

Studies on Fusarium wilt of Watermelon by using Antifungal Agents: An Invitro in Sights

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ABSTRACT

Watermelon (*Citrullus lanatus*) is highly prone to *Fusarium* wilt, caused by a deadly soil-borne pathogen called *Fusarium oxysporum* f. sp. *niveum* (FON). Changes in production practices in crops, reduction in usage of fumigation with methyl bromide, crop rotation activities, and increasing human population, led to a resurgence of more virulent strains of FON. When resistant varieties are unavailable, the disease must be managed through a combination of integrated disease control practices. We conducted *in vitro* screening of botanicals where *Allium cepa* exhibited maximum inhibition of 80.32 and 82.62 percent at 5 and 10 percent concentrations, respectively. *In vitro* evaluation of bioagents revealed the maximum percent inhibition by *T. harzianum* (83.03%) followed by *B. subtilis*, *A. flavus*, *P. fluorescence*, *T. viride*, and *A. niger*. Fungicides by Poisoned Food Technique showed that a combination of carbendazim +mancozeb was effective in mycelial inhibition of 99.74 and 99.81 percent at 500 and 1000 ppm concentration respectively. The knowledge obtained from these *in vitro* findings can be applied in the field at a large scale for reducing and managing watermelon wilt disease.

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INTRODUCTION

Watermelon [*Citrullus lanatus* (Thunb.) Matsum and Nakai)] is a popular summer vegetable fruit belonging to the gourd family Cucurbitaceae with a chromosome number of $2n=2x=22$ (Edwards *et al.*, 2003). The crop originated in the Kalahari deserts of tropical Africa. In India, major watermelon-producing states are Uttar Pradesh, Andhra Pradesh, Karnataka, West Bengal, Orissa, Maharashtra and Tamil Nadu. The plant is an annual trailing creeper with pinnately-lobed leaves and yellow flowers, and it has a special kind of berry, which is botanically called a 'pepo'. The fruit is deep green with a thick rind having gray or light green vertical stripes. Black seeds are embedded inside a red color fleshy fruit (Levi *et al.*, 2001). Watermelon is a warm-season crop preferably grown in a temperature range of 24-27°C. For better accumulation of sugars in fruits, cool nights and warm days are needed. Crop is grown in sandy loam soil with a pH range of 6.5-7.5 (Kumar *et al.*, 2013).

Diseases are more common in watermelon, which deteriorates quality and productivity. Among the most destructive soil-inhabiting fungal pathogens limiting watermelon production is *F. oxysporum* f. sp. *niveum*. This was the second described *Fusarium* wilt after cotton wilt by Erwin F Smith (Martyn, 2012). They suggested that the asexual stage of the pathogen was *Neocosmospora vasinfectum* Atk., a cotton wilt-causing pathogen. However, this was proved wrong later. Snyder and Hansen (1940) revised the *Fusarium* section and reduced ten species into one, *Fusarium oxysporum*. Thus, watermelon wilt fungi became *Fusarium oxysporum* f. sp. *niveum*. Major symptoms of the disease include chlorosis, defoliation, cortical rot, damping off of seedlings, sudden progressive wilt of older plants, necrotic lesions on the roots, and vascular browning. (Fig.1). The severity of the disease is determined by the type of soil, irrigation, application of fertilizer, crop management practices, and seedling preparation. *Fusarium* wilt disease is more common in monocropping systems, where the watermelon crop is cultivated continuously in a shorter

rotation for a longer period such as in China (Everts and Himmelstein, 2015).

The importance of *Fusarium* wilt of watermelon is increasing throughout the world because production practices for watermelon have changed dramatically over the past two decades. To date, four races of *F. o. f. sp. niveum* are described in the literature (race 0, 1, 2, & 3). Race 3 was recently described and is reported to be pathogenic on all cultivars. No commercial watermelon cultivars are available with resistance to the highly aggressive race 2 and newly identified race 3 of FON. Thus, there is a need to search for alternative and environmentally friendly strategies to control this disease. The application of fungicides is a general management tactic for diseases. Biological control through the use of plant extracts and bioagents has been considered a good alternate solution which is safer, ideal, cheap, and eco-friendly when compared with chemicals (Eziashi *et al.* 2007).

