An Optimized Inoculation Method of Fusarium Wilt (*Fusarium oxysporum* f. Sp. cubense) Causal Agent of Banana Wilt Disease in Ethiopia

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ABSTRACT

Banana is among the most important fruit crops in Ethiopia and is produced widely by small scale farmers. Despite its importance and cultivation, its production is, however, hampered by different biotic and abiotic factors. Fusarium wilt of banana caused by soil-borne fungus *Fusarium oxysporum* f. sp. *cubense* (Foc) is becoming an important production constraint of the crop. In Ethiopia it was reported in 2014 in potential banana producing areas mainly in Dale, Aleta Chuko and Samen Bench in Southern Nations, Nationalities and Peoples Regional State. The general management option to this disease is replacing susceptible cultivars by resistant/tolerant ones. Use of resistant/tolerant varieties is the most cost-effective and sustainable method of managing the disease. A reliable and rapid screening method is needed to select resistant/tolerant banana varieties. In Ethiopia there is no developed protocol to artificially inoculate banana fusarium wilt in order to screen resistant/tolerant variety. An optimized inoculation methods by root dipping into an adjusted suspension of Foc inoculum concentration 1×10^6 conidia/ml on acclimatized three months old tissue cultured plantlets of susceptible variety (poyo) has enabled to observe typical symptom of FW by tenth days of inoculation and an overall result was obtained within three months. This protocol facilitates the way to produce symptoms of fusarium wilt in banana and screen resistant/tolerant variety released in Ethiopian Research system. Therefore, this paper presents the details of the protocol and achievements of the study in major banana growing areas of Ethiopia.

1. INTRODUCTION

Fusarium wilt is one of the most notorious diseases that affect banana plants. The disease is caused by the soil-borne hyphomycete, *Fusarium oxysporum* f. sp *Cubense* (*Foc*) (Ploetz, 2015). In terms of crop destruction, Fusarium wilt ranked the foremost devastating plant diseases such as wheat rust and potato blight (Carefoot and Sprott 1969). In the period 1890-1960, some 40,000 hectares of the susceptible banana cultivar Gros Michel (grown for export) were destroyed or abandoned in Central and South America and the Caribbean because of race 1 of *Foc*. Based on its virulence in specific banana cultivars (Ploetz, 1990,1994), the pathogen can be classified into three races: 1, 2 and 4. Production and productivity of banana is high in Northern and Southern part of Ethiopia, however diseases caused by fungi, bacteria and nematode affect banana plantation (Jones 2000). Among others fusarium wilt and sigatoka distributed across main banana producing region of the country. The soil-borne fungus, *Fusarium oxysporum* is the causal agent of vascular wilt, a disease that affects a large variety of economically important crops worldwide (Ortoneda et al., 2004). Other than Fusarium wilt burrowing nematode, banana anthracnose, cigar end rot, bacterial wilt and black sigatoka were important disease of banana in Ethiopia. Fusarium wilt and Black sigatoka diseases were reported in 2014 around Tepi Agricultural Research Centre which is potential in banana production areas. From assessed area fusarium wilt was sever in Dale woreda (84%), Aleta Chuko (36%) and Samen Bench (36%) (un published) districts. Use of resistant genotypes is the best control practice for Fusarium wilt (FW) of banana also called Panama disease. Genetic improvement plays an important role in transferring disease resistance alleles to the genetic background of elite genotypes. In addition, field tests are time consuming (3 years) and demanding in terms of manpower and space. Hence, a rapid and reliable method of inoculation is vital for early detection of resistant genotypes. Several protocol to infect banana with fusarium wilt under green house condition have been reported. The present paper describes the way to produce symptoms of fusarium wilt in banana variety by considering several factors and to optimize efficient protocol for further research on identifying resistant or tolerant variety of released and pipe line banana varieties.

2. MATERIAL AND METHODS

2.1 Study area

The experiments were carried out at Melkassa Agriculture Research Center (MARC) located at 8°24'985 N latitude and 39°19'529 E longitudes, with an altitude of 1,550meter above sea level. The annual average maximum temperature is 27°C the minimum

average temperature 20°C and minimum relative humidity was 50 percent and the maximum relative humidity was 80 percent. The investigations were carried out under greenhouse condition.

