Plant Genetic Resources: Characterization and Utilization

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Exploiting phenotypic and genotypic diversity against *Colletotrichum truncatum* in chilli hybrids developed using resistant breeding lines[‡]

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Research Article

[‡]This article has been updated since its original publication. A notice detailing this change can be found here: https://doi.org/10.1017/S147926212400011X

Cite this article: Herath HMSN, Rafii MY, Ismail SI, Jaafar JN, Ramlee SI (2024). Exploiting phenotypic and genotypic diversity against *Colletotrichum truncatum* in chilli hybrids developed using resistant breeding lines. *Plant Genetic Resources: Characterization and Utilization* **22**, 37–44. https://doi.org/10.1017/S1479262123001144

Received: 4 April 2022
Revised: 28 December 2023
Accepted: 29 December 2023
First published online: 1 February 2024

Keywords:
disease resistance; fruit inoculation; hybrids; parents; polymorphic markers

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Abstract

In an effort to control anthracnose disease, one of the major problems that has been faced by farmers, 14 chilli hybrids and their parents were screened phenotypically using the fruit inoculation method under laboratory conditions. Genotypic screening of 14 chilli hybrids and their parents was done by the identified polymorphic markers, HpmsE 051 and HpmsE 082. Based on the phenotypic and genotypic data, chilli hybrids, H1, H2, H3, H4, H6, H7, H8, H9, H11 and H12 were identified as resistant chilli hybrids against anthracnose disease caused by the *C. truncatum*. Molecular markers, HpmsE 051 and HpmsE 082 could be utilized as polymorphic markers to isolate resistant genotypes against *C. truncatum*.

Introduction

Chilli (*Capsicum annuum* L.) plant belongs to the family *Solanaceae* (chromosome number, 2n = 2x = 24) is one of the most cultivated spices and vegetable crops (Siddappa *et al*., 2019; Thakur *et al*., 2019; Sindhusha and Rawat, 2020). Chilli has been cultivated on more than 2.0 million hectares and the annual global production for chilli is about 36 million tonnes. Asian region itself contributes to almost 24.6 million tonnes of hectares with 1.3 million cultivated lands (FAO STAT, 2021). Chilli hybrids are very popular among farmers around the world due to their high yielding ability and other quality characteristics. The development of hybrid varieties allows to combine desired and most important traits from two selected parents like disease resistance (Sahid *et al*., 2020; Anilkumar, 2021).

Fresh fruit yield in chilli is affected by genetic and environmental factors (Meena *et al*., 2020). Further, chilli genotypes are often susceptible to many diseases and it results in low productivity of chilli cultivation (Kothari *et al*., 2010). The yield of chilli fruit is affected by anthracnose, a devastating fungal disease, worldwide. Anthracnose disease has been identified as one of the major constraints in chilli production reported globally (Chunying *et al*., 2015; Ananthan *et al*., 2018; Mishra *et al*., 2019a; Zhao *et al*., 2020). Application of fungicides and integrated disease management to control this disease is not a long-lasting and sustainable solution (Chunying *et al*., 2015). Development of anthracnose disease-resistant chilli varieties is the most economical and environmentally friendly method to control this disease (Mishra *et al*., 2019a). In the process of development of anthracnose disease-resistant chilli varieties, only the phenotypic evaluation is not enough. Confirmation that resistant varieties carry the resistant gene using the molecular markers provides a more scientific base to phenotypic observations (Zhao *et al*., 2020).

According to Kim *et al*., (2008) and Lee *et al*., (2011), the pattern of inheritance of anthracnose disease resistance is very complex and it varies with the *Colletotrichum* isolate and source of resistance. Among these *Colletotrichum* species, *Colletotrichum truncatum* is the most prevalent species in major chilli growing areas resulting in a huge decline in the quality and quantity of the harvest (Noor and Zakaria, 2018; Silva *et al*., 2019; Welideniya *et al*., 2019). Based on previous studies, resistance against *C. truncatum* in *C. annuum* L. is controlled by single dominant gene (Park *et al*., 1990; Ridzuan, 2018; Mishra *et al*., 2019a, 2019b). In contrast, Mashuk *et al*., (2009) reported that resistance against *C. truncatum* in *Capsicum* Chinense is controlled by recessive genes.

