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Dynamic association of *Fusarium verticillioides* with maize and its biological control

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Abstract

Fusarium verticillioides have distinct host - pathogen association with maize. Dynamics of disease from necrotrophic pith disintegrating and slow evading soil inhabitant to harmless endophytic association was unravelled in different inoculation techniques. Internodal region of maize stalk as dominant niche of post flowering stalk rot inducing *F. verticillioides*, when subjected to isolation in potato dextrose agar expressed white fluffy mycelium turning purple in incubation. Microscopic observation recorded few single septate and abundant aseptate oval shaped micro-conidia from monophialides, macro-conidia were falcate shaped and were observed to be rare. Chlamydo-spores were absent. Several artificial inoculation techniques were implied to analyse the varying response of the plant with respect to different inoculation sites, age and method of inoculation. Toothpick method of inoculation expressed typical post flowering stalk rot symptoms indistinct to farmer's field and recorded highest percent disease intensity of 89.63. Pith disintegration in toothpick inoculation technique was supplemented by injury, susceptible reproductive stage and internodal stalk region of maize. Hence, toothpick method of inoculation of *F. verticillioides* was indicated as an ideal technique for the laboratory analysis of disease. Underdeveloped plants with rotten roots, slight pith discolouration and 5.18 percent disease intensity manifested in soil inoculation technique indicated slow above ground growth of fungus along with its soil inhabiting nature. Endophytic association of *F. verticillioides* with maize during absence of stress was revealed in spore injection technique with zero percent disease intensity. Pith disintegration and mycelium infected cobs induce disease severity of post flowering stalk rot of maize. Efficacy of different fungal and bacterial antagonists were tested against the mycelial growth of *F. verticillioides* using dual culture technique. *Trichoderma harzianum* isolates recorded higher growth inhibition exceeding 70 per cent. Growth inhibition of *Trichoderma viride* isolates ranged from 51.48 to 64.07 per cent. Sardarkrushinagar isolates of *T. harzianum* and *T. viride* exhibited superior reduction. *Bacillus subtilis* and *Pseudomonas fluorescens* recorded 61.86 and 43.70 per cent inhibition. These free living bio-agents being highly interactive in soil, root and foliar environment serves as an effective biopesticide for the control of *F. verticillioides* in maize.

Keywords: Maize, necrotrophic, soil inhabitant, endophytic, growth inhibition, bio-agents

Introduction

Maize (*Zea mays*) also known as corn, is an important cereal crop belonging to the family *Poaceae*. Maize is thought to be originated from ancient wild grass teosinte, from Mexico and Gautamela. It is the third most important crop in the world with its wider adaptability to different agro-climatic conditions. Hence, it is grown in tropical as well as temperate regions. Population increasing at a rapid rate can gain spiralling demand on maize among cereals due to its easy availability, starch content and its nutritional status. Diseases account for 9 percent of yield loss of maize in global sector (Oerke, 2005) [1]. Among several diseases, the most widely spread devastating disease of maize is post flowering stalk rot. The post flowering stalk rot manifests as internal decay and discolouration of tissue that blocks water and nutrient translocation thereby reducing yield and can even lead to crop lodging (Khokhar *et al.*, 2014) [2]. Maize production in India is severely declined by post flowering stalk rot (Sharma *et al.*, 1993) [3]. Post flowering stalk rot of maize was first reported by Palmel (1914) [4] from United States of America and *Fusarium verticillioides* (Saccardo) Nirenberg was identified as the causal organism of the disease. The species *Fusarium moniliforme* is accepted as *F. verticillioides*. Helgard Nirenberg rejected *F. moniliforme* and transferred *Oospora verticillioides* to *Fusarium verticillioides* (Sacc.) Nirenberg, retaining Saccardo as the original author (Deepa and Sreenivasa, 2017) [5]. The epithet "*verticillioides*" describes whorled nature of the conidiophore. The species has also been designated as mating population A of *Fusarium fujikuroi* species complex (formally known as *Gibberella fujikuroi* species complex). General