2.2 Culture media and Inoculum production

Samples with a symptom of fusarium wilt were collected from Melkassa Agricultural Research Centre, Sidamo Zone and brought to MARC plant pathology Laboratory. Samples were washed with bleach and distilled water to remove saprophytic fungi to get pure culture. In this experiment potato dextrose agar (PDA) was used to grow pathogen. After seven days of incubation, pathogens developed from cultured samples were identified based on their conidia shape and other fruiting structures with the help of stereomicroscope and descriptors. From actively growing PDA cultures of *fusarium spp*, aseptically the spore were placed at the centre of other Petri dish with diameter of 9cm containing media for purifying the pathogen. Then inoculate plates were incubated in mould incubator with adjusted temperature of 26±2 and relative humidity above 75% for 10 days. Seventeen day old culture were identified by morphology and conidial shape under stereomicroscope and scraped in to cylinder. Then the pathogen were shaken on shaker for twelve hour in order to get uniform conidia. Haemocytometer and hand tally counter were used to adjust suspension.

2.3 Planting materials and Observations

Acclimatized three month old tissue cultured plantlets of banana variety (poyo) which is susceptible to all race of fusarium were used to see the effectiveness of the optimized protocol to inoculate *fusarium wilt*. Plants grown in a disease-free environment with no contact with *Foc*, which may eventually provoke accidental or cross-contamination. Plantlet were 15-25 cm height and shows no nutrient deficiency. Treated and untreated seedlings were managed with optimum agronomic practice to get normal farmers practice except the pathogen case. Untreated seedlings were isolated from inoculated(treated) one to minimize cross contamination of test pathogen.

2.4 Optimized protocol to inoculate *fusarium wilt* of banana on banana seedlings

1. Susceptible variety was selected and multiplied by tissue culture and grown for three month under aseptic condition.
2. Three months old seedlings were brought to Melkassa Agricultural Research Centre, plant pathology laboratory and seedlings were removed from the pot and washed neatly with tap water when necessary.

3. 17 day old purified culture were adjusted with a spore concentration of 1×10^6 spore/ml and excess roots were trimmed to favour pathogen penetration and during all step avoid plant stress and keep plants with adequate water levels.
4. Leave the plant root contact with suspension adjusted appropriately for 40 minutes and remove from suspension and dry the seedlings for ten minutes before planting in to pot.
5. Transplant immersed seedling to the pot size of 20 cm diameter and 15cm height with full of sun sterilized soil media which is composed with sandy soil, animal dung and forest or fertile soil in the ratio of 1:1:2 respectively.
6. Incubate infected and untreated seedlings at 26 ± 2 °C and with 60%-80% Relative humidity for 48 hours for both treated and untreated seedlings.
7. After removing the seedlings from the incubation, water was applied regularly to maintain the substrate at field capacity level and all treatments were at recommended condition except inoculated pathogen.
8. Evaluation of treatments were started after ten day post inoculation with parameter of plant height, leaf number, infected leaf number, disease severity and incidence.

Figure 1: Symptom of *fusarium wilt* fungi on banana and purified pathogen, A (sample of diseased banana leaf), B (cultured and purified fusarium wilt) and C-macro and micro conidia of (*Fusarium oxysporum* f. *Sp. cubense*) of banana under microscope

Figure-2: Procedures of inoculation (A. pathogen harvesting, B. soaking trimmed root, C. drying/sticking infested seedling, D. transplanting)

2.5 Disease rating

Disease development in the greenhouse was evaluated 4–9 weeks after inoculation using a modified version of the disease severity rating scale for Fusarium wilt of banana (Carlier et al., 2002). The rating scale ranged from 0 to 5, with plants showing no internal symptoms scoring 0 and plants showing 100% vascular discoloration scoring 5. In the glass house disease incidence was scored according to the presence or

absence of external disease symptoms. Healthy plants were given a value of 0, while diseased plants were scored as 1.

2.6 Temperature and relative humidity

To determine the effect of temperature and relative humidity on fungal growth, mold incubator were used in laboratory and in glass house humidifier were used to adjust the normal environment for the pathogen.

