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Virulence test of colletotrichum species isolates on some mango (*Mangifera indica* L.) varieties in Western Ethiopia

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Abstract
Colletotrichum species are the most important and prevalent pathogen causing great yield losses to mango growers in the country. Characterization of the causal agent virulence variability of the pathogen on their host response is not well documented in southwestern part of Ethiopia. Therefore, the aims of the current study were to investigate pathogenicity of *Colletotrichum* species (isolates) and to assess pathogenic variability on local and commercially cultivated mango varieties in Ethiopia. Isolates of *Colletotrichum* species collected from nine locations in southwestern Ethiopia from mango leaves, panicles and fruit were studied for pathogenicity. Artificial inoculation of 5 varieties of Tommy Atkins, Keitt, Kent, Apple mango, Vandyke and local variety was made by wounding detached mango fruits, leaves and seedlings under greenhouse condition. The pathogenicity test result on detached mango fruits showed the presence of three virulence level among *Colletotrichum* isolates as highly pathogenic (62.5%), pathogenic (25%) and mildly pathogenic (12.5%). Virulent isolate tested on mango seedlings scored (13.6mm) lesion diameter. Accordingly, local mango varieties, Kent and Vandyke were categorized as susceptible varieties, while Tommy Atkins, Keitt and Apple mango varieties were intermediately susceptible. Since this result was solely based on laboratory experiment, we recommend further research to be conducted at field conditions on those varieties and including other mango varieties in the country.

Keywords: colletotrichum, Ethiopia, keitt, kent, mango, pathogenicity

Introduction

Materials and Methods

*Colletotrichum*s species isolates were retrieved from the leaves, panicles and attached fruit of mango showing typical symptoms of anthracnose. The isolates were taken from nine location of mango grown at home gardens southwest Ethiopia. After which the isolates were grown on potato dextrose agar medium (PDA). After confirming the spores, the cultures were purified by single spore isolation technique (Dhingra and Sinclair, 1993) [2]. The fungi were identified on the basis of morphological characteristics through mycological key suggested by Barnett *et al*. (1972) [1]. Suspension of conidia was prepared by suspending mycelia scraped from seven days old of *Colletotrichum* species isolates were separately added in 3-milliliter sterile distilled water and shacked vigorously for 3 minutes. The resulting suspensions were filtered through 2-layer of cheese cloth. The concentrations of spore suspension were adjusted to (1 x 10^6 spore's ml $^{-1}$) or conidia using haemocytometer.

Artificial inoculation methods were commonly used to test the pathogenicity of a fungal species *in vitro*, as it is easy to control environmental conditions (Ekbote *et al.*, 1997) [3]. Pathogenicity tests on detached fruits, leaves and seedling of mango varieties (cv. Tommy Atkins, Keitt, Kent, Apple mango, Vandyke and locale mango) collected from Melkasa Agricultural Research Canter was made. Detached fruits and leaves were washed under running tap water for 60 seconds followed by surface sterilization by immersing the fruits in 70% ethanol for 3 minutes, then rinsed three times in sterile distilled water for 2 minutes and dried with sterile tissue paper and then air dried.

Inoculations of isolates on physiologically matured detached mango fruits were done. , Surface sterilized fruits were placed in a plastic box having three fruits per treatment with tissue paper then sprayed with sterilized water to maintain at least 95% relative humidity (Than *et al.*, 2008a) [29]. The samples were inoculated using the wound inoculation method which included pin-pricking the fruits to a 3 mm depth with a sterile needle in the middle portion of fruit, and then placing 20µl of conidia suspension on to the wound (Than *et al*., 2008a, b) [30]. Control fruits were inoculated with 20µl of sterile distilled water. The inoculated samples were incubated in the plastic containers at 25°C under controlled conditions. The plastic box was removed after 48hrs and fruits were kept at the same temperature. Fruits were monitored for the onset of symptoms for 5-11 days. Pathogenicity test on detached leaves free from anthracnose symptom were collected from greenhouse grown seedlings of Vandyke, Keitt, Tommy Atkins, Kent, Apple mango and local mango varieties. The leaves were properly washed, and