MATERIAL AND METHODS

Isolation of fungus

The fungus was isolated from roots of infected watermelon plants (cv. Sugar Baby) collected from the All India Coordinated Research Project on Vegetables, Mahatma Phule Krishi Vidyapeeth, Rahuri, Maharashtra, India by the standard technique under aseptic conditions. Infected tissues of the root were cut into small pieces of 1-2 mm size and surface sterilized with 0.1% sodium hypochlorite solution for 30 seconds and washed repeatedly in sterile distilled water and placed in Petri plates containing sterilized Potato Dextrose Agar medium under aseptic conditions of laminar-air-flow cabinet and incubated at 27±2 °C. The culture thus obtained was purified by single spore isolation and identified as *Fusarium oxysporum* f. sp. *niveum* based on morphological description by Barnett (1960). Identification of the pathogen was done based on a pathogenicity test, microscopic examination, and by comparing cultural and morphological characteristics of the pathogen with those described by previous workers. The pure culture of the pathogen was mounted on a clean glass slide in 0.1 % lactophenol cotton blue, covered with a cover slip, and observed under a compound microscope. Pathogenicity test was performed by using soil inoculation technique where the pathogen was multiplied on sand maize medium by 90 g of sand, 10 g of maize meal, and 20 ml of distilled water in each 250 ml conical flask. The medium was autoclaved at 121°C for 15 minutes. These flasks were inoculated with a bit of actively growing fungal culture and incubated at 25°C for 15 days. The fungal soil mixture was prepared by hand mixing the contents of each flask with the required quantity of autoclaved field soil under hygienic conditions. Inoculum from mass multiplied culture of the pathogen was inoculated in sterilized pots. Healthy and sterilized watermelon seeds of cv. Sugar Babies were sown in pots. Surface sterilized seeds sown in uninoculated sterilized soil served as a check. These pots were watered regularly. Observations on disease

incidence were taken. Re isolation was made from the infected parts of the watermelon plants and the culture obtained was compared with the original one. The organism was reisolated from the roots of artificially inoculated and diseased plants showing the typical symptoms of wilting. The fungus growth obtained was transferred to a potato dextrose agar medium for comparison with the original culture of the test pathogen.

Cultural studies:

The morphological and cultural variability of the pathogen *F. oxysporum* f. sp. *niveum* was studied on ten different solid media. The media used were Potato dextrose Agar (PDA), Richard's Agar, Czapek's Dox Agar, Corn Meal Agar, Oat Meal Agar, Sabouraud's Dextrose Agar, V8 juice Agar, Host Leaf extract Agar, Conn's Agar, Ashby's Mannitol agar.

Spore Morphology studies:

A loopful of the culture of *F. oxysporum* f. sp. *niveum* obtained from each of 12 days old culture media was placed on the slide and mixed thoroughly with lactophenol to obtain uniform spread and a coverslip was placed to observe under the microscope. The sporulation was graded as follows.

Sporulation = No of Conidia/ microscopic field (400 X)

Sporulation	Remark	Range
++++	Excellent	>75
+++	Good	50-75
++	Moderate	25-50
+	Poor	1-25
-	No sporulation	00

In vitro Evaluation of Fungicides:

Five systemic fungicides (Carbendazim 50% WP, Mancozeb 75% WP, Hexaconazole 5%SC, Propiconazole 25%EC, Chlorothalonil 75%WP) and one combination of systemic and contact fungicide (Carbendazim 12%EC + Mancozeb 63% WP) were evaluated at 500 and 1000 ppm concentrations against pathogen on PDA medium.