Table 1: Average Temperature and Relative humidity during inoculation

Time	Average Temperature(°C)		Average Relative Humidity(%)	
	Before inoculation	After inoculation	Before inoculation	After inoculation
8:00am	26	22	75	60
1:00pm	26	30	75	51
4:00pm	26	29	75	55

3. RESULTS AND DISCUSSION

3.1 Results

3.1.1 Effectiveness of *Foc* Inoculation Methods.

Untreated seedlings did not show wilting symptoms, while inoculated seedlings showed various degrees of wilting at 10 days after inoculation. The colonies of *Foc* were successfully re-isolated from inoculated poyo showing the symptoms of yellowing as seen in Figure 3(f- re cultured fusarium spores under microscope). Compared to other inoculation methods the optimized inoculation method under this experiment shows the highest disease intensity and score of the symptoms (Table 2). Ten seedlings of poyo variety were evaluated for this experiment and typical symptom development of Fusarium wilt were observed successfully. The incubation period (the period from inoculation to the first symptoms appearance) varied depending on inoculation procedures, banana genotypes used, aggressiveness of the *Foc* isolate or environmental conditions.

3.1.2 Effects of *Foc* on disease incidence and severity

The *Foc* inoculation by dipping the plants at 1×10^6 conidia/ml were equally able to cause the wilting symptoms on ten tested poyo seedlings. At 10 days after

inoculation, the infected seedlings showed the score of the symptoms was lower as shown in second score in (Table 2). Both at 3rd and 4th score of DAI, inoculation with 10^6 conidia/ml resulted an equal percentages of plants showing the symptoms (Table 2). However, the damages due to *Foc* infection has reached xylem tissues. Transversal sections of *Foc* un inoculated banana root in the control was presented in Figure 3A shows no symptom. Phenotypes of banana plants at 60 DAI showing wilting symptom score 5 or all inoculated seedling were dead (Figure 4). This shows the success of inoculation protocol in Ethiopian condition.

3.1.3 Response of 10 poyo Cultivars to *Foc* Infection.

Un-inoculated poyo plants did not show wilting symptoms due to *Foc* infection. After inoculation of *foc*, percentages of banana seedling showing the symptoms were 100% (figure 4). All tested poyo cultivars showed higher average of score of wilting symptom and disease intensities than the control (Table 2). Based on disease intensities, ten poyo cultivars tested were identified as either very susceptible to *foc*. Plant height on pre and post inoculation revealed high difference among seedlings as shown (fig, 5, 6 1a and 2d) respectively. On inoculated seedlings plant height decreases and plant number four on 3rd score already dead (fig 6, 2d). Plant height in un inoculated seedling increases about 10cm in period of experimentation (fig 6, 2d).

Figure- 3: Banana seedling with various degrees of wilting symptoms at 60 days after inoculation with *F. oxysporum f.sp. cubense*. a – e stages of wilting after inoculation, based on criteria developed by Epp (1987) and f- re cultured fusarium spores under microscope

Table 2: Mean disease severity and incidence

Treatments	Mean disease severity					Disease incidence
	1st score	2nd score	3rd score	4th score	5th score	0 and 1 for the occurrence of disease
Inoculated	0	2	3.75	3.75	4.6	1
Un inoculated	0	0	0	0	0	0

Figure- 4: Effect of *foc* on three month old banana seedling and re cultured spore (A (Dead seedling at the completion of the experiment, and B(re isolated foc))

Figure-5: Evaluation of disease incidence and plant height in different day intervals before and after inoculation of pathogen

Figure-6: Disease severity after development of disease symptom and plant height after inoculation, DS(disease severity), PH(plant height), Foc (fusarium inoculated seedling), cont (control/un inoculated).