In the process of developing anthracnose disease-resistant chilli hybrids, both phenotypic selection and genotypic selection are equally important. Phenotypic selection can be practised as a field experiment or laboratory experiment (Garg *et al*., 2013). Various molecular markers

are utilized in genotypic selection (Srivastava and Mangal, 2019). Molecular markers such as Amplified Fragment Length Polymorphic markers, Sequence Characterized Amplified Region, Cleaved Amplified Polymorphic Sequence, Simple Sequence Repeats (SSR) associated with anthracnose disease resistance in chilli have been identified by many researchers (Voorrips *et al.*, 2004; Lee *et al.*, 2011; Ying *et al.*, 2015; Suwor *et al.*, 2017; Mishra *et al.*, 2019b). These identified molecular markers can be utilized to breed the cultivars with resistance to anthracnose disease (Lee *et al.*, 2011). Among the identified markers, SSR markers are widely applied in plant breeding. These markers are highly polymorphic, multiallelic and monolocus. Therefore SSR markers are applied by the researchers to increase the efficiency of chilli breeding against anthracnose disease (Nanda, *et al.*, 2016;.Suwor *et al.*, 2017; Ly *et al.*, 2020). According to the study conducted by Ridzuan, (2018) using the resistant genotypes of *C. annuum* L, AVPP0805 and AVPP9813 developed by the Asian Vegetable Research and Development Centre, Taiwan, SSR markers, HpmsE 082 and HpmsE 051 were linked markers for anthracnose disease caused by the *C. truncatum.* Therefore, there is a possibility to identify *C. truncatum* resistant parent lines of *C. annuum* L further using these markers.

Homozygous parent lines are used as parents in the production of hybrid varieties. Qualities of the hybrid varieties depend on these parent lines (Shuro, 2017). Efforts have been taken to develop chilli hybrids against anthracnose using the parent lines with anthracnose disease resistance (Ridzuan, 2018). Herath *et al.* (2022) have identified four resistant parents of *C. annuum* L against *C. truncatum.* Chilli hybrids developed using these parents have applied to this study with the purpose of identification of new chilli hybrids resistant to anthracnose disease caused by the *C. truncatum* by the phenotypic and genotypic selection.

Materials and methods

Fourteen single cross chilli hybrids (Table 1) were developed using four resistant parents (MICH PL CA 2018/3, MICH PL CA 2018/20, MICH PL CA 2018/21 and MICH PL 35) against *C. truncatum,* previously identified through the fruit inoculation of *C. truncatum* and three susceptible parents (MICH PL CC 2018/33, MICH PL 21, MICH PL CC 2018/17) previously identified through the fruit inoculation of *C. truncatum* (Herath *et al.*, 2022). These 14 single cross chilli hybrids, their parental inbred lines and two commercial varieties (SJ2- 461, Kulai 907) as check varieties were used for this study (Table 1). The experiment was conducted in the glasshouse facility belonging to the Faculty of Agriculture, University Putra Malaysia (UPM) in two locations (Field 10 and Field 15). The experiment was conducted from August 2020 to December 2020. At the beginning of August 2020, seeds of chilli genotypes were sown in 50-cell seed trays filled with peat moss. Polybags (35 × 35 cm) were filled with a 4.5 kg soil mixture of 1:1 compost and topsoil to transplant the seedlings.

After one month, seedlings were transplanted in the poly bags inside plant houses at Field 10 (GPS location $2^0$58'54.0"N latitude and $101^0$42'53.8"E longitude) and Field 15 (GPS location $2^0$98'33.4"N latitude and $101^0$72'49.2"E longitude) under the randomized complete block design with three replicates with the spacing of 60 cm between rows and 45 cm within the row. Each replicate contained three plants per treatment. Chemical fertilizer application was done following recommendations of the Malaysian Agricultural Research and Development Institute (MARDI) (MARDI, 1997). As a fertilizer, 18 g of N, 3 g of P and 15 g of K were applied per each plant in total as one basal dressing and three top dressings. Sufficient irrigation was supplied throughout the study period.

Table 1. Chilli hybrids evaluated at two locations (Field 10 and Field 15)

Given name of the chilli hybrids	Cross combination
H1	MICH PL CC 2018/33 × MICH PL CA 2018/3
H2	MICH PL CC 2018/33 × MICH PL CA 2018/20
H3	MICH PL CC 2018/33 × MICH PL CA 2018/21
H4	MICH PL CC 2018/33 × MICH PL 35
H5	MICH PL CC 2018/33 × MICH PL CC 2018/17
H6	MICH PL 21 × MICH PL CA 2018/3
H7	MICH PL 21 × MICH PL CA 2018/20
H8	MICH PL 21 × MICH PL CA 2018/21
H9	MICH PL 21 × MICH PL 35
H10	MICH PL 21 × MICH PL CC 2018/17
H11	MICH PL CA 2018/20 × MICH PL CC 2018/33
H12	MICH PL CA 2018/20 × MICH PL CC 2018/17
H13	MICH PL CC 2018/33 × MICH PL 21
H14	MICH PL 21 × MICH PL CC 2018/33

