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DN Shukla
Department of Plant Pathology, RPCAU, Pusa, Samastipur, Bihar, India

Pankaj Tiwari
Department of Plant Pathology, T.D. P.G. College, Jaunpur, Uttar Pradesh, India

SK Singh
Department of Plant Pathology, RPCAU, Pusa, Samastipur, Bihar, India

Rohit Tiwari
RML Avadh University, Ayodhya, Uttar Pradesh, India

Corresponding Author:
DN Shukla
Department of Plant Pathology, RPCAU, Pusa, Samastipur, Bihar, India

Perusal and propriety of inoculation techniques against the *Fusarium oxysporum* f.sp. *cubense* causing Panama wilt of banana

DN Shukla, Pankaj Tiwari, SK Singh and Rohit Tiwari

Abstract
Banana (*Musa* spp.) is one of the most important fruit crops grown in tropical and sub-tropical region throughout the world. They are produced in 135 countries and territories across the tropics and subtropics. Panama wilt incited by *Fusarium oxysporum* f.sp. *cubense* TR4 strain B_2 was first time identified in Dwarf Cavendish group of banana cultivar by sending culture to Agharkar Research Institute, Pune from Koshi belt of banana producing area of Bihar. Therefore, establishment of etiology is more important for the suitable management of this new variant. In this view, different types of inoculation techniques were tested against this new variant in which the inoculums mixed with sterilized soil @ 5% (w/w) was found statistically superior over all the inoculation techniques in relation to early symptom expression in different banana cultivars *viz.*, Malbhog (AAB), Alpan (AAB), Kothia (AAB), Robusta (AAA) and Grand Naine (AAA) against the own relative isolates (Iso. M, Iso. A, Iso. K, Iso. G and Iso. R).

Keywords: *Fusarium*, TR 4

Introduction
Banana and plantains are considered as one of the major leading fruit crop for millions of people in the various developing countries of tropical and sub-tropical region. Banana cultivation is old as old Indian civilization and perceived to be one of the earliest fruit crops cultivated by people from pre historic times in India with great socio-economic importance. Banana provides most economical source of carbohydrates and provides much balanced diet than any other fruit in term of nutritional point of view with 67 to 137 calories per 100 grams. Banana fruit is also suggested for kidney diseases, ulcer and gastro-enteritis. As a diet it is easy to digest, because it is nearly fat free, rich source of carbohydrates with calorific value of 67g per 100g of fruit and is free from Sodium, building it a salt free diet (Singh, 2007).

Banana and Plantains is the 4th important food crop at the world level in term of gross value. It is produced in >130 countries in tropical and subtropical regions of the world. Banana and plantains are cultivated in 5.49 million hectares with a total production of 113.28 million tonnes and 20.62 ton/ha productivity. This fruit crop is widely cultivated in both tropical and sub-tropical region comprising Tamil Nadu, Orissa, Bihar, Eastern UP, West Bengal, Assam and North Eastern states. In Bihar, there are two distinct banana growing area *viz.*, old Vaishali region and new north-eastern (Koshi) region. Tall group of banana cultivars are mainly cultivated in old Vaishali belt. It is being grown commercially in the districts of Muzaffarpur, Vaishali, Samastipur and Darbhanga. In 2017-18, banana was grown in 31.07 thousand hectares with 1396.39 thousand metric ton production and 44.94 ton/ha productivity (Horticulture Statistics at a glance, Govt. of India 2018).

Banana crop is affected by a number of various diseases like Panama wilt, Sigatoka leaf spot, Bunchy top of banana and other infestation caused by many pathogen and insect. Now a days, global banana production is threatened by the new variant of *Fusarium oxysporum* f.sp. *cubense* causing Panama wilt of banana. This highly virulent strain become more devastative due to absence of effective resistant cultivars, fungicides, plant products and bio-agent for the better management of this disease. It has been observed in different countries like Taiwan, Malaysia, Indonesia, Philippines, Australia, Oman, Jordon, Mozambique, Lebanon, Pakistan, Laos and Vietnam. In India, severe incidence of TR 4(10-50%) was first time recognize in Cavendish group of bananas in Bihar (Katihar and Purnia districts), Uttar Pradesh (Ayodhya district), Madhya Pradesh (Burhanpur district) and Gujarat (Surat district).