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symptoms of fusarium stalk rot include, rotting of roots, crown and lower internodes, pith disintegrates but vascular bundles remain intact. High plant density, corn borer infestation, heavy cloudiness and moisture stress are several factors responsible for symptom development (Parry *et al.*, 1995) [6]. In India, Fusarium stalk rot is most common in Rajasthan, Jammu and Kashmir, Karnataka, Tamil Nadu, Uttar Pradesh, Bihar, Andhra Pradesh, West Bengal, Punjab and Madhya Pradesh, where scarcity of water coupled with high soil temperature is seen (Singh *et al.*, 2012) [7]. Very meager information is available on various aspects of this disease. Hence, it has been felt necessary to generate scientific information on several aspects of this disease

Materials and Methods

All the experiments were conducted in the laboratory of Department of Plant Pathology, Chimanbhai Patel College of Agriculture, Sardarkrushinagar Dantiwada Agricultural University, Sardarkrushinagar, Gujarat.

Isolation, Purification and Identification of *Fusarium verticillioides* (Saccardo) Nirenberg

Diseased samples were collected from farmer's field of Sabarkantha district, Gujarat. Tissues from root, internode and leaves of infected samples were isolated on potato dextrose agar (PDA). Purification is done by single spore isolation

(Niemeyer and Andrade, 2015) [8] and hyphal tip method (Leslie and Summerell, 2006) [9]. Cultural and morphological characteristics of the pathogen were identified using standard descriptions of Leslie and Summerell (2006) [9].

Proving pathogenicity of *F. verticillioides* using different inoculation techniques

Several techniques namely, toothpick method, spore injection technique and soil inoculation technique were used to analyse the pathogenicity of *F. verticillioides*. Development of disease symptoms was observed regularly on artificially inoculated plants and observations on disease severity using 1-9 disease rating as suggested by Shekhar and Kumar (2012) [10], where 1 = Healthy or trace/slight discoloration at the site of inoculation, 2 = 50% of the inoculated internode is discoloured, 3 = 75% of the inoculated internode is discoloured, 4 = 100% of the inoculated internode is discoloured, 5 = Less than 50% discoloration of the adjacent internode, 6 = More than 50% discoloration of the adjacent internode, 7 = Discoloration of three internodes, 8 = Discoloration of four internodes and 9 = Discoloration of five internodes or plants prematurely killed. Based on these grades, Percent Disease Intensity (PDI) was calculated using the formula of Mc Kinney (1923) [11] as detailed below. Reisolation was made by tissue isolation technique and the isolate was compared with original one.

$$PDI = \frac{\text{Sum of individual rating}}{\text{Total No. of leaves examined} \times \text{Maximum disease score/grade}} \times 100$$

Toothpick method

Toothpick method was followed as given by Afolabi *et al.*, (2008) [12]. Toothpicks colonised by *F. verticillioides* were used as inoculum for stalk inoculation. Wooden toothpicks were boiled three times and then air dried alternately. Dried toothpicks were packed in screw capped jars. Prior to

autoclaving, potato dextrose broth was added. The level of broth was adjusted to one-third length of toothpicks after autoclaving. Later on, the sterilised jar was seeded with fungus and incubated at 27±1° C. The toothpicks colonised with test pathogen were inserted into the stalk region of the plants (second internode), just after the flowering stage.

