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Cross pathogenicity of *Botryodiplodia theobromae*, an original isolate from guava fruits on the different cultivars of mango

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Abstract
The objectives of this study was to determine the cross pathogenicity of *Botryodiplodia theobromae*, an etiological agent of post-harvest stem end rot, on the fruits of different cultivars of mango. In the present investigation, the pathogen *B. theobromae* was able to cause virulence in the different cultivar of mango namely safeda, kesar and dasheri during the post-harvest storage stage. Disease ability of pathogen was measured in the loss in physiological weight (PLW %), diameter of disease lesions and disease incidence (%) under both controlled and ambient conditions. Different cultivar of mango showed susceptibility to the attack of pathogen, when inoculated via plug inoculation. In the background of huge post-harvest wastage by fungi, this is unique kind of study revealing how stem end rot pathogen is responsible for inducing spoilage in the several mango cultivars. These finding can be good platform for applying correct type of control strategy for minimizing spoilage caused in mango and other fruits due to rot causing fungal pathogens.

Keywords: *Botryodiplodia theobromae*, mango, PLW, disease incidence

Introduction
Mango (*Mangifera indica* L.), belonging to a family *Anacardiaceae*, is a very important fruit crop of India. It is considered as king of fruits due to its delicious nature and abundance of vitamins, minerals and nutritional content. This fruit is an important component of India's exportable commodities and is a major source of foreign exchange. Additionally, it is also vital part of the diet in many different regions of globe (Mukherjee, and Litz, 2009) [15]. Worldwide, it is mostly grown in tropical and subtropical climatic regions including subcontinent countries. However, mango is vulnerable to a numerous diseases occurring almost at every phases of its growth and development including post-harvest handling stage (Alemu, 2014) [3]. The attack of various fungal and bacterial pathogens leads to either crop loss at preharvest stage or losses in terms of quality, quantity and export during postharvest phage of crop (Prakash, 2004) [18]. Among biotic agents, fungi are predominantly responsible for the cause of post-harvest decay/spoilage, which results in the considerable economic losses. The pathogens spores and inoculums residing on either mango tree or fruit specifically produce decay and fruit rot during postharvest storage (Diedhiou *et al*. 2007) [6].

In India, postharvest stem-end rot disease of various fruits, caused by *Botryodiplodia theobromae* (Syn: *Lasiodiplidia theobromae* Pat.) is the most widespread fungal disease of great economic value. *B. theobromae* (asexual state, *Botryosphaeria rhodina*) is an opportunistic plant pathogen that causes different types of plant diseases worldwide (Faber *et al*. 2007) [11]. It has been estimated that this pathogen attacks to more than 280 plant species including mango, guava, banana, cocoa, and yam with a varied pathological effects on its hosts (Domsch *et al*. 2007; Khanzada *et al*. 2006) [7, 13]. Stem end rot in guava and mango fruits, crown rot in banana, rots in yam and cassava, stem rot in pawpaw, leaf spot in citrus and collar rot in peanuts are some major diseases caused by *B. theobromae* pathogen (Twumasi *et al*. 2014) [20]. In mango fruits, typical symptoms of stem end rot diseases caused by *B. theobromae* includes appearance of dark brown to black spot on the tip of mango and subsequently it is extended on entire fruit (Maqsood *et al*. 2014) [14].

More often, fruits crops such as guava, mango, are often cultivated in close proximity, so it is always important to find out whether the different hosts can act as sources of pathogens inoculum for infecting the other dissimilar host. Therefore, the purpose of present study was to determine the cross pathogenecity/virulence potential of *B. theobromae*, an original isolates of stem end rot pathogen isolated from guava fruits on three different cultivar of mango.

Materials and Methods

Growth and maintenance of plant pathogenic fungus

A virulent strain of pathogenic fungi responsible for causing postharvest disease in mango and guava were used in the present study. Pure strain of test fungal pathogen viz., *Botryodiplodia theobromae* (ITCC No-7740) were purchased from Indian Type Culture Collection (ITCC), Division of Plant Pathology, ICAR-IARI, New Delhi. This fungal pathogen is etiological agent of stem end rot disease in harvested mango and guava fruits respectively. Fungal culture was maintained and preserved on potato dextrose agar (CAB, 1968) [4] medium at 4 °C for further studies.

