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Original Research Paper

Evaluation of screening methods for anthracnose fruit rot resistance in chilli (*Capsicum* spp.)

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ABSTRACT

Anthracnose fruit rot caused by *Colletotrichum* spp. is a serious production constraint causing severe marketable yield loss in chilli. Field evaluation of chilli accessions for resistance to *Colletotrichum* spp. depends on various factors affecting disease expression such as edaphic conditions, temperature, rainfall, humidity and other variables that are difficult to control, therefore considered less accurate. Also, high chances of cross contamination with different *Colletotrichum* species leads to inconclusive assays for specific pathogen species and isolate. To identify a stable and reliable screening method, various chilli accessions were subjected to *in vitro* pin-prick and non-wounding spray methods using a specific pathogen isolates. When chilli accessions were screened against *C. gloeosporioides* isolate '*IHRCg-1',* the *in vitro* pin- prick method showed positive correlation with the non-wounding spray method, except in the accession PBC80. The change in bioassay influenced the disease reaction pattern in the accession PBC 80, probably the pin pricks break the basal cuticle defense mechanism that was retained in spray inoculation method indicating varied resistance pattern. However, in the accession PBC 81 stable resistance pattern was observed against isolates of both species *viz*., *C. truncatum 'IIHR Ct-1'* and *C. gloeosporioides 'IIHR Cg-1'* and in the accession PBC 80 against *C. truncatum 'IIHR Ct-1'* in both the inoculation methods that depicted the expression of resistance genes during both methods of inoculation. Based on disease development pattern, the red ripe chilli expressed a variant reaction to infection by *C. truncatum* and *C. gloeosporioides*. The peak anthracnose infection at 10 DAI and 14 DAI is an accurate duration to record *'IIHR Cg-1'* and *'IIHR Ct-1'* infection, respectively on chilli ripe fruit for assaying the resistance.

Keywords: Anthracnose, chilli, pin-prick, non-wounding spray, *Colletotrichum truncatum, C. gloeosporiodes*

INTRODUCTION

Chilli (*Capsicum* spp.) is one of the most important and widely cultivated spice crops. Currently, India being the largest consumer, producer and exporter of day chillies and its products constituting approximately 42.3% of the total world production accounting for 2.05 million tonnes (FAOSTAT, 2021). In India, Andhra Pradesh produces 0.63 million tonnes of chilli from 0.23 million hectares followed by Telangana (0.55 million tonnes in 0.18 million ha) and Indian chilli exports amounting for 8581.88 crores rupees (SB, 2023).

The anthracnose fruit rot or dieback caused by *Colletotrichum* species is one of the major constraints in chilli production which affects both at pre and post-harvest stages (Saxena et al., 2016). At least 24 species of *Colletotrichum* are known to be the pathogens of chilli anthracnose disease (Mongkolporn & Taylor, 2018). Amongst them, three primary species *viz*., *Colletotrichum truncatum* (Syn. *C. capsici* Syd. Butler and Bisby), *C. gloeosporioides* and *C. scovillei* (Syn. *C. acutatum*) are very serious. All developmental stages are targeted by *C. gloeosporioides* (Sharma et al., 2005; Katoch et al., 2017), while, *C. truncatum* causes major damage at the ripe fruit stage of the plant (Saxena et al., 2014). Typical symptoms include dark spots, and sunken necrotic tissue with concentric rings of acervuli on fruits (Mistry *et al*., 2010). The disease is aggressive in major chilli growing belts causing 25-30% loss across the nation (Lakshmesha et al., 2005) which annually sums up to US$ 491.67 million (Garg et al., 2013).

The main sources of resistance to anthracnose have been identified in different accessions of *Capsicum baccatum* L. and *C. chinense* Jacq. (AVRDC, 1999

and Kim et al., 2008a). Accurate, fast, economic and repeatable screening methodology is crucial in breeding programmes to develop resistant varieties (Galvan, 2010). Therefore, the present study evaluates the effectiveness of available chilli anthracnose fruit rot screening protocols and incubation period.

MATERIALS AND METHODS

Plant material

Six *Capsicum* spp. accessions (Table 1) were screened against virulent isolates of C. *truncatum 'IIHR Ct-1'* and *C. gloeosporioides 'IIHR Cg-1'* under field and *in vitro* conditions for anthracnose fruit rot resistance.

