cultivars collected in the state of Minas Gerais, Brazil. Annual Report of the Bean Improvement Cooperative 53:224–225

Pinto JMA, Pereira R, Mota SF, Ishikawa FH, Souza EA (2012) Investigating phenotypic variability in *Colletotrichum lindemuthianum* populations. Phytopathology 102:490–497

R Core Team (2015) R: A language and enviroment for statistical computing. R Foundation for Statistical Computing, Vienna. Available at: www.R-project.org

Schoonhoven A, Pastor-Corrales MA (1987) Standard systems for the evaluation of bean germoplasm. CIAT, Cali

Shaner G, Finney RE (1977) The effect of nitrogen fertilization on the expression of slow-mildewing resistance in Knox wheat. Phytopathology 67:1051–1056

Silva KJD, Souza EA, Ishikawa FH (2007) Characterization of *Colletotrichum lindemuthianum* isolates from the state of Minas Gerais, Brazil. Journal of Phytopathology 155:241–247

Vanderplank JE (1968) Disease resistance in plants. Academic Press, New York

Zuiderveen GH, Padder BA, Kamfwa K, Song Q, James DK (2016) Genome-wide association study of anthracnose resistance in andean beans (*Phaseolus vulgaris*). PLoS ONE 11:e0156391