Fruits sample collection and Surface sterilization of fruits

Fruits of three different Mango cultivars viz., kesar, dasheri and safeda were collected from the local market of Abohar (Punjab), India. All these fruits of different cultivars with attributes of healthiness, physiologically mature but unripe, freshly harvested, no treated and unwaxed etc., were utilized for artificial plugs inoculations in later stage of experiments. All collected fruits from market were washed 2-3 times thoroughly under running tap water and air dried at room temperature. Following air drying, fruits were immersed in freshly prepared 1% v\v sodium hypochlorite (NaOCl) solution for 2-3 minutes in order to reduce the surface contamination. After surface sterilization, these fruits were again rinsed in sterile distilled water for three times and were again left to air dry in a sterile laminar air flow chamber kept in the laboratory.

Preparation of pathogen inoculum

The inoculum of stem end rot pathogen was prepared by growing pure culture on freshly prepared Potato dextrose agar (PDA) media. Starter cultures were prepared by incubating 5-mm^2 disc of growing *Botryodiplodia theobromae* culture for two days on PDA media. After incubation at 28±2 ºC for two days, mycelial plugs (8 mm diameter) were removed using sterilized stainless steel cork borer from actively-sporulating areas in plate and were further used for inoculation into the holes created on the fruits.

Assay for pathogenicity by plug inoculation technique

An artificial plug inoculation technique was used to determine the pathogenicity of the pathogenic strains on three different varieties of mango fruits. The pathogenicity of the fungal strains were ascertained and confirmed by Koch's postulates. For this, surface decontaminated fruits were aseptically inoculated with the test organism into an incision cut made on the skin of the fruits. The inoculation involved aseptically punching of circular holes of about 5 mm deep and 8 mm diameter on the broadest side of mango fruits using hot-oven sterilized cork borer (8 mm diameter). The resulted craters on the surface of fruits served as sites for pathogen inoculation. The inoculums consisted of 8-mm^2 disc of fungus mycelia plug, cut with cork borer (8 mm diameter) from the actively growing region of pure culture in PDA plate. This fungal disc was placed into the deep holes created on each fruits. Further, these pathogen plug inoculated holes on each fruits were covered with parafilm. The control treatments were performed in a similar manner except that plug of PDA medium instead of pathogens mycelia plug was used for inoculation in holes. Each treatment had six fruits per single mango cultivar for both ambient and controlled storage conditions. These treated six fruits of each cultivar were incubated upright at controlled conditions in BOD incubator at 25 ºC and at room/ambient temperature.

Observation recorded

Fruit weight, percentages loss in physiological weight (PLW %), diameter of disease lesions on fruits and disease incidence were started to measure after 24 hr incubation and the observation was recorded up to 9 days of storage of fruits for both controlled and ambient conditions. At the end of experiments, isolations of pathogen were made from the disease fruit to confirm that developed diseased lesions in fruits were due to external artificial inoculation and infection by stem end rot pathogen.

The details of the recorded observations in the experiment are given as below.

Physiological loss in weight (PLW %)

The weight of fruits in each treatment was recorded at initial days and subsequently at different days of storage intervals. Initial weight of each fruits was subtracted from respective fruit weight noted on different days of storage intervals and appraisals were made for physiological loss in weight (PLW) percentages. The physiological loss in weight was calculated on fresh weight basis by following formula:

$$\text{PLW (\%)} = \frac{\text{Initial fruit weight-Fruit weight on the day of observation}}{\text{Initial fruit weight}} \times 100$$

Disease lesion diameter (mm): The disease lesion developed on both opposite site of fruit hole due to an external artificial inoculation of stem end rot pathogen (*B. theobromae*) was measured. Lesion diameters in millimeter (mm) were noted for 9 days and their mean was calculated for each days of observation.