Earlier, a virulent strain of *Fusarium oxysporum* f.sp. *cubense* affecting Cavendish group of banana has been reported (Mustafa *et al.,* 2011) [4] and the first time TR 4 race of *Fusarium oxysporum* f.sp. *cubense* was recorded in cv. Grand Naine (AAA) in Uttar Pradesh (Damodaran *et al.,* 2018) [2]. Recently, a highly virulent strain of *Fusarium oxysporum* f.sp. *cubense i.e.* TR 4 strain B_2 has been observed which affected Cavendish group of banana cv. Grand Naine (AAA) in Koshi belt of Bihar (Shukla and Singh, 2019) [17].

Purwati *et al*., (2008) [5] reported that the method of inoculation by wounding ababca roots followed the sub merging the injured plant in suspension of *Fusarium oxysporum* f.sp. *cubense* conidia(10_6 conidia/ml) for 2 hours before planting was the most effective method for causing the wilt.

Weiming *et al*., (2018) [10] conducted an experiment for the study on the defence related gene in three model plant species *i.e. Aradiopsis thaliana*, tobacco and tomato and these plants were applied to test their susceptibility to *Fusarium oxysporum* f.sp. *cubense* TR 4. In Ethiopia, inoculation techniques optimized with TR 4 in 2014 with inoculum concentration at 1×10^6 conidia/ml and observed typical symptom of *Fusarium* wilt by 10 days of Inoculation (Gabrekiristos *et al*., 2018) [1].

Material and Methods

Isolation and purification of the pathogen

Banana plant of diiferent cutivars *viz.,* Malbhog (AAB), Alpan (AAB), Kothia (ABB), Grand Naine (AAA) and Robusta (AAA) showing characteristic symptoms of Panama wilt samples were washed thoroughly in several times of tap water for 5 minutes to evacuate dirt and cut into small bits of 2-3 mm dimension. These bits were surface sterilized by dipping in 0.1 $Hgcl_2$ solution for 30 seconds followed by washing 2-3 times with sterilized distilled water to remove traces of $Hgcl_2$ and dried between 2 sterilized filter paper, then placed aseptically on PDA slants and Petri plates with the help of inoculating needle in aseptic condition and incubated at 28±2º. These all isolates were categorised/grouped in a five different types (Iso.M, Iso.A, Iso.K, Iso.G, Iso.R) based on mycelial growth pattern and pathogenicity. For molecular characterization, the culture (Iso.G) was sent National Fungal Culture Collection of India (NFCCI), Pune.

Pathogenicity of different isolates had been confirmed by two methods as described below

Inoculum mixed with sterilized soil

Sand maize medium was prepared by mixing 100 g maize grain flour, 50 g sand, 50 ml distilled water in 250 ml conical flask and sterilized at 15 psi for one hr in three consecutive days. Flasks were subsequently inoculated with 5 mm diameter discs of culture of the test fungus and incubated at 25-30 °C. The fungal isolates multiplied on sand maize medium was added to soil in pots at 5% (w/w). Soil mixture without inoculum served as control. Each pot was planted with disease free banana sucker and development of Panama wilt was observed from symptomatic plants to fulfil Koch's postulates; there by the pathogenicity was established.

Root immersed in conidial suspension

Tissue culture plant of banana cv. Grand Naine (AAA) transferred to pot for hardening as well as to grow them in the poly house until they attained the desired size. The plantlets were watered with tap water every other day. After 45 days of acclimatization, the test plantlets were carefully uprooted and healthy white roots were selected for inoculations by immersion in the appropriate conidial suspension. The plants without immersion were considered as control. All the test plantlets kept in polyhouse for maintenance and observation.