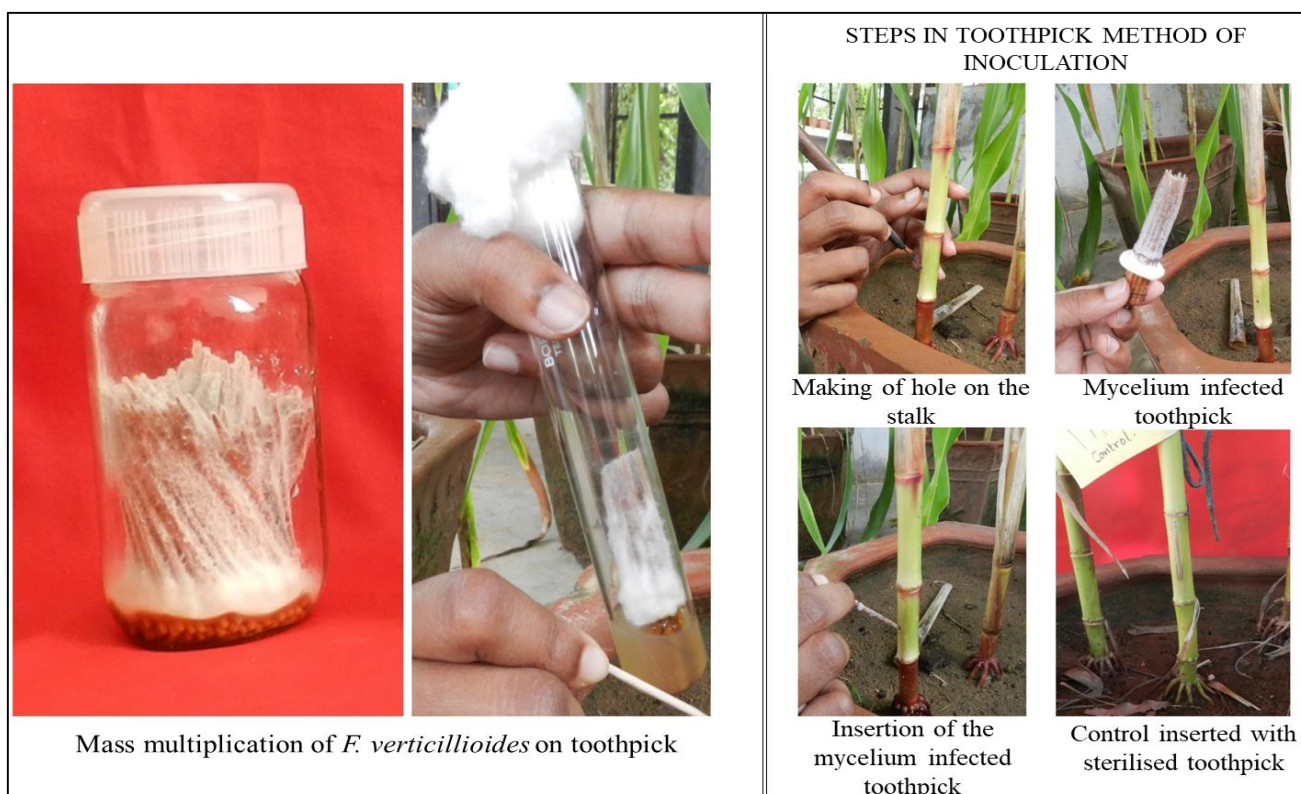


Fig 1: Toothpick Method of inoculation

Spore injection technique

For spore injection technique, spore suspension of *F. verticillioides* was injected into the first internode of the three-week-old plant as described by Shin *et al.*, (2014) [13]. The Petri-plates with culture was flooded with sterilised distilled water and the surface was scraped to harvest conidia. Mycelial fragments were separated by filtration using muslin cloth. The conidia pelleted by centrifugation was washed and diluted to get a concentration of 10^6 conidia/ml in sterilised distilled water.

Soil inoculation technique: Soil inoculation technique was

followed as suggested by Kala *et al.*, (2016) [14]. The fungus *F. verticillioides* was mass multiplied in sand maize meal medium and incubated for 7 days at a temperature of $27 \pm 1^\circ \text{C}$. Sand maize meal medium was prepared by mixing 90 g of riverbed sand, 10 g of maize meal and 20 ml of distilled water in 250 ml conical flask. The sterilised medium in the flask was inoculated by a bit of actively growing fungal culture and incubated at $27 \pm 1^\circ \text{C}$ for 15 days. The fully grown fungal inoculum was thoroughly mixed with sterilised soil in 1:10 ratio and was filled in pots. The soil was moistened for one week before sowing of the seeds.