Five red ripened fruits (40–45 days after flowering) from each treatment, and replicate were harvested separately from the chilli plants at the glasshouses at two locations (Field 10 and Field 15) for the fruit inoculation as two different experiments. Anthracnose disease severity assessment was conducted with the randomized complete block design with three replicates. *C. truncatum* was isolated using chilli fruits from three chilli cultivated field with severe anthracnose infestation and confirmed through molecular identification (gene bank accession numbers; MT995064 and MW030430) (Herath *et al.*, 2022). Nine days old *C. truncatum* cultures collected and confirmed by molecular level were incubated at room temperature (28°C–30°C) were used to prepare the conidial suspension of *C. truncatum.* Fruit inoculation with 1 µl conidial suspension of *C. truncatum* was done following Montri *et al.* (2009) using a Micro injector (micro syringe model 1705 TLL with a dispenser, PB 600-1Hamilton). The concentration of the conidial suspension was adjusted as 5×10^5 conidia·mL^{-1} using a haemocytometer (Marienfeld, Germany). Inoculated fruits were incubated in plastic boxes (13 cm × 13 cm × 7 cm), on four layers of white tissue moistened with 10 ml of sterilized distilled water. Data were collected on lesion size and fruit size after nine days of fruit inoculation.

After assessing 14 SSR markers and two STS markers, two polymorphic markers HpmsE 051 and HpmsE 082 were identified for genotypic selection. Total genomic DNA of chilli hybrids and parental inbred lines were extracted following Cetyltrimethyl Ammonium Bromide method according to Doyle and Doyle

https://doi.org/10.1017/S1479262123001144 Published online by Cambridge University Press

(1987). The polymerase chain reaction (PCR) included 7.5 µl of PCR master mix (1st base ex10 2X PCR master mix), 1 µl of template DNA, 1 µl of each primer. The reaction was adjusted to 15 µl with nuclease-free water. Amplification was performed using T100 Thermal Cycler (Bio-Rad, USA) following 3 min of initial denaturation, 30 s at 95°C of denaturation, 30 s at 55°C of annealing, 1minute at 72°C of extension, 10 min at 72°C for final elongation and cooled down to 4°C. Gel electrophoresis was done using 2% agarose gel in TBE buffer and set to run at 90 V for 60 min. Agarose gel was visualized under Gel Doc™ XR with molecular image software (Bio-Rad, USA).

Data analysis

Per cent lesion size relative to the overall size of the fruit ([lesion area/fruit area]*100) was estimated and anthracnose severity score was given according to 0–9 scale described by Montri *et al.*, (2009). Statistical Analysis Software (SAS) version 9.4 were used for the data analysis. Arcsin square root transformation was done for the data before analysis since data were not normally distributed. Data were checked for normality using the Shapiro–Wilk test. Mean separation for disease severity data was done by Duncan Multiple Range Test by using the transformed data after confirming the significant difference among genotypes (hybrids, parents and check varieties) for anthracnose disease by the analysis of variance. Under the molecular marker analysis, individuals that are similar to the amplified product size of parental inbred lines were categorized as homozygous [resistant (R) or susceptible (S)] and the individuals that showed resemblances with both parental inbred lines were classified as heterozygous.

Results

Table 2 showed the percentage mean of anthracnose disease severity (%) and resistance level of chilli hybrids, parents and check varieties harvested from the glasshouses at Field 10 and Field 15. Figure 1 showed the lesion development on chilli hybrid fruits and commercial varieties after 9 days of fruit inoculation. Fruits of chilli hybrids, H1, H2, H3, H4, H6, H7, H8, H9, H11 and H12 harvested from the glasshouses at Field 10 and Field 15 showed <2% of disease severity at both green mature fruit and red ripened fruit stage. From the total of 14 developed chilli

Table 2. Percentage means of anthracnose disease severity against *Colletotrichum truncatum* and resistant level of chilli hybrids, parents and commercial varieties