Poisoned Food Technique

A recommended dose of fungicide was added to molten PDA just before pouring it into plates. For each sterilized plate, twenty milliliters of medium with the desired concentration of fungicide were poured. Suitable checks were laid for comparison. Five-millimeter mycelial disc of fungus was taken from the periphery of 10-day-old culture placed in the center of the plate, and incubated at 28± °C. Growth of the fungus was measured by measuring its diameter in two directions and the average value was recorded. The growth reading was recorded when the growth of the fungus in the control plate was full. Percent inhibition of growth was calculated using the formula given by Vincent (1947).

fungus growth in control plates - Growth in treated plates
 Percent inhibition = ----- x 100
 Growth of test fungus in control plates

In vitro Evaluation of Bioagents:

Four fungal antagonists viz., *Trichoderma viride*, *T. harzianum*, *Aspergillus niger*, *A. flavus*, and bacterial antagonist *Pseudomonas fluorescens* and *Bacillus subtilis* were evaluated against pathogen by applying the dual culture technique given by Dennis and Webster, 1971 in three replications

Dual Culture Technique

Seven days old cultures of the bio-agents and fungus grown on PDA were used for the study. Discs of 5 mm diameter of PDA along with culture growth of the test fungus and bio-agents were cut out with sterilized cork borer. Then two culture discs were placed at equidistance and exactly opposite to each other on solidified PDA medium in sterilized plates and were incubated at 27 ± 2°C. The plates inoculated only with culture discs of the test fungus were maintained as a control. Observations on linear mycelial growth of the test fungus and bio-agents were recorded at intervals of 24 hours and continued till untreated control plates were fully covered with the mycelial growth of the test fungus. Percent inhibition of the test fungus by the bio-agents over untreated control was calculated by a formula given by Arora and Upadhyay, 1978.

Colony growth in control plate - Colony growth in Intersecting plate
 percent Growth = ----- x 100
 Inhibition Colony growth in the control plate

In vitro Evaluation of Botanicals:

The leaf and bulb extracts from locally available plants such as *Azadirachta indica*, *Pongamia pinnata*, *Parthenium hysterophorus*, *Calotropis gigantea*, *Annona squamosa*, *Ocimum santum* and *Allium cepa* were used as treatments, and evaluated against pathogen by poisoned food technique given by Nene and Thapliyal, 1993. Fresh healthy plant parts (100 g of leaves/bulbs) collected from fields were washed with distilled water, air-dried, and crushed in 100 ml of distilled water (w/v). The crushed product was tied in a muslin cloth and the filtrate. The prepared solution gave 100 percent concentration, which was further diluted to the required concentrations of 5 and 10 percent. A quantity of 12.5 and 25 ml of each plant extract was separately mixed thoroughly with autoclaved and cooled PDA medium in conical flasks of 250 ml capacity to obtain desired concentrations of 5 and 10 percent respectively. The PDA medium with separate plant extract was then poured (20 ml/plate) into sterile Petri plates (90 mm dia.) and allowed to solidify at room temperature. Upon solidification of PDA, all the treatments and control plates were aseptically inoculated by placing a 5 mm mycelial disc obtained from a week-old actively growing pure culture of fungus at the center of the plate. For each test plant extract and their respective concentrations, three replications were maintained. All these plates were then incubated at 27 ± 2°C temperature for a week or till the untreated control plates were fully covered with the mycelial growth of the test fungus.

Observations on radial mycelial growth of the test fungus were recorded at 24 hr. intervals and continued till the growth of the test pathogen in the control plate was fully covered. Percent inhibition of the test pathogen was calculated by the formula given by Vincent (1927).

Growth of test fungus in control plates - Growth of test fungus in treated plates
 Percent Growth = ----- x100
 Inhibition test fungus growth in treated plates

Statistical Analysis

The data was analyzed statistically by using a completely randomized design. Percent data was first transformed to the arc sine values before analysis and the treatments were compared to the means of critical difference at a 1 percent level of significance (Panse and Sukhatme, 1967).

$$S. E = \sqrt{2 EMSS / r}$$

Where,
 S. E. = Standard error
 EMSS = Error means of sum of square
 r = Number of replications
 C. D. = t error * S. E.

Where,
 C. D. = Critical difference
 t = t value at error degrees of freedom

RESULTS AND DISCUSSION:

The characteristic symptoms observed were chlorosis, defoliation, cortical rot and damping off of seedlings, vascular browning, partial and complete wilting of plants (Fig. 1).



Fig.1 Fusarium wilt symptoms

Several researchers reported that extracellular cell wall degrading enzymes (endo polygalacturonase, cellulases, hemicellulases, pectinases, and others) of both host and pathogen origin were causing gums and gels that occlude the vessels, limiting water transport. Additionally, they reported that tyloses may be induced adding to the blockage resulting in permanent wilt and ultimately plant death. Di Basaran, *et al.* (2007) and Recorbet *et al.* (2003) provide excellent reviews of the current understanding of the underlying mechanisms and molecular determinants of pathogenicity of *Fusarium* wilt diseases.