surface sterilized before treatment. Then three leaves per treatments were placed on plastic boxes lined on the inside with moist tissue paper. Wound inoculations technique was performed following the protocol described by Oliveira Costa *et al.* (2010) [11]. Aqueous conidial suspensions of *Colletotrichum* isolates (1 x 10^6 spore's ml $^{-1}$) were prepared from 7-day-old cultures of each isolates. Leaves inoculated with sterile distilled water drops served as controls. Inoculated leaves were incubated in plastic boxes with moist tissue paper for 48 hours in the laboratory to ensure high humidity that creates favorable conditions for conidial germination and infection at room temperature. The inoculated leaves were monitored daily after 5 days of inoculation for lesions formation and measured accordingly.

Anthracnose development on seedling test, six mango varieties were arranged in the greenhouse and three attached leaves of all varieties having similar size were selected for inoculation. The selected leaves were washed, and surface sterilized by immersing the leaves in 70% ethanol for 3 minutes and then immersing three times in sterile distilled water for 2 minutes and drying with sterile tissue paper and then air dried (Sanders and Korsten, 2003) [22]. Then 20µl of conidia suspension was placed onto three spot on each leave, and the control tissues was similarly treated with distilled water. Inoculated seedling leaves were incubated in moist plastic sheet for 48 hours in greenhouse condition to ensure high humidity for establishing favorable conditions for conidial germination and infection.

Disease reactions of the hosts were evaluated by measuring the length, width and area of the typical anthracnose lesion which developed on tissues. Isolates were considered pathogenic when the lesion area advanced beyond the 4mm diameter initial injury. The pathogenicity of *Colletotrichum* strains was estimated according to the grading scale (Nova *et al.*, 2011) [10] presented in the (Table. 1) varying from 0 to 4. Lesion length (along the long axis of the tissues) and width (along the short axis of the tissues) were measured and evaluation of response of different mango varieties against anthracnose disease of mango seedling caused by *Colletotrichum* isolate at (Table. 2) greenhouse (Giblin *et al*., 2010) [4]. It was assumed that lesions grew in a circular manner so that their diameter (LD) was evaluated using the formula proposed by Sharma *et al*, (2005) [24].

$$LD\ (mm) = \frac{\text{Lesion length + lesion width}}{2}$$

The virulence of the isolates was evaluated by measuring the lesion length at 11 days after inoculation in two perpendicular directions on each leaves. Lesion diameter and Percent Disease Index (PDI) was calculated. The PDI was calculated by using the formula (Prabakar *et al.*, 2005) [16].

$$PDI = \frac{\text{Sum of all numerical ratings}}{\text{Total number of observations}} \times \frac{100}{\text{Maximum rating observed}}$$

The numerical values of percent disease index and latent period were used to calculate the virulence index using the following formula (Thakur and Rao, 1997) [28].

Virulence index (VI) = Percent disease index (PDI) $_{x}$ Latent period^{-1}

Table 1: Grading scale used for evaluation of the pathogenicity of *Colletotrichum* species isolates in mango fruits (Nova *et al.*, 2011)

Grade	Symptoms characteristics (lesions mm)	Classification of the isolates
0	Absence of lesions	Non-pathogenic
1	from 1 to 5 mm	Weakly pathogenic
2	Lesions from 6 to 15 mm	Mildly pathogenic
3	from 16 to 25 mm	Pathogenic
4	Lesions over 25 mm	Highly pathogenic

Table 2: Grading scale used for evaluation of response of different mango varieties against anthracnose disease of mango seedling caused by *Colletotrichum* isolate at greenhouse (Giblin *et al*., 2010)

Scale rate	Symptom lesion(mm)	Host response
0	no symptom	Immune
1	1mm lesion	Highly resistance
2	2-3mm lesion	resistance
3	3-5mm lesion	intermediate
4	lesion>5mm	susceptible
5	spread all area of leaves	highly susceptible

Statistical analysis

The laboratory and greenhouse experiments were arranged in Completely Randomized Design (CRD) with three replications. The experiment was carried out twice for each experiment. Single and interaction effects of factors

were determined using the general liner model (GLM) procedure of (SAS, 2008) [3]. Whenever significant interaction was observed between factors; the level of one factor was compared at level of other factors. Mean values among treatments were compared by the LSD at □ = 5% level of significance.