	Location 1 - Glasshouse at Field 10 UPM			Location 2 - Glasshouse at Field 15 at UPM		
Genotypes	Disease severity	Disease score	Resistant level	Disease severity	Disease score	Resistant level
H1	0.96 c	1	R	0.96 efgh	1	R
H2	0.82 c	1	R	1.39 e	1	R
H3	0.27 c	1	R	0.28 h	1	R
H4	0.41 c	1	R	0.43 gh	1	R
H5	11.89 b	5	MS	33.37 a	9	HS
H6	1.14 c	1	R	0.70 efgh	1	R
H7	1.15 c	1	R	0.91 efg	1	R
H8	1.41 c	1	R	0.49 fgh	1	R
H9	1.46 c	1	R	1.30 e	1	R
H10	19.70 a	9	S	27.54 bc	9	HS
H11	1.35 c	1	R	1.27 ef	1	R
H12	1.25 c	1	R	1.30 e	1	R
H13	25.17 a	9	HS	27.48 bc	9	HS
H14	25.79 a	9	HS	22.32 d	9	S
MICH PL CA 2018/3	1.18 c	1	R	0.66 efgh	1	R
MICH PL CA 2018/20	1.35 c	1	R	1.30 e	1	R
MICH PL CA 2018/21	1.42 c	1	R	0.99 efg	1	1
MICH PL CC 2018/33	37.13 ab	9	HS	30.79 ab	9	HS
MICH PL 35	1.27 c	1	R	1.11 efg	1	R
MICH PL 21	30.25 a	9	HS	23.82 dc	9	S
MICH PL CC 2018/17	33.72 a	9	HS	35.44 a	9	HS
SJ2- 461 (check)	28.42 a	9	HS	34.86 a	9	HS
Kulai 907 (check)	26.45 a	9	HS	32.16 a	9	HS

Within the column, the means followed by the same letters are not significantly different at $p = 0.05$. Anthracnose disease severity as disease score of 0 – highly resistant (HR), 1- resistant (R) 1-2% ,3- moderately resistant (MR)>2-5% -,5- moderately susceptible (MS) >5-15% ,7- susceptible (S) >15-25%, 9 - highly susceptible (HS) >25%.

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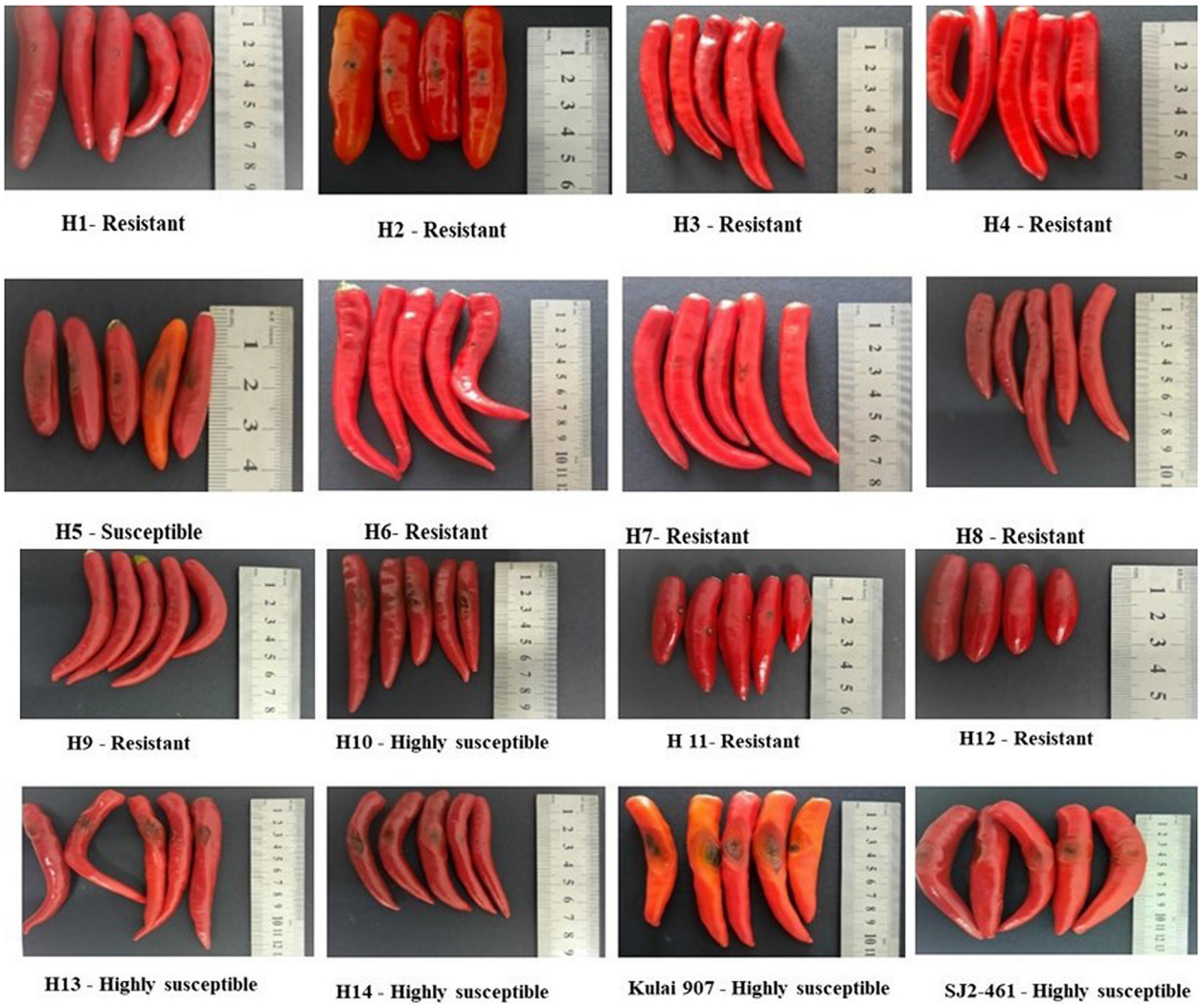