The pathogen was isolated by using standard procedures on a PDA medium which produced a pale yellow to white cottony color growth. (Fig. 2).



Fig.2 Fusarium culture on PDA

A microscopic view showed profusely branched hyaline mycelium with microconidia, macroconidia, and chlamydo spores. Microconidia were one-celled, hyaline elliptic to ovoid in shape. Macroconidia were sickle-shaped, hyaline, slightly curved, septate (1-5 septa) and varying in size. Both macro and microconidia were formed from conidiophores on PDA. (Fig. 3).

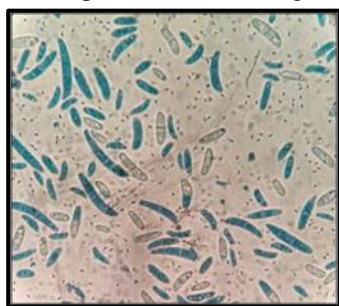


Fig.3 Micro and macroconidia of Fusarium

Chlamydo spores were spherically borne terminal and intercalary, hyaline to light brown color was observed. Dutta *et al.* (2017) recorded morphological characteristics of *F. oxysporum* f. sp. *niveum* in a similar way that pathogen produced asexual spores such as microconidia, macroconidia, and chlamydo spores. They described macroconidia as fusiform (boat-shaped) and often have three to five cells. Chlamydo spores were small, single-celled, and kidney-shaped.

The characters such as colony color, shape, margin, and meal colony diameter in mm were recorded. The colony color observed on all media was mostly white and it varied from pale yellow to pinkish color in some media also. Mycelial growth was observed as puffy, profuse, loose cottony white, and circular to irregular. There was a good growth of the pathogen on most of the media however no growth was observed on Ashby's mannitol agar. On 5th and 7th day, Potato Dextrose Agar medium showed significantly highest mean mycelial growth of 54.28 mm and 86.32 mm respectively. The least growth was observed in the case of Oatmeal agar at 22.60 mm and 34.12 mm. The colony growth in the case of other media was as follows Corn meal agar- 48.32 mm and

50.23 mm, Richard's agar- 44.28 mm and 57.34 mm, Czapek's dox agar-43.08 mm and 53.41 mm, Watermelon leaf extract agar-42.60 mm and 58.22mm, Conn's agar- 40.22 mm and 50.63 mm, V8 agar-34.04 mm and 45.65 mm, Sabouraud maltose agar- 28.80 mm and 36.84 mm respectively in 5th and 7th days.(Fig 4)



Fig 4 Cultural studies on ten medias

Sporulation was observed on all media except Ashby's mannitol agar (T1). Excellent (++++) sporulation was found on mediums such as Potato dextrose agar (T6), Richard's agar (T7), Oatmeal agar(T5), and Sabouraud maltose agar (T9). Media such as Czapek's dox agar(T2) and Cons agar (T3) showed good (+++) sporulation. V8 agar(T8) and Watermelon leaf extract agar (T10) showed moderate (++) sporulation. Whereas, poor (+) sporulation was observed on Corn meal agar.

Nikam *et al.* (2011) studied similarly the morphological characters of *F. oxysporum* f. sp. *ciceri* on various media and revealed that maximum mycelial growth was obtained on host leaf extract medium of susceptible cultivar JG-62 (90mm) followed by Richard's agar (86mm) and Potato dextrose agar (83mm). All three media showed significantly good mycelial growth and sporulation. The least growth was observed on Oatmeal agar (71mm). Shinde (2019) reported that Potato dextrose agar showed maximum mycelial growth of 86.33 mm with excellent sporulation while conducting similar research on cultural characters of *F. oxysporum* f. sp. *niveum* on ten different solid media.