Table 1 : Field evaluation of chilli accessions against *Colletotrichum truncatum 'IIHR Ct-1'*

Accession	Disease Severity	Disease reaction
PBC80 (*Cb*)	1.0[c]	R
PBC81 (*Cb*)	0.8[c]	R
IHR4491 (*Cb*)	35.3[a]	HS
Solan Bharpur (*Ca*)	28.70 [a]	HS
EC382053 (*Ca*)	35.6 [a]	HS
EC399573 (*Ca*)	29.0[b]	HS

R-resistance, HS- highly susceptible

*values are *arc sine* transformed before analysis. Means the same letter are not significantly different in Duncan's multiple range test (p d" 0.05)

Isolation and identification of pathogen

A small tissue piece (5x5 mm) was taken at the edge of the infected area, washed in sterile distilled water, surface-disinfected in 70% ethanol for 30 sec and 1% (v/v) sodium hypochlorite (NaOCl) for 1 min, rinsed three times in sterile distilled water and placed on potato dextrose agar (PDA, Himedia, India) amended with cocktail of antibiotics *i.e.* streptomycin, tetracycline, ampicillin and chloramphenicol (100 µg/mL. Plates were incubated for five days at 25±1°C with a 12 h photoperiod provided by fluorescent light. The growing edges of fungal hyphae developing from the tissues were then transferred aseptically to PDA (Chowdappa et al., 2015). The pathogens were molecularly characterized using fungal ITS specific primers *viz*., ITS 1 and ITS 4 (White et al., 1990).

Inoculum preparation

The fully sporulated plates of two weeks old were loaded with sterile distilled water and conidia were gently scraped off from the plates. Spore density was made up to 10^5 spores mL^{-1} using haemocytometer (AVRDC, 1999). To reduce surface tension, Tween-20 was added to the inoculum (0.5 mlL^{-1}).

Open-field screening protocol

Field screening was performed in a randomized block design with three replications. Six accessions (Table 1) at the red ripe fruit stage were inoculated with *'IIHR Ct-1'* by spraying fresh spore inoculum of known density (Rajapakse & Ranasinghe, 2002; Susheela, 2012). The fruits were then immediately covered with a polyethene bag for four days followed by spraying of water twice a day post removal of plastic covering. Non-inoculated (spraying only with water and surfactant) was included as a control (Pedrosa et al., 2004). The symptoms were visually estimated and disease severity index (DSI) was calculated as per Montri et al., (2009) at two weeks after spraying the inoculum on fruit based on the mean percentage lesion size of fruits (Suwor et al., 2015).

In vitro assay

Fully matured red ripe fruits were surface sterilized with 1 per cent (v/v) sodium hypochloride for 5 mins. Further, twice washing with distilled water and then wiped dry with sterilized paper towels. Spore suspensions of virulent isolates of *'IIHR Ct-1'* and *'IIHR Cg-1'* were inoculated by pin prick method to infilter the inoculums (with 5 µl droplets) into fruit (Kim et al., 1989) while 5 µl droplets of sterilized water were kept as control. Fruits were then incubated at 25±1°C with a 12 h dark/light cycle in a small moist chamber (relative humidity > 90%) created by spreading layers of moistened paper towel in acrylic boxes. Anthracnose symptoms at the inoculation sites were evaluated as per AVRDC (1999). Similarly, disease severity was calculated.

RESULTS AND DISCUSSION

Detection and identification of *C. truncatum* and *C. gloeosporioides* isolates

The isolated pathogen from the naturally infected host was identified as *C. truncatum* and *C. gloeosporioides* on the basis of morphological, pathogenicity and molecular assays (Fig. 1). The ITS sequence of the *'IIHR Cg-1'* with accession MN873009 showed 99.6% similarity with accession MG282163 (Saini et al., 2017b) in NCBI-BLAST, while the accession MN873012 of *'IIHR Ct-1'* isolate exhibited 98.7%