Figure 1. Anthracnose lesions produced after 9 days of inoculation on chilli hybrids.

hybrids, 10 showed a resistant response against anthracnose disease. According to Montri *et al*., (2009), <2% of anthracnose disease severity denotes the resistance against the disease. Therefore, these hybrids could be grouped as resistant chilli hybrids.

Among the parents, MICH PL CA 2018/3, MICH PL CA 2018/20, MICH PL CA 2018/21 and MICH PL 35 that were included as resistant parents the in crossing programme exhibited resistant response at both fruit stages with <2% of anthracnose disease severity in case of chilli fruits harvested from both Field 10 and Field 15. All the other developed hybrids (H5, H10, H13, H14) and parents (MICH PL CC 2018/33, MICH PL 21, MICH PL CC 2018/17) exhibited >15% or >25% of disease severity. According to Montri *et al*., (2009) >15% and >25% of anthracnose disease severity indicate the susceptible and highly susceptible responses respectively.

Commercial imported chilli hybrid, SJ2-461 had > 28% of disease severity at both fruit stages in the case of Field 10 and Filed 15 indicating the highly susceptible nature of this hybrid to the anthracnose disease caused by the *C. truncatum*. Similarly, local open-pollinated commercial chilli variety, Kulai 907 exhibited >26% of disease severity in the case of the chilli fruits harvested at both Field 10 and Filed 15. Therefore, Kulai 907 was a highly susceptible genotype for the anthracnose disease caused by the *C. truncatum*.

When considering the screening of chilli hybrids using the markers, HpmsE 051 and HpmsE 082 (Fig. 2 and Fig. 3), 10 hybrids were showed heterozygous nature for anthracnose disease resistance. Those hybrids were, H1, H2, H3, H4, H6, H7, H8, H9, H11 and H12. Amplified product size of the other four hybrids, H5, H10, H13, H14 were similar to the amplified product size of susceptible parental inbred lines and it indicated that these hybrids were susceptible to the anthracnose disease.

When comparing both phenotypic and genotypic data (Table 3) hybrids, H1, H2, H3, H4, H6, H7, H8, H9, H11 and H12 that showed heterozygous nature for anthracnose disease under the markers HpmsE 051 and HpmsE 082 were resistant to anthracnose disease caused by the *C. truncatum* based on the phenotypic evaluation. When one parent is resistant to *C. truncatum* in a cross, resulting hybrids were resistant to anthracnose disease caused by the *C. truncatum*. When both parents were susceptible to anthracnose disease caused by the *C. truncatum,* resulting hybrids were susceptible to the disease (H5, H10, H13 and H14).