Evaluation of Inoculation techniques

Suitability of inoculation techniques for quick and easy development of disease was proved in poly house at Horticultural Research Farm of RPCAU, PUSA Samastipur. Healthy banana suckers were grown in pots and inoculated by different inoculation techniques. The data was recorded based on the incubation period, percent wilt index and percent vascular wilt index after that data was analysed by CRD design. Following four methods were used for inoculation during the course of this experiment:

a) Cut sucker dip method

- Cut sucker of one month old banana plant was dipped up to 2 hours in 500 ml of spore suspension (10^6 spore/ml) solution.
- Inoculated plants were planted on sterilized soil medium in pot.
- Observations were recorded

b) Whole sucker dip method

- One month old banana suckers were dipped up to 2 hrs in 500 ml of spore suspension (10^6 spore/ml) solution.
- Inoculated banana suckers were planted in pots containing sterilized soil.
- Observation of symptoms were recorded.

c) Making hole in psuedostem and filled with test pathogen

- Making hole with scissor in the psuedostem of potted banana plants and insert 21 days old 5 mm disc of pathogen grown on PDA after that it sealed with wax.
- Observation was recorded periodically.

d) Root dip method

- Root tips of one month old banana plants were cut up to one cm and wounded root were dipped for 2 hrs in 500 ml of conidial suspension (10^6 spore/ml) and planted into the pots.
- Observations based on the periodic symptom expression was recorded.

Result and Discussion

Opinion and interpretations about the pathogen by Agharkar Research Institute, Pune

The tested fungal strain showed 100% sequence similarity with *Fusarium oxysporum* f.sp. *cubense race* 4 strain B_2 of NCBI accession number LT 571434.1 after sequence analysis.

Standardization of inoculation technique for quick and easy development of Panama wilt disease

A field trial was conducted under pot condition in different banana cultivars *viz.,* Malbhog (AAB), Alpan (AAB), Kothia (ABB), Grand Naine (AAA) and Robusta (AAA). These banana cultivars were transplanted in pot and inoculated by own respective isolate of *Fusarium oxysporum* f.sp. *cubense* using different methods of inoculation techniques for the early

development of Panama wilt. Observation was recorded based on the incubation period, percent wilt index and percent vascular wilt index in each banana cultivars. The control plants were showed no wilting symptoms, while inoculated plant was showed various degree of percent wilt index and percent vascular wilt index based on the incubation period.

Effect of various inoculation techniques on the development of Panama wilt of banana in cv. Malbhog (AAB) under pot condition

The result showed that maximum wilt index (47.3%) and vascular wilt index (44.3%) were observed in Inoculum mixed with sterilized soil @ 5% w/w (T_5) followed by Root dip inoculation in 10^6 spores/ml (T_4) with wilt index (43.2%) and vascular wilt index (38.5%), making hole in psuedostem and insert 21 days 5 mm old disc of pathogen grown on PDA after that sealed with wax (T_3) with wilt index (36.3%) and vascular wilt index (32.3%) and whole sucker dip in spore suspension of 10^6 spores/ml (T_2) with wilt index (32.3%) and vascular wilt index (28.4%). The lowest wilt index (28.2%) and vascular wilt index (26.3%) were observed in cut sucker dip in spore suspension of 10^6 spores/ml ($T_{1)}$. Regarding incubations period, the minimum incubation period was recorded in T_5 (24-30 days) followed by T_4 (28-34 days), T_1 (30-37 days) and T_3 (32-38 days). The maximum incubation period was recorded in T_2 (35-39 days). Data pertaining to different observations indicate that all treatments were showed considerable variation and T_5 was found statistically highly superior over all treatments. The differences between T_2 and T_1 varied significantly. Data are presented in (Table 1).