Disease incidence

Disease incidence was assessed by counting the number of diseased lesion developed in artificially inoculated holes to the overall total number of inoculations per treatments (Tucho *et al*. 2014) [19]. Percent (%) disease incidence was calculated by formula = Number of diseased lesion observed in total inoculation/Total number of inoculations per treatments × 100

Results

Physiological loss in weight, disease lesion diameters and disease incidence were measured after 24 hr incubation and the observation was noted for up to 9 days. Pathogen isolation was made from the fruit to confirm that lesions was developed due to infection by stem end rot

Effect of plug inoculation of *B. theobromae* on the physiological loss in weight (%)

Physiological loss in weight (PLW) is one of the key parameter signifying the ability of the harvested fruits to retain its freshness and firmness during extended period of storage under controlled or ambient conditions. Application of

artificial inoculation of stem end rot pathogen inoculums on three different mango cultivars resulted in the slightly significant differences regarding physiological loss in weight at all days of storage. As depicted in table 1 and 2, Kesar mango fruits stored in controlled condition exhibited maximum physiological weight losses (24.70%) at the end of 9 storage days as compared to fruits stored in the room/ambient storage (22.19%). In contrary to this, fruits of dasheri and safeda cultivars stored under ambient conditions showed maximum storage losses of 33.34% and 18.66% respectively, which was slightly higher than that of fruits (29.97% PLW losses for dasheri and 14.14% PLW for safeda losses) stored in controlled condition. Thus is clear that with the advancement of storage duration, the physiological weight loss was steadily increased in all the mango cultivars under both controlled and ambient storage.

Table 1: Effect of stem end rot disease development on physiological weight loss (%) in different cultivar stored under controlled condition

Mango cultivars	Kesar		Dasheri		Safeda	
Storage days	Weight (gm) of mangoes	Percentage (%) loss in physiological weight	Weight (gm) of mangoes	Percentage (%) loss in physiological weight	Weight (gm) of mangoes	Percentage (%) loss in physiological weight
Initial day	191.01±14.45	-	224.85±7.61	-	250.42±10.65	-
1	187.68±14.18	1.74	218.67±7.68	2.75	246.07±10.81	1.74
2	186.10±14.16	2.57	215.78±7.73	4.03	244.20±10.79	2.48
3	183.17±14.18	4.11	211.30±7.74	6.03	240.50±10.77	3.96
4	179.20±14.16	6.18	204.93±8.03	8.86	236.13±10.71	5.71
5	176.27±13.64	7.72	197.37±6.29	12.22	232.93±10.62	6.98
6	169.50±11.49	11.26	184.27±4.86	18.05	228.87±10.23	8.61
7	161.80±10.39	15.29	174.57±3.78	22.36	224.47±10.00	10.36
8	153.23±12.19	19.78	168.77±3.44	24.94	221.13±9.73	11.70
9	143.83±15.37	24.70	157.47±5.54	29.97	215.00±8.94	14.14

Table 2: Effect of stem end rot disease development on physiological weight loss (%) in different cultivar stored under ambient conditions

Mango cultivars	Kesar		Dasheri		Safeda	
Storage days	Weight (gm) of mangoes	Percentage (%) loss in physiological weight	Weight (gm) of mangoes	Percentage (%) loss in physiological weight	Weight (gm) of mangoes	Percentage (%) loss in physiological weight
Initial day	242.17±17.35	-	209.58±9.72	-	349.90± 35.00	-
1	238.18±16.91	1.65	201.84±9.23	3.69	343.23±34.83	1.91
2	234.03±16.36	3.36	194.01±8.86	7.43	336.57±34.47	3.81
3	229.50±15.74	5.23	186.57±8.67	10.98	330.03±34.20	5.68
4	224.93±15.65	7.12	179.30±8.52	14.45	323.30±33.97	7.60
5	220.93±15.26	8.77	172.30±8.36	17.79	316.00±33.12	9.69
6	215.23±15.69	11.12	164.83±8.34	21.35	308.90±33.07	11.72
7	208.30±17.24	13.99	157.07±8.16	25.06	300.67±32.69	14.07
8	199.23±17.77	17.73	149.80±7.86	28.52	292.97±32.22	16.27
9	188.43±17.46	22.19	139.70±7.60	33.34	284.60±31.15	18.66

Values are mean ± SE of 3 replications

Effect of plug inoculation of *B. theobromae* on the disease lesion diameter (mm)

Figure 1 and 2 shows the effects of plug inoculation of *B. theobromae* on the development of diseased lesions on different cultivar of mango stored under controlled and ambient condition. The diameter of lesion developed around the artificially inoculated plug was measured and recorded on a daily basis. At the end of 9 days of storage under the controlled conditions, it can be noted that fruits of safeda mango showed less disease lesion diameter (55.00 mm) due to stem end rot plug inoculation as compared to kesar (63.33mm) and dasheri (81.67mm). Complete disease development due to plug inoculation was observed after 3-4 days. The progress of disease lesion development was observed faster in mangoes stored under ambient conditions than the controlled conditions storage. As shown in figure 2, diameter of disease lesion developed on safeda dasheri and kesar was 118.67mm, 125.67 and 131.67 mm respectively. The figure below shows lesion development due to stem end rot plug inoculation after 3 days incubation.