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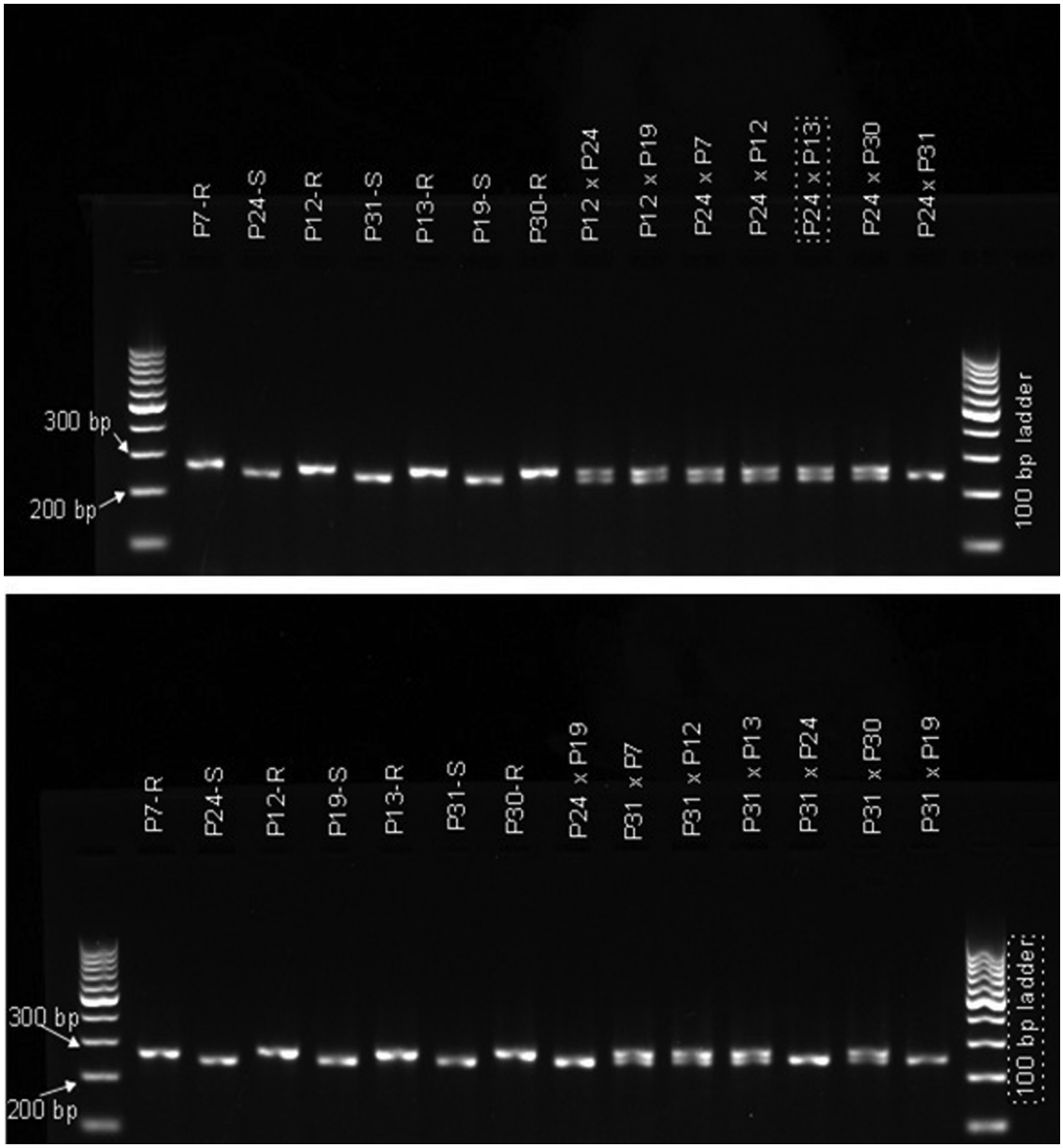

Figure 2. Screening of developed 14 chilli hybrids using marker HpmsE 051(262 bp) (R: resistant, S: susceptible, P7: MICH PL CA 2018/3 (R), P24: MICH PL CC 2018/33 (S), P12: MICH PL CA 2018/20 (R), P31: MICH PL 21 (S), P13: MICH PL CA 2018/21 (R), P19: MICH PL CC 2018/17 (S), P30: MICH PL 35 (R), H1 (P24 × P7), H2 (P24 × P12), H3 (P24 × P13), H4 (P24 × P30), H5 (P24 × P19), H6 (P31 × P7), H7 (P31 × P12), H8 (P31 × P13), H9 (P31 × P30), H10 (P31 × P19), H11 (P12 × P24), H12 (P12 × P19), H13 (P24 × P31), H14 (P31 × P24).