Effect of various inoculation techniques on the development of Panama wilt of banana in cv. Alpan (AAB) under pot condition

The result showed that maximum wilt index (45.4%) and vascular wilt index (40.3%) were observed in inoculum mixed with sterilized soil @ 5% w/w (T_5) followed by making hole in psuedostem and insert 21 days 5mm old disc of pathogen grown on PDA after that sealed with wax (T_3) with wilt index (41.3%) and vascular wilt index (38.5%), root dip inoculation in 10^6 spores/ml (T_4) with wilt index (34.3%) and vascular wilt index (30.3%) and whole sucker dip in spore suspension of 10^6 spores/ml (T_2) with wilt index (30.4%) and vascular wilt index (28.4%). The lowest wilt index (26.3%) and vascular wilt index (24.2%) were observed in cut sucker dip in spore suspension of 10^6 spores/ml ($T_{1)}$. Regarding incubations period, the minimum incubation period was recorded in T_5 (25-30 days) followed by T_3 (27-34 days), T_4 (31-38 days) and T_2 (32-39 days). The maximum incubation period was recorded in T_1 (35-40 days). Data pertaining to different observations indicate that all treatments were showed considerable variation and T_5 was found statistically highly superior over all treatments. The differences between T_3 and T_4 were recorded as significant and T_1 was considered as highly inferior to the T_5. Data are presented in (Table 2).

Effect of various inoculation techniques on the development of Panama wilt of banana in cv. Kothia (ABB) under pot condition

The result showed that maximum wilt index (44.3%) and vascular wilt index (40.3%) were observed in inoculum mixed with sterilized soil @ 5% w/w (T_5) followed by Root dip inoculation in 10^6 spores/ml (T_4) with wilt index (35.5%) and vascular wilt index (32.4%), making hole in psuedostem and insert 21 days 5mm old disc of pathogen grown on PDA after that sealed with wax (T_3) with wilt index (30.5%) and vascular wilt index (28.4%) and cut sucker dip in spore suspension of 10^6 spores/ml ($T_{1)}$ with wilt index (28.4%) and vascular wilt index (26.4%). The lowest wilt index (24.3%) and vascular wilt index (20.4%) were observed in whole sucker dip in spore suspension of 10^6 spores/ml (T_2). Regarding incubation period, the minimum incubation period was recorded in T_5 (26-32 days) followed by T_4 (29-36 days), T_3 (34-40 days) and T_1 (36-41 days). The maximum incubation period was recorded in T_2 (37-41 days). Data pertaining to different observations indicate that all treatments were showed considerable variation and T_5 was found statistically highly superior to remaining all treatments. The differences between T_4 and T_3 varied significantly and T_4 closely followed by T_3. Data are presented in (Table 3).

Effect of various inoculation techniques on the development of Panama wilt of banana in cv. Robusta (AAA) under pot condition

The result showed that maximum wilt index (49.4%) and vascular wilt index (46.1%) were observed in inoculum mixed with sterilized soil @ 5% w/w (T_5) followed by making hole in psuedostem and insert 21 days 5mm old disc of pathogen grown on PDA after that sealed with wax (T_3) with wilt index (44.2%) and vascular wilt index (41.0%), root dip inoculation in 10^6 spores/ml (T_4) with wilt index (41.3%) and vascular wilt index (39.3%) and cut sucker dip in spore suspension of 10^6 spores/ml ($T_{1)}$ with wilt index (40.1%) and vascular wilt index (35.3%). The lowest wilt index (37.4%) and vascular wilt index (32.4%) were observed in whole sucker dip in spore suspension of 10^6 spores/ml (T_2). Regarding incubations period, the minimum incubation period was recorded in T_5 (22-28 days) followed by T_4 (25-33 days), T_1 (26-35 days) and T_3 (28-37 days). The maximum incubation period was recorded in T_2 (31-39 days). Data pertaining to different observations indicate that all treatments were showed considerable variation and T_5 was found statistically highly superior over all treatments. The differences between T_3 and T_1 were varied significantly and T_2 was considered as highly inferior to the T_5. Data are presented in (Table 4).