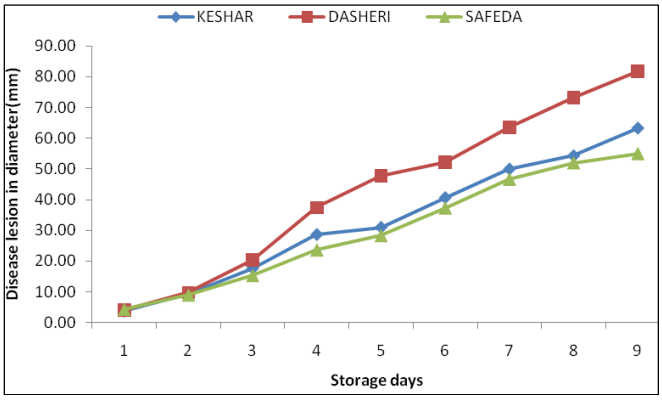

Fig 1: Effect of plug inoculation on disease lesion (mm) development in different mango cultivar stored under controlled condition

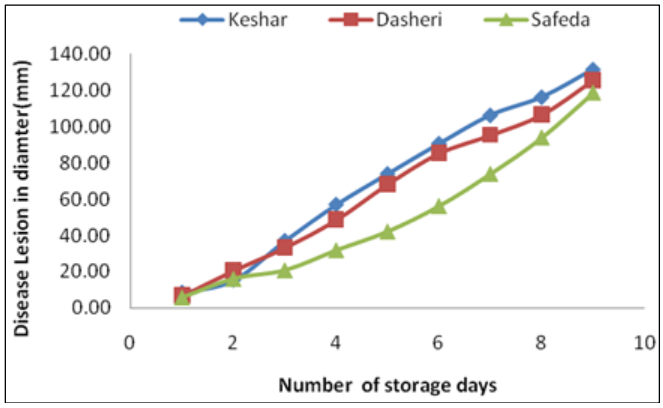

Fig 2: Effect of plug inoculation on disease lesion (mm) development in different mango cultivar stored under ambient conditions

Effect of plug inoculation of *B. theobromae* on the disease incidence

This pathogen was effective in causing disease symptoms and rotting of all three different types of mango fruits used in the study. The pathogen was so aggressive that almost all the mango started to show the symptoms of disease development when stored in both controlled and ambient condition (data not shown). In terms of percentage disease development, fruits of kesar mango achieved 100% disease development after 24 hours incubation under both controlled and ambient condition, dasheri showed 100% disease development after 24 hours incubation in controlled condition and after 72 hours in ambient storage, while complete disease symptoms in all fruits of safeda was observed after 24 hours incubation in ambient condition and after 48 hours in controlled storage. Figure 3 shows the effect of plug inoculation on development of stem end rot disease symptoms in fruits of different mango cultivar.

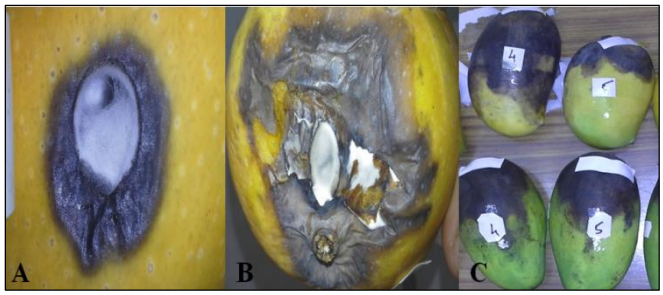

Fig 3: Effect of plug inoculation on development of stem end rot disease in mango cultivar. Image A. Establishment of inoculated pathogen mycelium in artificially prepared plug Image B and C. Successive spread of disease infection and spoilage of mango fruits by artificially established pathogens inoculums