Among the different fungicides tested, it was observed that at 500 ppm, the percent inhibition of the test pathogen ranged from 51.11 to 99.74 percent. Fungicide carbendazim 12 % WP + mancozeb 63 % WP was statistically found best which inhibit 99.74 percent mycelial growth followed by fungicide carbendazim 50 % WP (83.51 %), chlorothalonil 75 % WP (83.07 %), hexaconazole 5 % SC (62.22 %), mancozeb 75 % WP (60.92 %) over untreated control. Propiconazole 25 % EC was found less effective with minimum mycelial inhibition per cent of 51.11.

At 1000 ppm, the percent inhibition of test pathogen *F. oxysporum* f. sp. *niveum* ranged from 56.11 to 99.81 percent. The treatment of fungicide carbendazim 12 % EC + mancozeb 63 % WP was again found significantly highest percent inhibition of 99.81 percent among all other fungicides used, which was followed by treatment

of carbendazim 50 % WP (84.44 %), chlorothalonil 75 % WP (84.25 %), hexaconazole 5 % EC (66.66 %), mancozeb 75 % WP (66.29 %) and propiconazole 25 % EC (56.11 %) against percent inhibition of test pathogen over untreated control. The least percent inhibition was in the treatment of propiconazole 25 % EC (56.11 %). (Table 1). The results are in conformity with the previous works of Mukherjee and Tripathi (2000), and Parmer (2014).

Table 1. Inhibition of fungus by different fungicides

Tr. No.	Treatments details	Per cent inhibition (ppm)*	
		500	1000
T ₁	Carbendazim 50 % WP	83.51 (66.04)	84.44 (66.76)
T ₂	Mancozeb 75 % WP	60.92 (51.30)	66.29 (54.50)
T ₃	Hexaconazole 5 % SC	62.22 (52.07)	66.66 (54.73)
T ₄	Propiconazole 25 % EC	51.11 (45.63)	56.11 (48.50)
T ₅	Chlorothalonil 75 % WP	83.07 (65.70)	84.25 (66.61)
T ₆	Carbendazim 12 % WP + Mancozeb 63 % WP	99.74 (87.07)	99.81 (87.50)
T ₇	Control	0.00 (0.00)	0.00 (0.00)
	S.Em. ±	0.18	0.17
	CD at 1 %	0.78	0.71

For botanicals, the results revealed that at 5 percent concentration, fungal percent inhibition ranged from 45.89 percent to 80.32 percent. Significant maximum percent inhibition was recorded in *Allium cepa* (80.32 %), which was followed by *Parthenium hysterophorus* (79.72 %), *Calotropis gigantea* (69.92 %), *Azadirachta indica* (64.82 %), *Ocimum santum* (62.64 %), *Annona squamosa* (61.55 %). The least inhibition was observed in *Pongamia pinnata* (45.89 %) against the test pathogen over the untreated.

At 10 % concentration, the pattern of botanical efficacy was found the same as, in the case of 5 % concentrations, the mycelial growth inhibition of the test pathogen ranged from 60.25 to 82.62 percent. Onion (*Allium cepa*) showed significant maximum percent inhibition of 82.62 percent followed by *Parthenium hysterophorus* (80.81 %), *Calotropis gigantea* (72.88 %), *Azadirachta indica* (68.40 %), *Annona squamosa* (62.44 %), *Ocimum santum* (60.55 %). The least inhibition was observed at *Pongamia pinnata* (60.25 %) against the test pathogen untreated. The results are in conformity with the previous works of Farrag *et al* (2013) and Bharath *et al.* (2006). (Table 2)

Table 2. Inhibition of fungus by different by botanicals

Tr. No.	Treatments details	Per cent inhibition*	
		5 %	10 %
T ₁	<i>Azadirachta indica</i>	64.82 (53.60)	68.40 (85.31)
T ₂	<i>Pongamia pinnata</i>	45.89 (49.87)	60.25 (50.91)
T ₃	<i>Parthenium hysterophorus</i>	79.72 (58.26)	80.81 (64.01)
T ₄	<i>Calotropis gigantea</i>	69.92 (56.92)	72.88 (58.61)
T ₅	<i>Annona squamosa</i>	61.55 (51.67)	62.44 (52.2)
T ₆	<i>Ocimum sanctum</i>	62.64 (52.61)	60.55 (51.09)
T ₇	<i>Allium cepa</i>	80.32 (63.60)	82.62 (65.36)
T ₈	Control	0.00 (0.00)	0.00 (0.00)
	S.E m. ±	0.20	0.17
	CD at 1 %	0.85	0.69