Mass multiplication in sand maize meal medium

Inoculated soil

Fig 2: Soil inoculation technique

Efficacy of different bio-agents against *F. verticillioides* in vitro

Management of plant pathogens using bio-agents is an integral component of plant disease management. In the present investigation, two *Trichoderma harzianum* isolates (Sardarkrushinagar and Junagadh), three *Trichoderma viride* isolates (Sardarkrushinagar, Junagadh and Navasari) as well as two bacterial antagonists namely *Pseudomonas fluorescens* and *Bacillus subtilis* were tested for its efficacy against *in vitro* growth of *F. verticillioides* using dual culture technique on PDA medium. Sterilised PDA (20 ml) was poured aseptically in 90 mm diameter sterilised Petri-plate. Mycelial disc of size 5 mm from seven days old culture of the bio-agents and *F. verticillioides* were cut aseptically from the periphery of the colony with the help of sterilised cork borer and placed on to a solidified PDA approximately 70 mm away from each other. The culture of *F. verticillioides* was subjected alone for growth and comparison. All inoculated Petri-plates were incubated at $27 \pm 1^\circ \text{C}$ temperature in an incubator. Observations on radial growth in each Petri-plate was measured periodically and final observation was recorded when control plate is fully covered with the mycelial growth of *F. verticillioides*. The experiment was conducted using completely randomized design with factorial concept and data were statistically analysed using Duncan's New Multiple Range Test. Colony diameter was measured along the two diagonals passing through the colony by excluding the initial diameter (5 mm) of bit. Colony diameter was measured when the control treatment with pathogen reached full growth. The

per cent growth inhibition (PGI) of the fungus in each treatment in comparison with control was calculated by the following equation (Bliss, 1934) [15].

$$\text{PGI} = \frac{C-T}{C} \times 100$$

Where,

PGI = Percent growth inhibition
C = Colony diameter in control (mm)
T = Colony diameter in treatment (mm)

Results and Discussion

Isolation, Purification and Identification of *Fusarium verticillioides* (Saccardo) Nirenberg

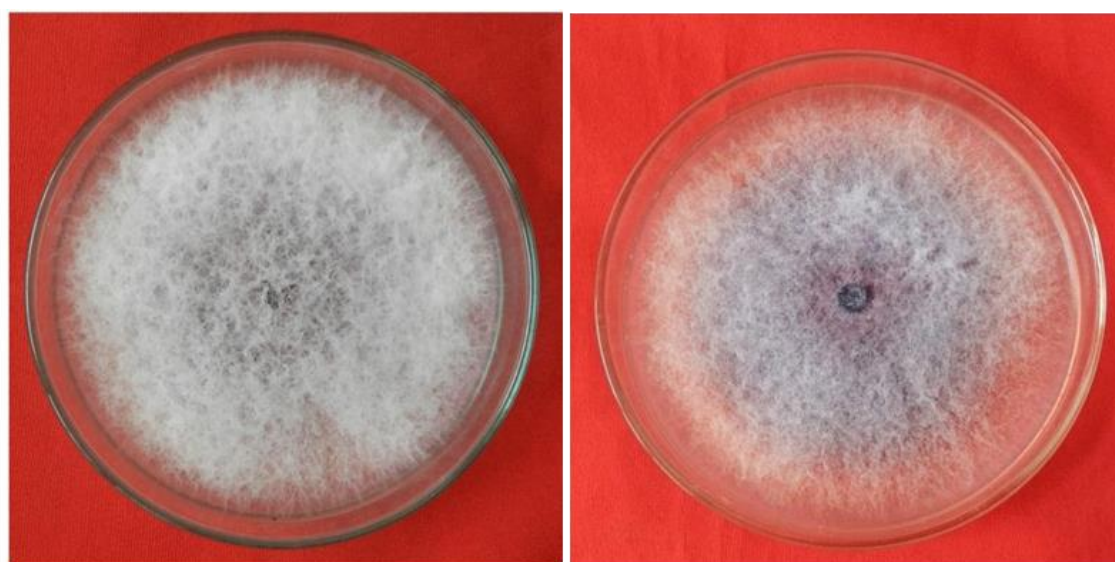
Among six samples collected, five samples yielded *F. verticillioides* from the internodal tissues of the stalk region. However, one sample yielded *F. verticillioides* from both root and internodal stalk region tissues. Thus, it can be concluded that *F. verticillioides* mainly occupies internal stalk region of maize as all the samples of internode yielded pathogen. Koehler (1990) [16] mentioned that *Fusarium moniliforme* was most frequently isolated from the stalk region. Chehri *et al.*, (2010) [17] reported frequent isolation of *F. verticillioides* among several isolates of *Fusarium* sp from the stalk and root tissues of diseased maize samples at a frequency of 75 and 25 percent. Li *et al.*, (2019) [18] isolated *F. verticillioides* from ear (38.8%), kernels (38.1%) and stalk region (49.5%) of maize. Thus, results obtained in the present investigation are more or less in accordance with the findings of previous researchers as

the predominant occurrence of *F. verticillioides* in the stalk region of maize.