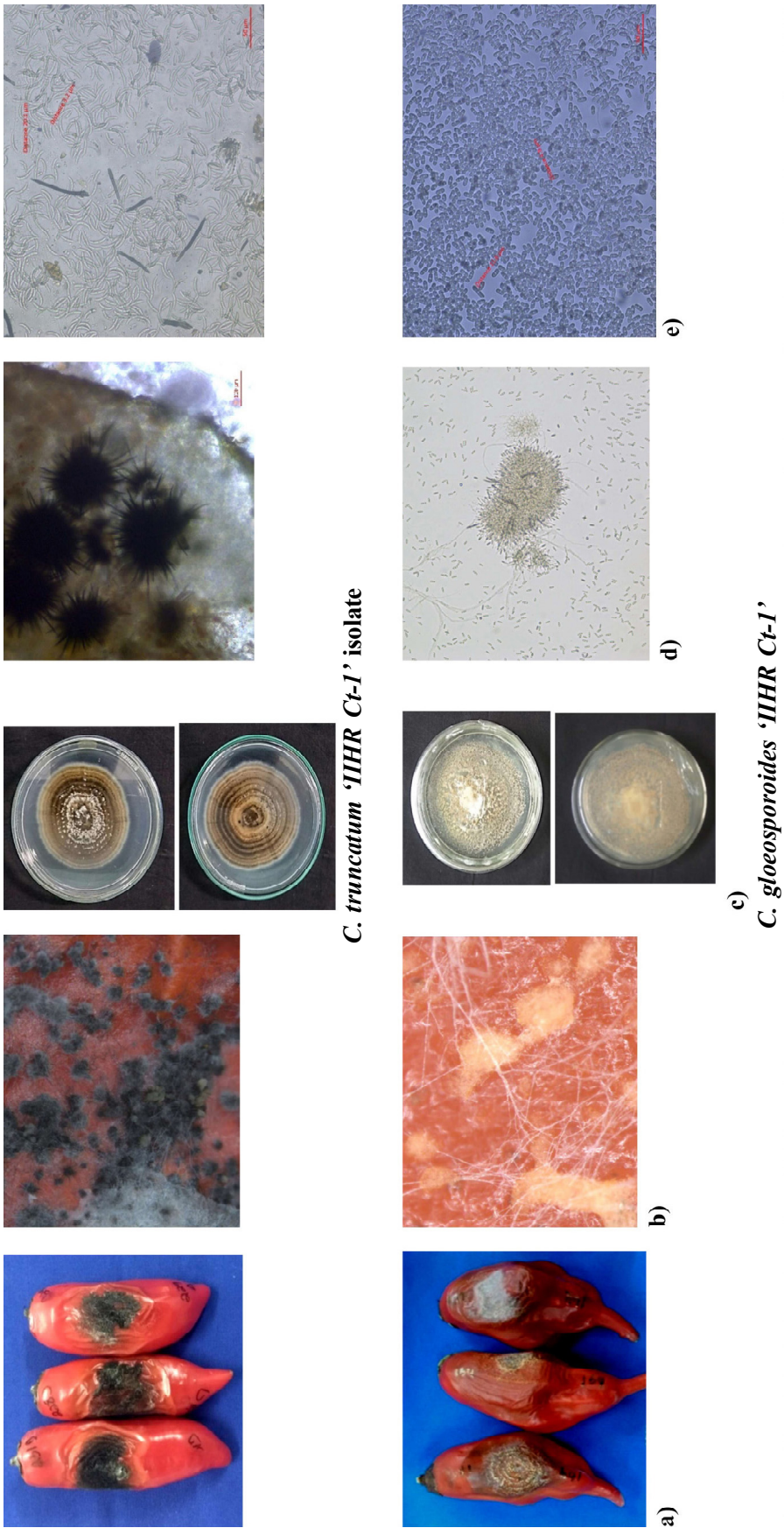

Fig. 1 : Morphological confirmation of *Colletotrichum* species, (a) Anthracnose infected fruits, (b) Stereo microscopic view mycelial and acervuli on fruit surface, (c) Upper colony and lower colony surface of culture, (d) Acervuli of *Colletotrichum* species 100 *µm*, (e) Conidia of *Colletotrichum* species. Bars 50

similarity with accession MG204566 (Saini et al., 2017a). The anthracnose species *viz.*, *C. truncatum 'IIHR Ct-1'*and *C. gloeosporioides 'IIHR Cg-1'* were able to cause infection in susceptible accessions (Table 1 & 2).

Reaction of different accessions against *C. truncatum 'IIHR Ct-1'* and *C. gloeosporioides 'IIHR Cg-1'* isolates

In artificial non-wounding method of field screening, the accessions, PBC 80 and PBC 81 conferred resistant to *'IIHR Ct-1'* showing 1.0 and 0.8 DSI, respectively, while, IHR4491 and *C. annuum* accessions were found susceptible (Table 1). Under pinprick method, the accessions *viz.*, PBC 80 and PBC 81 exhibited resistance to *'IIHR Ct-1'* which confirmed the positive correlation of both screening methods (Susheela, 2012). In the present study, PBC 81 exhibits resistant reaction with 1.80% DSI, while, PBC 80 exhibited susceptible reaction (9.57% DSI) against *'IIHR Cg-1'* in pin prick method (Table 2). This finding contradicts the report of Mongkolporn et al. (2010) that 'PBC80' exhibited resistant reaction to 11 isolates of *C. gloeosporioides* in wounding assay. However, Mahasuk et al. (2013) reported that the resistance reaction changes based on inoculation methods. The pin pricking method confirmed the presence of resistance genes and bypasses the pseudo resistance as a result cuticle and jasmonates signaling pathway that activates the pathogenic related (PR) proteins which in fact is present in the non-wounding method of inoculation (Ro et al., 2021). Therefore, the 'PBC 81' exhibited resistant to both *Colletotrichum* spp., while, 'PBC80' lacks resistance to *'IIHR Cg-1'* isolate.

Anthracnose disease progression under controlled condition

The post-inoculation period of incubation determines the progression of anthracnose disease in chilli fruit. The disease development started at 3 DAI (Fig. 2) which is in agreement with the previous studies (Garg et al., 2013; Mishra et al., 2019) and progressed further in subsequent days irrespective of the genotypes and *Colletotrichum* spp.