Results and Discussion

The finding revealed that the interaction between *Colletotrichum* species isolates and tested mango varieties showed significant effect on the pathogenicity test of the physiologically matured detached mango fruits ($p < 0.0001$). Among eight isolates of *Colletotrichum* tested, maximum disease severity index 30.5, 33.3, 30.5, 30.4, were recorded in CGm-F, CSC, CBK-F, CAD-F and CBL-L, respectively (Table 3) on Vandyke mango variety while the severity index was not significantly different from each other. The result indicated that lower severity (percent disease index), was observed on isolate CMn-w (11.1 %) on Apple mango (Table 3). According to their pathogenicity, *Colletotrichum* isolates based on mean lesion diameters were grouped into three as highly pathogenic, pathogenic and mildly pathogenic (Table 4).

Table 3: Disease severities index (%) of *Colletotrichum* isolates on detached physiological matured mango fruits

	Varieties					
Isolates	**Vandyke**	**Keitt**	**Tommy Atkins**	**Kent**	**Apple mango**	**Local mango**
CGm-F	30.5^{ab}	33.3^{a}	33.1^{a}	33.2^{a}	32.8^{a}	33.3^{a}
CAd-F	30.4^{ab}	33.1^{a}	33.2^{a}	33.1^{a}	33.1^{a}	33.0^{a}
CBk-F	30.5^{ab}	33.2^{a}	27.7^{bc}	33.3^{a}	25.2^{cd}	33.2^{a}
CMn-w	25.3^{cd}	25.2^{cd}	16.7^{f}	25.1^{cd}	11.1^{g}	30.5^{ab}
CBm-F	20.5^{cd}	16.7^{f}	16.6^{f}	27.2^{bc}	30.1^{ab}	16.7^{f}
CG-L	19.4^{ef}	25.0^{cd}	22.2^{de}	22.1^{de}	27.7^{bc}	19.4^{ef}
CSC	33.3^{a}	33.1^{a}	33.0^{a}	33.3^{a}	33.3^{a}	33.0^{a}
CBL-L	30.5^{ab}	33.2^{a}	27.8^{bc}	30.1^{ab}	27.4^{bc}	29.1^{ab}
Control	0.0^{h}	0.0^{h}	0.0^{h}	$0.\ 0^{h}$	0.0^{h}	0.0^{h}

Means followed by the same letter are not significantly different at □= 5% level from each other. LSD values for the interactions comparisons were: 4.8 and CV=10.4%.

Isolates CGm-F, CAd-F, CBK-F, CSC and CBL-L were highly pathogenic, but isolates CGL and CMn-w were pathogenic, while isolate CBm-F was mildly pathogenic except on Kent variety (Table 4).

Table 4: Pathogenicity of *Colletotrichum* isolates on detached physiological matured mango fruit lesion diameter (mm)

	Varieties					
Isolates	**Vandyke**	**Keitt**	**Tommy Atkins**	**Kent**	**Apple mango**	**Local mango**
CGm-F	25.3^{h-l}	45.3^{abc}	35.6^{f}	42.0^{cde}	41.6^{cde}	47.6^{ab}
CAd-F	24.0^{i-l}	38.3^{d-f}	30.0^{gh}	38.0^{def}	34.3^{fg}	41.6^{cde}
CBk-F	26.0^{h-j}	34.0^{fg}	26.0^{hij}	34.7^{fg}	23.0^{i-l}	35.0^{fg}
CMn-w	18.0^{m-p}	21.0^{j-n}	12.6^{q-s}	20.3^{k-n}	20.0^{l-o}	25.3^{h-l}
CBm-F	20.3^{k-n}	10.0^{rs}	11.5^{p-s}	24.6^{h-l}	6.0^{s}	10.3^{rs}
CGL	14.7^{o-r}	22.3^{i-l}	16.0^{q-r}	14.7^{o-r}	25.7^{h-k}	14.3^{o-r}
CSC	29.7^{gh}	36.7^{fg}	33.7^{fg}	30.0^{gh}	50.0^{a}	43.3^{bcd}
CBL-L	27.0^{hi}	46.0^{abc}	26.3^{hij}	35.0^{fg}	47.7^{ab}	41.3^{cde}
Control	0.0^{t}	0.0^{t}	0.0^{t}	0.0^{t}	0.0^{t}	0.0^{t}

Means followed by the same letter are not significantly different at □= 5% level from each other. LSD values for the interactions comparisons were 5.5 and CV=12.1%.