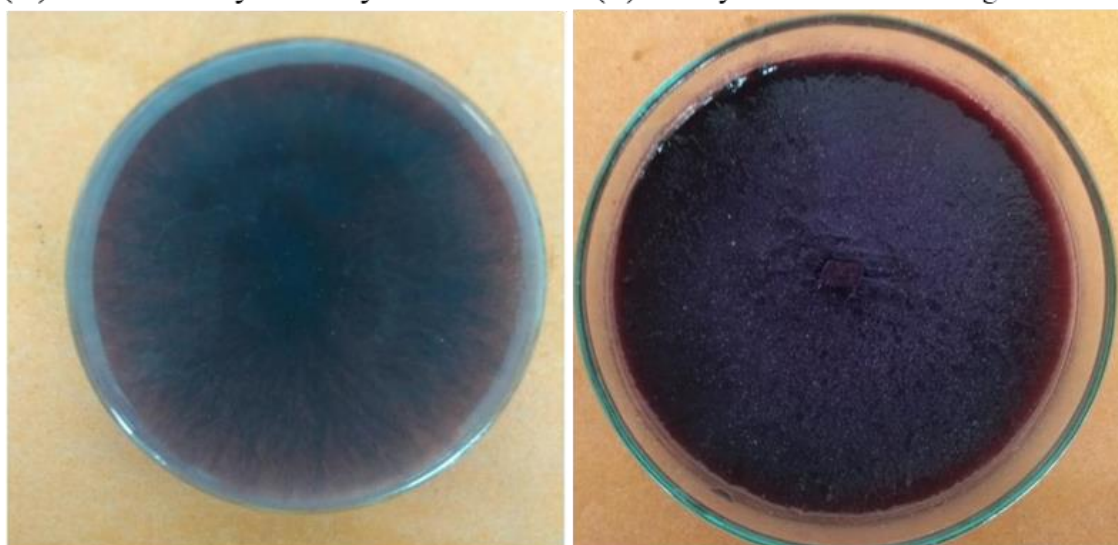
Identification

Morphological and cultural characteristics of the fungal isolate (Fig. 3 and 4) cultured on PDA and water agar (WA) medium at a temperature of $27 \pm 1^\circ\text{C}$ were compared with standard descriptions of Leslie and Summerell (2006) and was identified as *Fusarium verticillioides* (Saccardo) Nirenberg. Cultural characters of the isolate were studied by culturing it on PDA medium at $27 \pm 1^\circ\text{C}$ (Fig. 3). The fungal isolate initially produced white fluffy colonies which gradually turned violet to dark purple after ten days of incubation. Dark purple pigmentation was clearly visible at the bottom side of the Petri-plate within seven days of incubation as well as in the culture incubated at 4°C . The radial growth of mycelium attained an average size of 90 mm with circular margin in seven days of incubation. The culture produced aerial mycelium without any undulations. Microscopic observation (Fig. 4) revealed the hyaline and septate hypha of *F.*

verticillioides. Culture on the WA medium displayed the verticillate arrangement of conidiophore as well as the production of micro-conidia from monophialides. Oval shaped and flattened base, aseptate micro-conidia were produced abundantly in chains with an average size of $11.33 \times 1.5 \mu\text{m}$, while oval shaped, single septate micro-conidia with an average size of $9.7 \times 2.4 \mu\text{m}$ were produced in less proportion. Macro-conidia were produced rarely. They were 2-3 septate, long and slender, slightly falcate shaped with an average size of $38.24 \times 2.78 \mu\text{m}$. The chlamydospores were absent. Therefore, *F. verticillioides* was considered to be the causal organism of post flowering stalk rot of maize. Low proportion of single septate micro-conidia along with abundant aseptate micro-conidia in *F. verticillioides* were reported by Hirata *et al.*, (2001) [19]. Jat *et al.*, (2017) [20] reported $10.34 \times 1.5 \mu\text{m}$ single septate micro-conidia and $40.24 \times 6.67 \mu\text{m}$ macro-conidia in *F. verticillioides*. Thus, the result obtained corroborate with the findings of previous researchers.