Depending upon the host-pathogen interaction, the peak disease progression was recorded at different days after inoculation. Despite, the peak progression of *'IIHR Cg-1'* at 14 DAI, the infection rate is at par

Table 2 : *In vitro* differential reaction of chilli genotypes against *Colletotrichum gloeosporioides 'IIHR Cg-1'* and C. *truncatum 'IIHR Ct-1'*

Accession	3 DAI			7 DAI			10 DAI			14 DAI		
	C. g 'IIHR Cg-1'	Disease reaction	*C. t* 'IIHR Ct-1'	*C. g* 'IIHR Cg-1'	Disease reaction	*C. t* 'IIHR Ct-1'	*C. g* 'IIHR Cg-1'	Disease reaction	*C. t* 'IIHR Ct-1'	*C. g* 'IIHR Cg-1'	Disease reaction	*C. t* 'IIHR Ct-1'	Disease reaction
PBC80 (*Cb*)	0.37^{e}	HR	0.10^{e}	2.94^{d}	MR	0.30^{e}	9.03^{d}	MS	0.97^{d}	9.57^{d}	MS	1.90^{e}	R
PBC81 (*Cb*)	0.03^{f}	HR	0.10^{e}	0.53^{f}	R	0.47^{f}	1.67^{f}	R	1.07^{d}	1.80^{f}	R	1.57^{e}	R
IHR4491 (*Cb*)	1.53^{c}	R	1.70^{b}	15.23^{b}	S	10.56^{a}	37.67^{a}	HS	22.57^{a}	40.67^{a}	HS	37.10^{a}	HS
Solan Bharpur (*Ca*)	4.20^{a}	MR	3.43^{a}	18.73^{a}	S	10.03^{b}	29.10^{b}	HS	12.50^{b}	31.30^{b}	HS	31.00^{b}	HS
EC382053 (*Ca*)	0.43^{d}	HR	1.53^{c}	1.80^{e}	R	6.37^{c}	5.97^{e}	MS	10.21^{c}	7.10^{e}	MS	22.80^{d}	S
EC399573 (*Ca*)	1.80^{b}	R	0.27^{d}	5.83^{c}	MS	2.70^{d}	21.40^{c}	S	9.80^{d}	22.63^{c}	S	27.33^{c}	HS

HR: highly resistant; R: resistance, MR: moderately resistant; MS: moderately susceptible; S: susceptible; HS: highly susceptible

*Values are *arc sine* transformed before analysis. Means the same letter are not significantly different in Duncan's multiple range test (p d" 0.05)

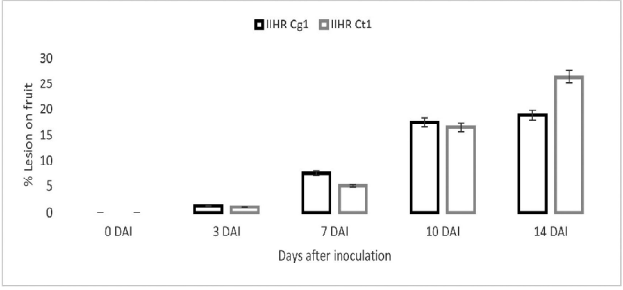

Fig. 2 : Anthracnose (*C. gloeosporioides* 'IIHR Cg-1' and *C. truncatum* 'IIHR Ct-1) progression on chilli fruit (Bar indicates standard error @ 5%)

with 10 DAI (Fig. 2) indicating 10 DAI as an appropriate to record anthracnose disease progression (Mishra et al., 2019). Alternatively, *'IIHR Ct-1'* inoculated fruits exhibited a significant difference disease progression between 10 DAI and 14 DAI (Table 2). Hence, observation for *C. truncatum* inoculated fruits were recorded at 14 DAI in agreement with Souza et al. (2019) & Ro et al. (2021).

CONCLUSION

Selection of precise screening assays is prerequisite in resistant breeding program. Both screening methods (field spraying and microinjection) substantiate equal disease reactions against the susceptible *C. baccatum* 'IHR4491' and *C. annuum* accessions. However, the wound inoculation or pin prick method is the desired bioassay for anthracnose fruit rot screening. Pin prick method provides chances of double inoculation, exhibited distinguishable symptoms, produces highly reproducible results, remove pseudo resistance and exerts the resistance gene. Additionally, the field spray method resulted in mixed species infection and thus, is not desirable for specific *Colletotrichum* spp. studies. Moreover, the experimental findings stabilized the disease reaction of the genotypes with respect to the incubation period by standardizing the incubation period at 10 DAI and 14 DAI for *C. gloeosporioides* and *C. truncatum*, respectively.

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