Effect of various inoculation techniques on the development of Panama wilt of banana in cv. Grand Naine (AAA) under pot condition

The result showed that maximum wilt index (51.3%) and vascular wilt index (48.4%) were observed in inoculum mixed with sterilized soil@5% w/w (T_5) followed by Root dip inoculation in 10^6 spores/ml (T_4) with wilt index (46.0%) and vascular wilt index (44.4%), making hole in psuedostem and insert 21 days 5mm old disc of pathogen grown on PDA after that sealed with wax (T_3) with wilt index (43.9%) and vascular wilt index (41.4%) and cut sucker dip in spore suspension of 10^6 spores/ml ($T_{1)}$ with wilt index (42.6%) and vascular wilt index (37.6%). The lowest wilt index (39.3%) and vascular wilt index (34.5%) were observed in whole sucker dip in spore suspension of 10^6 spores/ml (T_2). Regarding incubations period, the minimum incubation period was recorded in T_5 (20-28 days) followed by T_4 (24-32 days), T_1 (27-35 days) and T_3 (28-37 days). The maximum incubation period was recorded in T_2 (30-38 days). Data pertaining to different observations indicate that all treatments

were showed considerable variation and T_5 was found statistically highly superior over all treatments. The differences between T_2 and T_1 varied significantly and T_4 was closely followed by T_3 in all observation. Data are presented in (Table 5).

Sowmya (1993) [9] demonstrated pathogenicity of *Fusarium oxysporum* f.sp. *cubense* by inoculating pure culture of the fungus on both injured and uninjured healthy roots of Puttable variety of banana. The examination demonstrated that the indications grew uniquely on harmed roots following 15 days of incubation. Sarvanana *et al.*, (2005) [6] directed a trial and build up an appropriate technique to quantify the virulence of *Fusarium oxysporum* f.sp. *cubense* in banana cv. Rasthali. The treatments comprised: sand inoculum of the isolate at 10% w/v of tank soil (T_1); sand inoculum of the isolate at 15% w/v of tank soil (T_2); corm injection of the isolate at 3 ml per plant + sand inoculum of the isolate at 10% w/v of tank soil (T_3); sucker dipping for 30 minutes (10^6 cfu/ml) + sand inoculum of the isolate at 10% w/v of tank soil (T_4); sucker dipping for 30 minutes (T_5); infected plant parts at 10% w/v of tank soil (T_6); and infected soil (10^5 cfu/g soil) at 10% w/v of soil (T_6). Five isolates of Foc (1-5) were isolated from diseased suckers and used for this study. After 3 months, corm injection (T_3) had induced the most noteworthy percent wilt index followed by sucker dipping (T_4). The highest vascular discolouration incidence was also recorded in T_3 and T_4.

Table 1: Effect of various inoculation techniques on the development of Panama wilt of banana in cv. Malbhog (AAB) under pot condition

Treatment		Iso. M (Isolate from Malbhog) *		
		Incubation period (days)	**Percent wilt index (1-5)**	**Percent vascular wilt index (1-6)**
T_1	Cut sucker dip in spore suspension (10^6 spores/ml)	30-37	28.2	26.3
T_2	Whole sucker dip in spore suspension (10^6 spores/ml)	35-39	32.3	28.4
T_3	Making hole in psuedostem and insert 21 days 5 mm old disc of pathogen grown on PDA after that sealed with wax	32-38	36.3	32.3
T_4	Root dip inoculation (10^6 spores/ml)	28-34	43.2	38.5
T_5	Inoculum mixed with sterilized soil @ 5%(w/w)	24-30	47.3	44.3
T_6	Control		0.0	0.0
CD at 5%			1.73	1.88
S.Em. (±)			0.56	0.60
C.V. (%)			3.09	3.68

*Mean of three replications

Table 2: Effect of various inoculation techniques on the development of Panama wilt of banana in cv. Alpan (AAB) under pot condition

Treatment		Iso. M (Isolate from Alpan) *		
		Incubation period(days)	**Percent wilt index (1-5)**	**Percent vascular wilt index (1-6)**
T_1	Cut sucker dip in spore suspension (10^6 spores/ml)	35-40	26.3	24.2
T_2	Whole sucker dip in spore suspension (10^6 spores/ml)	32-39	30.4	28.4
T_3	Making hole in psuedostem and insert 21 days 5mm old disc of pathogen grown on PDA after that sealed with wax	27-34	41.3	38.5
T_4	Root dip inoculation (10^6 spores/ml)	31-38	34.3	30.3
T_5	Inoculum mixed with sterilized soil @ 5%(w/w)	25-30	45.4	40.3
T_6	Control		0	0
CD at 5%			1.56	1.57
S.Em. (±)			0.50	0.50
C.V. (%)			2.93	3.24