(A) White colony of 7 days old culture (B) 10 days old culture turning violet



(C) Bottom view of 10 days old culture (D) Purple pigmentation in culture at 4°C

Fig 3: Cultural characters of *F. verticillioides*

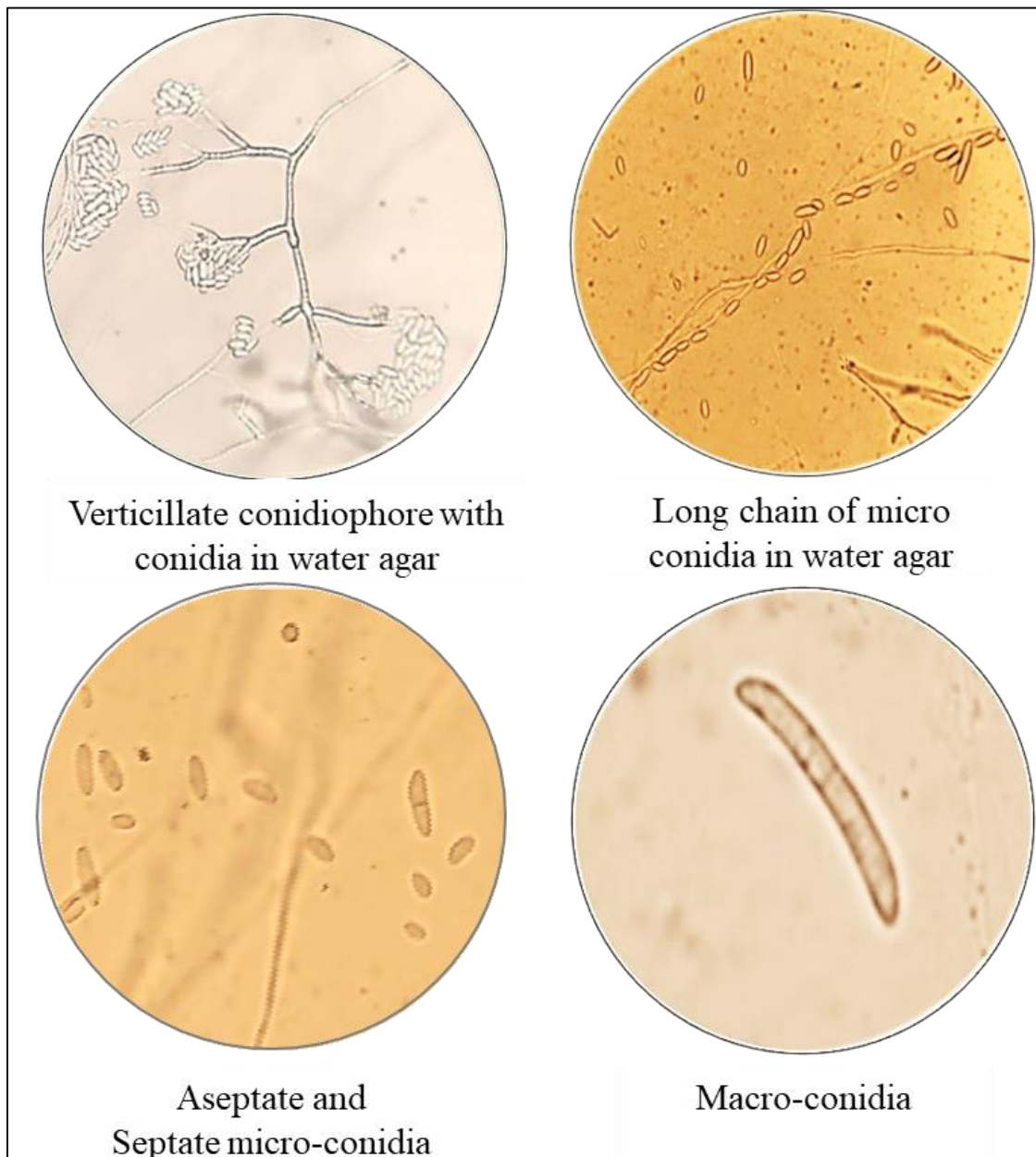


Fig 4: Morphological Characters of *F. verticillioides*

Proving pathogenicity of *F. verticillioides* using different inoculation techniques