Discussion

Based on the phenotypic and genotypic data, new chilli hybrids (H1, H2, H3, H4, H6, H7, H8, H9, H11 and H12) developed using resistant breeding lines could be isolated as anthracnose disease-resistant chilli hybrids that carry the resistant gene. Further testing of these hybrids is needed to check the yield performance to isolate the potential hybrids for commercial cultivation with anthracnose disease-resistant character. In addition, these hybrids could be utilized to develop second cycle inbred lines that could be utilized as parents in the process of new anthracnose disease-resistant variety development. Even though, effort on the development of anthracnose disease-resistant varieties is very limited according to the available literature, this study provides information on the possibility of the development of anthracnose disease-resistant hybrids. Dominance nature of disease resistance of *C. annu*um against *C. truncatum* was observed in this study, because, when one parent is resistant to *C. truncatum*, resulting hybrid was anthracnose disease resistant.

However, based on the resistant germplasm of chilli, researchers have reported different findings regarding the inheritance of anthracnose disease resistance from past to present (1990–2021). Park, Kim and Lee (1990) found that inheritance of resistance to *C. truncatum* is controlled by a partial dominance gene in the *C. annuum* chilli accession, Chungryong. According to Lin *et al*., (2002), *C. annu*um breeding line, 83–168 was resistant to *C. truncatum* and inheritance of resistance was controlled by a single dominant gene. Ridzuan, (2018) observed the dominant

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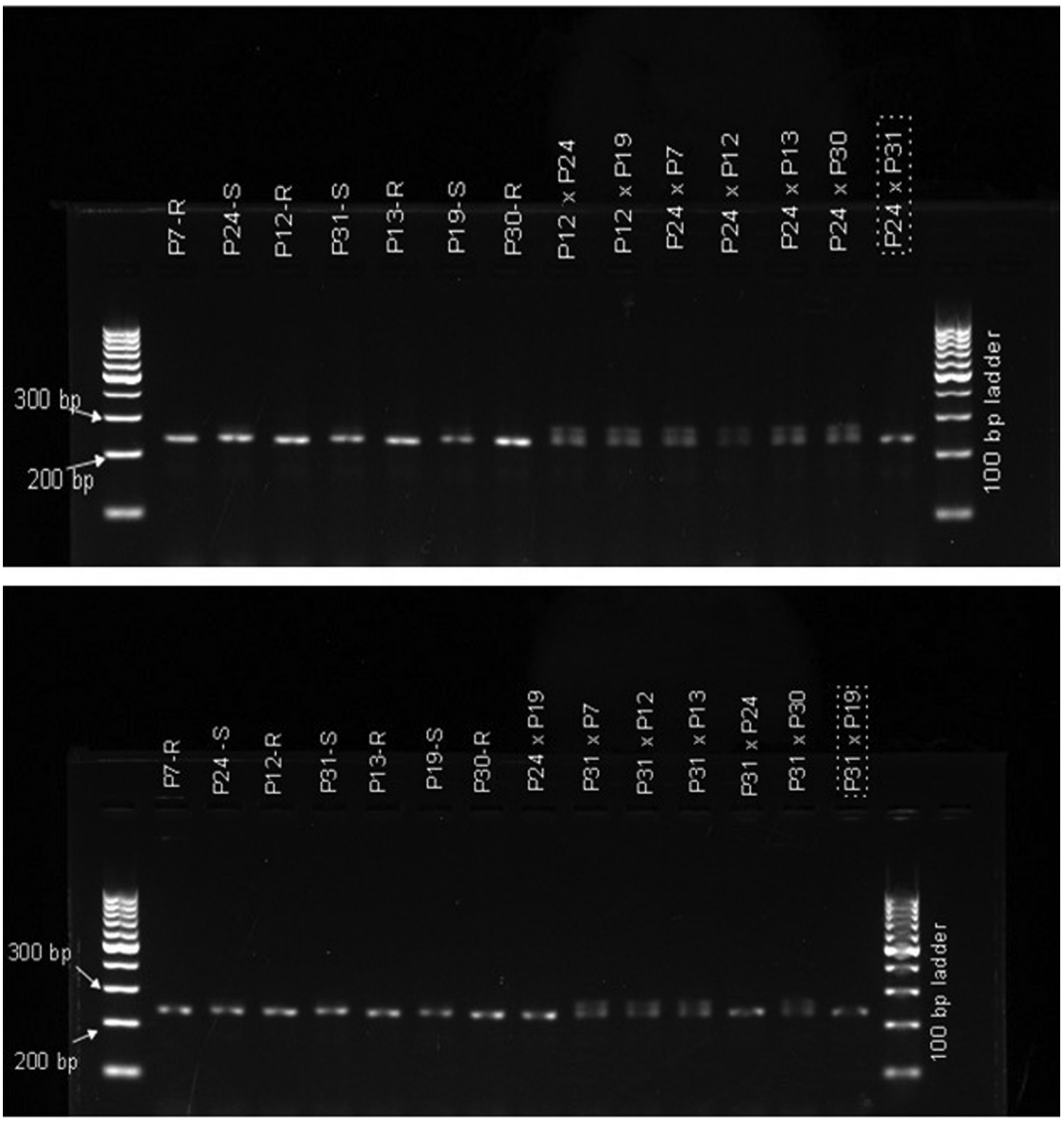