3.2 DISCUSSION

Inoculation method, age of plant, plant environment and amount of pathogen have impact for the development of disease symptom. In this experiment disease severity increases during different scoring stages. Under this experiment there is high variation among treatments and almost all inoculated banana seedlings show wilting symptom and lastly all seedling were died. Un inoculated seedlings showed no wilting symptom and showed normal growth with deep green leaf. The success of *Foc* infection in banana were the final results of a complex processes involving at least five stages, namely: (i) recognition by *Foc* of signals released by banana roots, (ii) *Foc* attachment on the banana root surface and penetration of *Foc* hyphae into root tissues, (iii) penetration of *Foc* hyphae into cortex tissues and degradation of physical defence system of root tissues, (iv) proliferation of *Foc* hyphae and production of microconidium in xylem tissues, and (v) secretion of fungal toxin and hydrolytic enzymes by *Foc* resulting in further tissue damages of the infected roots (Di Pietro et al. 2003). Wounding of root tissues assisted initial process of *Foc* infection in banana. Infection of *Foc* in banana might also through similar processes and wounding might assisted *Foc* infection.

In the field, banana plant tissues might be wounded because of fallen trees due to strong wind, pruning of suckers, damages due to sucking insects or nematode infection. (<http://www.plantmanagementnetwork.org/pub/php/management/banana/panama>).

Djatnika et al. (2003) stated that *Foc* is a soil-borne pathogen. It infects banana plants through lateral root hairs, and is able to infect root tissues through wounding due to infection of *Radopholus similis* nematode. *Foc* inoculation using optimized inoculation method resulted more plants showing *Foc* infection symptoms and higher disease intensity. Wounding of banana root tissues followed by dipping of the wounded plants in the suspension of *Foc* conidia in optimized method guaranteed high *Foc* infection. Wounding has been shown to assist fungal penetration into inoculated plant tissues (Yusnita & Sudarsono 2004; Sakamoto & Gordon 2006). Observed wilting symptoms of banana seedling were the results of blocking of xylem tissues by *Foc* mycelia and by induction of thylosis because of activities of secreted enzymes and fusaric acid by *F. oxysporum* (Salerno et al. 2000). Infected root tissue into brownish or blackish colour could indicate the presence of damages in the root tissue of banana caused by *Foc* inoculation. Although root damages at 10 DAI reached only the yellowing the lower part of the leaf, the tested poyo cultivar has shown average scores of wilting symptom ranged from 0 to 2 in different scoring time. The functions of the cells were associated with water transportation and oxygen storage in roots. Cortex role did not directly related to water transportation. However, the cortex damaged might indirectly affect water

transportation from root to shoot and leaf tissues and result in wilting symptoms. At 30 DAI, the percentage of banana plants showing wilting symptoms and disease intensities due to *Foc* infection were higher than that at 10 DAI. The increase of disease intensity at 30 DAI might probably be the result of damages in root xylem tissues due to *Foc* infection. The xylem was responsible for water and nutrient transportation in plants. The damaged xylem might have direct effects on water transport from roots to leaves; therefore, it might also have direct effects on causing wilting symptoms. Under this research using developed optimized inoculation methods, all tested poyo cultivars were identified as very susceptible and un inoculated seedling shows no *Foc* infection.

4. CONCLUSIONS AND RECOMMENDATIONS

Based on the data from this research, it can be concluded that inoculation method by wounding banana roots followed by dipping wounded seedling on suspension of 1×10^6 *Foc* conidia/ml can be used to screen banana germplasms against *Foc*. Based on the value of observed disease intensities, ten poyo cultivar grown were identified as very susceptible to *Foc* infection.

This experiment confirms the protocol we followed to be efficient and reliable in terms of artificial inoculation of fusarium wilt of banana. Optimized protocol can be used for screening of banana varieties that can resist/tolerate fusarium wilt. Moreover, this protocol can also be tested for onset fusarium wilt. This research recommend that at the end of inoculation process avoid visiting disease-free areas such as acclimatization rooms, or other banana experiments after working in *Foc* inoculation area and Once the experiment is finished, carry out extensive sterilization of pots, substrate and other tools used in the experiment. Take care of harvesting the pathogen and harvesting tools. Inoculated and un inoculated seedlings should be transplanted at different time. Start with untreated and proceed to treated one to remove contamination.

Introduction of banana materials should pass through quarantine system and Basic studies should have to be conducted on molecularly characterizing the races in the country. Varietal screening should be conducted to replace susceptible one and Country wide survey is important for early detection.

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