The pathogenicity test result on detached mango fruits showed that the virulence variability of *Colletotrichum* isolates, the result was in line by Pandey *et al.* (2011) [13] who reported *C. gloeosporioides* isolates produced the highest lesion growth compared to other isolates. It can be argued that variation in the isolates may be inherent since the isolates were collected from different sites. Hence, the physiological characters are influenced by environmental conditions through natural mutations which may be responsible for such variability in virulence. Similar results were obtained by Rojas-Martınez *et al.* (2008), who have showed differences in the virulence of the *C. gloeosporioides* isolates.

The virulent isolate CGm-F was used to test for varietal susceptibility on six different mango varieties namely Van-Dyke, Keitt, Tommy Atkins, Kent, Apple mango, and Local mango. The results revealed that, the range of lesion diameter on 5 DAI was from 8.4 to 14.4 mm (Table 5). Typical anthracnose lesions were produced by CGm-F isolate on all the varieties tested. The development of anthracnose symptoms on different varieties were statistically significant ($p < 0.001$) compared to lesion diameter at 5, 7, 9 and 11 days after inoculation on the fruits. The size of the lesions was gradually increased as day's progress. Eleven days after the inoculation, the lesion size was significantly higher in local mango (32.3 mm) followed by Keitt (31.7 mm) and Kent (29.9mm),

but there was no significant difference between them. The least lesion size was recorded in Tommy Atkins (23.9 mm), on 11 days after inoculation (Table 5). The present finding suggests that the *Colletotrichum* isolates were more pathogenic on detached fruits than seedlings leaves. This might be due to accessibility of substrates in fruits (Prusky and Plumbley, 1992) [18]. The authors further notes that the susceptibility of fruits to *Colletotrichum* infection is related to the level of antifungal inhibitors present in these fruits. At maturity, hydrolytic process is triggered and poses the depletion of starch, and degradation of insoluble pectin (Phan, 1987) [14].

Table 5: Reactions of *Colletotrichum* isolate CGm-F on detached mango fruits of different mango varieties expressed as diameter of lesion progress days after isolate inoculation (DAI).

	Disease reaction			
	Diameter of lesion(mm) Days after inoculation (DAI)			
Varieties	5DAI	7DAI	9DAI	11DAI
Vandyke	8.4^{d}	15.6^{d}	18.6^{d}	29.1^{ba}
Keitt	11.9^{b}	22.0^{a}	26.6^{a}	31.7^{ba}
Tommy-Atkins	10.7^{c}	18.5^{bc}	21.5^{c}	23.9^{c}
Kent	12.0^{b}	19.3^{b}	23.4^{cb}	29.9^{ba}
Apple Mango	14.4^{a}	20.2^{ba}	23.0^{cb}	28.2^{b}
Local Mango	11.8^{cb}	17.2^{dc}	24.0^{b}	32.3^{a}
Control	0.0^{e}	0.0^{e}	0.0^{e}	0.0^{d}
LSD 5%	1.1	1.9	1.9	3.4
CV (%)	19.9	21.5	17	21.2

*In a column, means followed by the same letters are not significantly different at □ = 5% level

Among the six different mango varieties tested, the lesion diameter was constantly increasing in local mango variety compared to others over the incubation period. In addition, it was considered susceptible to the virulent isolates and showed statistically significant result (P <0.001).

Pathogenicity tests on detached mango leaves**,** eleven days' after inoculation, all the isolates caused lesions (6.2-12.6 mm) on local mango variety detached leaves with statistically significant (P< 0.001) among them. On detached leaves the most aggressive isolate was CBL-L and CGm-F with a mean lesion diameter of 12.6 and 11.2 mm, followed by CAd-F with a mean lesion diameter of 10.7 mm respectively (Table 6). However, other isolates produced lesions (6.2-9.8 mm).