*Mean of three replications

Table 3: Effect of various inoculation techniques on the development of Panama wilt of banana in cv. Kothia (ABB) under pot condition

Treatment		Iso. K (Isolate from Kothia) *		
		Incubation period(days)	**Percent wilt index (1-5)**	**Percent vascular wilt index (1-6)**
T_1	Cut sucker dip in spore suspension (10^6 spores/ml)	36-41	28.4	26.4
T_2	Whole sucker dip in spore suspension (10^6 spores/ml)	37-41	24.3	20.4
T_3	Making hole in psuedostem and insert 21 days 5mm old disc of pathogen grown on PDA after that sealed with wax	34-40	30.5	28.4
T_4	Root dip inoculation (10^6 spores/ml)	29-36	35.5	32.4
T_5	Inoculum mixed with sterilized soil @ 5%(w/w)	26-32	44.3	40.3
T_6	Control		0.0	0.0
CD at 5%			2.25	1.85
S.Em. (±)			0.72	0.59
C.V. (%)			4.60	4.16

*Mean of three replications

Table 4: Effect of various inoculation techniques on the development of Panama wilt of banana in cv. Robusta (AAA) under pot condition

Treatment		Iso. M (Isolate from Robusta) *		
		Incubation period(days)	Percent wilt index (1-5)	Percent vascular wilt index (1-6)
T_1	Cut sucker dip in spore suspension (10^6 spores/ml)	26-35	40.1	35.3
T_2	Whole sucker dip in spore suspension (10^6 spores/ml)	31-39	37.4	32.4
T_3	Making hole in psuedostem and insert 21 days 5 mm old disc of pathogen grown on PDA after that sealed with wax	28-37	44.2	41.0
T_4	Root dip inoculation (10^6 spores/ml)	25-33	41.3	39.3
T_5	Inoculum mixed with sterilized soil @ 5% (w/w)	22-28	49.4	46.1
T_6	Control		0	0
CD at 5%			2.01	2.14
S.Em. (±)			0.64	0.69
C.V. (%)			3.14	3.69

*Mean of three replications

Table 5: Effect of various inoculation techniques on the development of Panama wilt of banana in cv. Grand Naine (AAA) under pot condition

Treatment		Iso. G (Isolate from Grand Naine) *		
		Incubation period (days)	Percent wilt index (1-5)	Percent vascular wilt index (1-6)
T_1	Cut sucker dip in spore suspension (10^6 spores/ml)	27-35	42.6	37.6
T_2	Whole sucker dip in spore suspension (10^6 spores/ml)	30-38	39.3	34.5
T_3	Making hole in psuedostem and insert 21 days 5mm old disc of pathogen grown on PDA after that sealed with wax	28-37	43.9	41.4
T_4	Root dip inoculation (10^6 spores/ml)	24-32	46.0	44.4
T_5	Inoculum mixed with sterilized soil @ 5% (w/w)	20-28	51.3	48.4
T_6	Control		0.0	0.0
CD at 5%			2.52	2.12
S.Em. (±)			0.81	0.68
C.V. (%)			3.77	3.44

*Mean of three replications

Conclusion

The efforts were made to find out the best inoculation techniques against the *Fusarium oxysporum* f.sp. *cubense* causing Panama wilt of banana, during the studies on different inoculation techniques for the quick and easy development of Panama wilt disease. The inoculums mixed with sterilized soil @ 5% (w/w) was statistically superior over all the inoculation techniques in relation to early symptom expression in different banana cultivars *viz.,* Malbhog (AAB), Alpan (AAB), Kothia (AAB), Robusta (AAA) and Grand Naine (AAA) against the own relative isolates (Iso. M, Iso. A, Iso. K, Iso. G and Iso. R).The early symptom expression was found in inoculums mixed with sterilized soil @ 5% (w/w) because inoculums build up to larger level in sand maize medium during inoculation, inoculums mixed in soil by mechanical means so that root hair and root tip were become injured. After that, tested fungus multiplied on wounded part and caused infection by early germ tube formation.

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