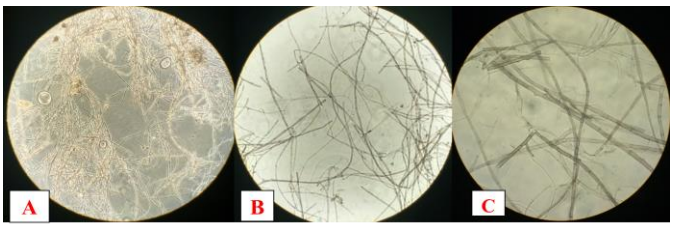

Fig 4: Light microcopy images of stem end rot fungal pathogen (*Botrydiploidia theobromae*) mycelia. Image A) 10X magnification; B) 40X magnification; C) 100X magnification

Re-isolation of pathogen and confirmation by microscopic observation

At the end of experiment, re-isolation of pathogen was carried out from the artificially inoculated and diseased fruits of all types of mango cultivar stored in both controlled and ambient condition.

Mycelia of re-isolated pathogen fruit tissue was observed under compound microscope. Figure 4 shows the mycelia photographs of pathogen mycelium observed at different magnification under light microscope. As per the basis of Koch's postulates tests, external infection of these mango fruits with mycelia plug of re-isolated pathogen was again able to cause disease.

Discussion

India, being an agricultural country, produces a variety of fruits round the year. Non-scientific methods of agricultural practices, poor storage facilities and unfavorable environmental conditions during pre-and post-harvest handling of fruits are accountable for the contamination, infection and colonization by post-harvest pathogens. These pathogens attack causes significant amount of spoilage in fruits during their postharvest operations such as handling, distribution and storage and also reduces shelf-life and produce quality (Dukare *et al*. 2018) [8]. A detailed exploration of the pathogenic behavior of fungal pathogens can predict its probable threat to mangos during its pre and post-harvest operations. An intensive study on the pathogenicity of this organisms showed that they can cause stem-end rot disease on all three cultivars.

The present research finding of artificial plug inoculation study confirmed that B. *theobromae* pathogen was able to cause disease symptoms and consequently spoilage/rotting in all the fruits of mango cultivars. Further, study also revealed that there was little difference in the virulence of *B. theobromae* pathogen towards these mango cultivars, suggesting disease causing aggressive nature of pathogen. Fruits of dasheri mango variety appeared to be more susceptible than other studied. Little difference in virulence ability of pathogen could be attributed to the adaptation to a less susceptible host cultivar, thus becoming more pathogenic to conquer defence mechanisms of the host's (Alahakoon *et al.* 1994) [2]. The physiological loss in weight during storage might be due to the extracellular diffusion of gas between internal tissue of fruits and its surrounding environment.

Cross-infection studies using B. *theobromae* isolates from subtropical fruit such as guava displayed that isolates could cause infection in most of the other host fruits. The preceding findings suggest that B. *theobromae* is a major spoilage organism and causes considerable rotting in the mango fruits under Indian conditions. This findings is similar with several earlier reports that confirms *B. theobromae* as a wide range pathogen causing rot diseases in broad spectrum of hosts (Pitt and Hocking, 2009; Opoku *et al*. 2007; Domsch *et al*. 2007; French, 2006) [17, 16, 7, 12].

Consistent disease lesion sizes were obtained when pathogen was externally inoculated from the stem end sites on broader part of fruits. The admission of a pathogen into a host is facilitated through wounds, or by means of enzymic and mechanical routes. Most of the pathogenic fungi are able to enter into hosts tissues by producing extracellular cell wall lytic enzymes (Chambost, 1986) [5]. From the available literature, it suggests that *Botryodiplodia* is mainly a post-harvest wound pathogen and penetrates the host through bruises and cuts. Our findings have shown that plug

inoculation of B. *theobromae* rapidly initiated spoilage of mango only when it was injected in fruits through plug created by cork borer, thereby substantiating the previous observations.

Conclusions

Under Indian conditions, a detailed investigation on identification of post-harvest spoilage losses in mango can assess the kinds of fungal pathogen attack and pathogenic behavior of various fungal pathogens during storage conditions. Such kinds of studies is needed for selection and applying suitable disease control measures such right fungicides, use of microbial biocontrol agents (Dukare *et al*. 2018) [8] and botanical extracts etc., for minimizing the post-harvest pathogen spoilage and losses. Biological control using microorganisms is the latest ecof-irendly approach for the management of phytopathogens of crops (Dukare *et al*. 2011, 2013) [9, 10]. Also, these types of study will also be helpful in disease resistance breeding programme in mango towards *B. theobromae.*

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