Table 6: Virulence variability in *Colletotrichum* isolates on detached on local mango leaves.

. Disease			
***Colletotrichum* isolates**	**Lesion diameter (mm)**	**Percent of disease index**	**Virulence index**
CGm-F	11.2^{b}	25.7^{ba}	2.7^{ba}
CAd-F	10.7^{bc}	24.1^{bc}	2.5^{bc}
CBk-F	9.8^{c}	23.1^{c}	2.4^{c}
CMn-w	6.8^{d}	19.6^{d}	2.0^{d}
CBm-F	6.7^{d}	20.3^{d}	2.1^{d}
CGL	6.2^{d}	18.4^{d}	1.9^{d}
CSC	9.3^{c}	22.8^{c}	2.4^{c}
CBL-L	12.6^{a}	26.6^{a}	2.8^{a}
Control	0.0^{e}	0.0^{e}	0.0^{e}
LSD 5%	1.35	2.4	0.2
CV (%)	22.2	14.3	14.35

* In a column, means followed by a common letter are not significantly different at □ = 0.05 level

All evaluated isolates caused typical anthracnose symptoms on the detached local mango leaves. The leaves that served as controls did not exhibit any symptoms. Some isolates were able to induce extensive lesions on the leaves at 100% of inoculated points. However, in some isolates the infection was only 5% of the inoculated points.

Isolates (CGm-F, CAd-F and CBk-F) caused rounded black spots, whereas the necrotic lesions caused by isolates (CMn-w, CBm-F and CGL) and isolates (CSC, and CBL-L) caused lesion surrounded by necrotic and black spots, respectively, indicating the region of growth of the fungus in the leaf tissue. Evaluation of virulence of isolates on mango seedling/attached leaves. The result of the study revealed that disease severity index was significantly varied and the disease severity ranged between 6.7–33.4% (Table 7). Disease severity and lesion developments interaction effect between isolates and mango varieties showed significant difference (P < 0.0001) at greenhouse.

Table 7: Disease severity (%) of *Colletotrichum* isolates on 2 years' different varieties of mango seedling/leaves

	Varieties					
Isolates	**Vandyke**	**Keitt**	**Tommy Atkins**	**Kent**	**Apple mango**	**Local mango**
CGm-F	24.6^{b-d}	17.7^{d-g}	13.3^{f-i}	33.3^{a}	28.8^{ab}	33.4^{a}
CAd-F	20.0^{c-f}	13.3^{f-i}	6.7^{i}	17.8^{d-g}	13.3^{f-i}	15.5^{e-h}
CBk-F	15.5^{e-h}	20.0^{c-f}	13.3^{f-i}	22.2^{b-e}	8.9^{hi}	24.5^{b-d}
CMn-w	15.5^{e-h}	24.4^{b-d}	13.3^{f-i}	13.3^{f-i}	13.3^{f-i}	28.8^{ab}
CBm-F	17.7^{d-g}	17.7^{d-g}	11.1^{g-i}	33.3^{a}	11.1^{g-i}	26.6^{a-c}
CG-L	22.2^{b-e}	11.1^{g-i}	6.7^{i}	24.4^{b-d}	13.0^{f-i}	15.5^{e-h}
CSC	22.1^{b-e}	13.3^{f-i}	13.3^{f-i}	22.2^{b-e}	13.3^{f-i}	22.2^{b-e}
CBL-L	22.3^{b-e}	13.3^{f-i}	17.6^{d-g}	20.0^{c-f}	33.3^{a}	33.3^{a}
Control	0.0^{j}	0.0^{j}	0.0^{j}	0.0^{j}	0.0^{j}	0.0^{j}

In a column, means followed by the same letter are not significantly different at □= 5% level from each other. LSD values for the interactions comparisons were 7.4, and CV=24.5%.

The isolates CGm-F, CMn-w and CBm-F (33.4mm, 28.8mm and 26.6mm) showed the highest disease severity Index on mango seedling respectively but the least severity index were recorded (6.7mm and 11.1mm) by isolates CG-L and CBm-T on varieties Tommy Atkins, Apple mango and Keitt.