Figure 3. Screening of developed 14 chilli hybrids using marker, HpmsE 082 (232 bp). (R: resistant, S: susceptible, P7: MICH PL CA 2018/3 (R), P24: MICH PL CC 2018/33 (S), P12: MICH PL CA 2018/20 (R), P31: MICH PL 21 (S), P13: MICH PL CA 2018/21 (R), P19: MICH PL CC 2018/17 (S), P30: MICH PL 35 (R), H1 (P24 × P7), H2 (P24 × P12), H3 (P24 × P13), H4 (P24 × P30), H5 (P24 × P19), H6 (P31 × P7), H7 (P31 × P12), H8 (P31 × P13), H9 (P31 × P30), H10 (P31 × P19), H11 (P12 × P24), H12 (P12 × P19), H13 (P24 × P31), H14 (P31 × P24).

gene action in the inheritance of anthracnose disease caused by the *C. truncatum* by the evaluation of F_2 segregation population resulted through the self-pollination of a cross between a resistant parent and susceptible parents. According to the study conducted using the *C. annuum* species, 'Punjab Lal' – Resistant parent × 'Arka Lohit'- susceptible parent found that monogenic dominant gene is responsible for the anthracnose disease caused by *C. truncatum* (Mishra *et al.*, 2019a). These findings are in conformity with our study. In contrast to these findings, Kim *et al.*, (2008) found that local Korean variety, Daepoong-cho belongs to the species, *C. annuum*, exhibited resistance to *C. truncatum* and further studies conducted by them revealed that this resistance is controlled by a single recessive gene. A study was conducted using *C. chinense* accession, PBC 932 and observed that three recessive genes namely, co 1, co 2 and co 3 were responsible for resistance to *C. truncatum* during the seedling, mature green fruit and red ripen fruit stages (Mashuk, *et al.*, 2009). Even though anthracnose disease is a devastating fungal disease in chilli, still it has difficult to find the responsible genes conferring disease resistance (Son *et al.*, 2021).

As observed by this study, markers, HpmsE 051 and HpmsE 082 were good polymorphic markers to isolate resistant genotypes of *C. annu*um (parents and hybrids) for the anthracnose disease caused by the *C. truncatum* for anthracnose disease-resistant breeding of chilli. Yi *et al.*, (2006), developed SSR markers based chilli linkage map and reported that, two markers, HpmsE 082 and HpmsE 051 were located on chromosome number 9 in the chilli genome. It implied that gene/genes located on chromosome number 9 are responsible for resistance against anthracnose disease caused by the *C. truncatum.*

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Table 3. Comparison of phenotypic and genotypic data

Genotype	Resistant level observed under phenotypic evaluation	Homozygosity or heterozygosity under marker analysis for anthracnose disease resistance in hybrids
H1	R	Heterozygous
H2	R	Heterozygous
H3	R	Heterozygous
H4	R	Heterozygous
H5	MS	Homozygous
H6	R	Heterozygous
H7	R	Heterozygous
H8	R	Heterozygous
H9	R	Heterozygous
H10	S	Homozygous
H11	R	Heterozygous
H12	R	Heterozygous
H13	HS	Homozygous
H14	HS	Homozygous

R, resistant; MS, moderately susceptible; S, susceptible; HS, highly susceptible.

Conclusion

Based on the phenotypic and genotypic data, chilli hybrids, H1, H2, H3, H4, H6, H7, H8, H9, H11 and H12 were identified as resistant chilli hybrids against anthracnose disease caused by the *C. truncatum* whereas hybrids, H5, H10, H13 and H14 were susceptible to anthracnose disease. Molecular markers, HpmsE 051 and HpmsE 082 validated in this study could be utilized as polymorphic markers to isolate resistant genotypes of *C. annuum* in the process of anthracnose disease-resistant variety development against *C. truncatum*.

Acknowledgements. The authors wish to thank Sri Lanka Council for Agriculture Research Policy for granting the PhD scholarship to H.M.S.N. Herath.

Competing interest. None.

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