Among all antagonists used *Trichoderma harzianum* showed significantly highest percent inhibition of 83.03 which was followed by *Bacillus subtilis* (76.48 %), *P. fluorescens* (75.37 %), and *Trichoderma viride* (66.85 %). The least percent inhibition 41.48 percent was shown by *Aspergillus niger*. (Fig. 7) (Table 3) Thus, results revealed that all bioagents that were evaluated *in vitro* against the test pathogen were antagonistic in action and significantly inhibited the growth of *F. oxysporum* f. sp. *niveum*. Beneficial microbes can also enhance plant growth by increasing the availability of nutrient supply and secreting phytohormones. The results are in conformity with the findings of Hamed *et al.* (2009) and Cao *et al.* (2011). Cao *et al.* (2011) revealed that the use of bacterial antagonist *Bacillus subtilis* SQR 9 under *in vivo* and *in vitro* conditions against *F. oxysporum* f. sp. *cucumerinum* gave successful results. Singh *et al.* (2014) conducted similar work on *in vitro* efficacy of bioagents viz., *T. viride*, *Gliocladium virens*, *T. harzianum*, *T. hamatum*, *Aspergillus niger*, *P. fluorescens* against *Fusarium* spp. by using dual culture techniques. The results revealed that seed treatment with *T. viride* and *T. harzianum* gave significantly better results. Several studies reported that *Trichoderma* spp. is the most widely studied biological control agents for soil-borne diseases and are also applied in post-harvest disease management (Woo *et al.*, 2014).

Table 3. Inhibition of fungus by bioagents.

	Bioagents	Per cent inhibition*
T ₁	<i>Trichoderma viride</i>	66.85 (54.8)
T ₂	<i>Trichoderma harzianum</i>	83.03 (65.7)
T ₃	<i>Aspergillus niger</i>	41.48 (40.1)

T ₄	<i>Aspergillus flavus</i>	76.29 (60.9)
T ₅	<i>Bacillus subtilis</i>	76.48 (60.98)
T ₆	<i>Pseudomonas fluorescens</i>	75.37 (60.24)
T ₇	Control	00 (0)
	S.Em. ±	0.15
	CD at 1 %	0.78

CONCLUSION

In conclusion, watermelon (*Citrullus lanatus*) stands as a vital vegetable fruit globally, belonging to the Cucurbitaceae family. While rich in water content and essential nutrients, Fusarium wilt, caused by *F. oxysporum* f. sp. *niveum*, poses a significant threat to its quality and productivity. Recognizing the soil-borne nature of the disease and its persistent presence in chlamydospores, this study aimed at integrated disease management through the evaluation of fungicides, botanicals, and bioagents under *in vitro* conditions. Analysis of the morphology and culture of *F. oxysporum* f. sp. *niveum* on different solid media revealed that PDA medium promotes robust sporulation and maximal colony diameter. The *in vitro* assessment revealed that carbendazim 12% WP + mancozeb 63% WP was the most effective fungicide, while *Allium cepa* (Onion) exhibited superior mycelial inhibition among botanicals. *T. harzianum* emerged as the most potent bioagent for disease suppression. The findings advocate for the adoption of eco-friendly combinations of botanicals and bioagents for *in vivo* testing in the field, emphasizing grafting, resistant rootstock identification, cover crop evaluation, and an enhanced understanding of Fusarium wilt suppression mechanisms. While progress has been made, the study encourages further exploration of biocontrol systems to effectively manage watermelon wilt disease, addressing the urgent need for sustainable agricultural practices. Furthermore, it is crucial to emphasize how quickly the eco-friendly solutions that have been developed must be put into practice in order to reduce the worldwide effects of Fusarium wilt on watermelon crops. The study also advocates collaborative efforts in the practical application of grafting, resistant rootstock identification, and cover crop evaluation, emphasizing the ongoing need for sustainable agricultural practices in the face of this persistent and detrimental disease.

Conflict of Interest Statement:

The authors declare that there is no conflict of interest with anyone. The authors also confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. It is further confirmed that the order of authors listed in the manuscript has been approved by all of us.

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