Table 8: Response of different mango seedling against anthracnose disease caused by *Colletotrichum* isolates under greenhouse condition lesion diameter (mm) on leaves.

		***Colletotrichum* isolates**						
Verities	**CGm-F**	**CAd-F**	**CBk-F**	**CMn-w**	**CBm-T**	**CG-L**	**CSC**	**CBL-L**
Vandyke	9.1^{b}	5.9^{h-l}	4^{n-s}	5.2^{i-n}	6.5^{e-h}	7.0^{d-g}	8.2^{b-d}	5.3^{h-m}
Keitt	6.8^{e-g}	4.0^{k-o}	3.3^{p-u}	4.9^{j-o}	4.1^{k-o}	3.2^{p-u}	3.3^{p-u}	3.3^{p-u}
Tommy-A	4.4^{m-q}	3^{q-v}	3.0^{q-u}	4.3^{m-q}	2.6^{t-v}	2.0^{v}	5.2^{i-o}	7.3^{c-f}
Kent	13.6^{a}	3.5^{p-v}	5.0^{j-o}	3.9^{q-s}	6.0^{g-k}	5.1^{i-o}	4.9^{j-o}	4.4^{m-q}
Apple M	7.4^{c-f}	2.7^{s-v}	3.0^{q-v}	3.3^{p-u}	2.9^{s-v}	2.4^{uv}	4.1^{m-r}	6.3^{f-i}
Local M	6.0^{g-k}	4.6^{l-p}	5.3^{h-m}	6.2^{f-j}	8.3^{bc}	4.9^{j-o}	5.1^{j-o}	8.3^{bc}

In a column, means followed by the same letter are not significantly different at □= 5% level from each other. LSD values for the interactions comparisons were 1.33, and CV=16%.

Greenhouse screening of five commercials and one local varieties of mango were performed in pots for the identification of resistance potential against anthracnose of mango caused by *Colletotrichum* isolates. Progress of the disease was measured in diameter of lesion (mm) along the inoculated leaves. The interaction effect between isolates and mango varieties showed significant difference ($P < 0.0001$). Maximum mean lesion diameter 13.6mm, 9.1 mm and 8.3mm was developed on Kent, Vandyke and local mango varieties after 11 days of inoculation respectively. Minimum mean lesion diameter was exhibited by Tommy Atkins (2mm) and Apple mango (2.4mm) respectively (Table 8). The result showed that most isolates, to varying degrees produced anthracnose symptoms on mango tissues. Isolates of *Colletotrichum* inoculated to detached mango fruit, in the laboratory were more aggressive on mango inoculated on detached leaves and mango seedling. This might be true because the leaf and fruit are very much different in physical structure and chemical composition. Each isolate of the pathogen studied was found to induce anthracnose on the different varieties of mangos. The findings are in agreement with the results of several authors that revealed *Colletotrichum* species are the causal agent of mango (Onyeani *et al.*, 2012) [12].

Varieties of mango seedling inoculated with *Colletotrichum* isolates in the greenhouse were infected and exhibited lesions typical of those observed in the field. Lesions began as dark brown, sunken, circular to irregular spots with distinctly gray centers. Re-isolation of *Colletotrichum* isolate CGm-F from inoculated seedling was consistent and confirmed the pathogenic role. According to the present results it is an intermediately susceptible to the pathogen. The result confirms the finding of Jayasinghe and Fernando, (2009) [5] who have report that most or all commercial mango varieties are susceptible to mango anthracnose, some are less susceptible than others, the variety Keitt, less susceptible than Kent, while Kensington Pride, the widely grown cultivar in Australia, is listed as moderately resistant to the disease.

Conclusion

Among eight isolates used, CGm-F was identified as virulent isolate based on lesion diameter, percent disease index and virulence index. Among six mango varieties, local mango and Kent were susceptible varieties. None of mango varieties namely Keitt, Kent, Vandyke, Apple mango, local mango and Tommy Atkins were resistant to the mango anthracnose disease in Ethiopia. Varieties of Tommy Atkins, Keitt and Apple mango were intermediately susceptible varieties. Varieties that have shown intermediate susceptibility in the present experiment may further be tested under different agro-climatic regions of mango producing areas.

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