Toothpick method

Most prominent symptoms of post flowering stalk rot were expressed with toothpick method of inoculation. All plants expressed vascular discolouration and pith disintegration. Vascular discolouration in three internodes by 4 plants, four internodes by 6 plants and more than five discoloured internodes in 5 plants yielded 89.63 percent PDI for tooth pick inoculation method (Table 1, Fig. 5C, 6 and 7). Infected plants showed yellowing and drooping of leaves, wilting and pith disintegration. Lower stalk region lost its rigidity and was able to squeeze between the fingers. Stalk, ear head and leaf sheath got infected with *F. verticillioides* mycelium (Fig. 8). Inoculation after silking stage, at the second internode by wounding as well as direct contact of the inoculum (mycelium and spore coated tooth pick) contributed to the prominent symptom expression. Drepper and Renfro (1990) [21] reported higher Disease Incidence (75 to 100%) of stalk rot by wounding techniques and established significant relation between wound diameter and disease severity. Koehler (1990)

[16] obtained the best results with similar technique and also mentioned the susceptibility of lower stalk region due to lower carbohydrate and sugar level at reproductive stage, easiness of toothpick inoculation, entry of pathogen facilitated by wound as well as hollow and soft stalk after infection. Toothpick method was suggested as an ideal technique to deliver pathogen inoculum by Ghimire *et al.* (2019) [22]. Similar symptoms were reported by Li *et al.*, (2019) [18] who mentioned it as systemic infection that reaches its peak after late milk stage to evade upper part of the plant and gets aggravated by wind. Thus, the results in the present investigation were in accordance with the findings of previous researchers.

Spore injection technique

None of the spore suspension inoculated plants showed vascular disintegration and did not differ with plants in control, even at maturity (Table 1, and Fig. 5d and 6). Thus, it is clear that spore injection technique failed to provide any kind of symptoms but yielded *F. verticillioides* on reisolation from inoculated tissue. Asymptomatic association of *F.*

verticillioides with the host was attributed to its endophytic nature in the absence of stress (Bacon *et al.*, 2008) [23], low inoculum load or restricted growth of fungus (Singh *et al.*, 2012) [7] or inoculation at the vegetative stage with high non-structural carbohydrate and sucrose level in the stalk region to suppress stalk rot (Soliman, 1979) [24]. Zhang *et al.* (2016) [25] also obtained asymptomatic plants on spore suspension inoculation with *Fusarium graminearum* and revealed the presence of hypha in the parenchyma cells away from the wounded site through fluorescent microscopy. Thus, results obtained agrees with the findings of previous researchers.

Soil inoculation technique

Seeds germinated normally in the *F. verticillioides* inoculated soil but developed as thin, short plants with chlorotic leaves and poor cob development in comparison to tall, green plants with well-developed cobs in control (Fig 5b). None of the plants exhibited vascular disintegration. Seven out of fifteen inoculated plants showed slight discoloration at the site of inoculation and rotting of roots with a PDI of 5.18 percent for soil inoculation technique (Table 1 and Fig. 6). Drepper and Renfro (1990) [21] reported low Disease Incidence (8 to 25%) of stalk rot by nonwounding techniques in comparison to inoculation involving wounding of tissues. Oren *et al.* (2003) [26] attributed chlorotic leaves and poor development of the

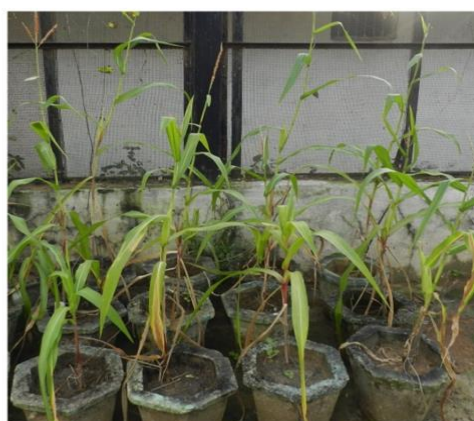
plants to the root colonisation of *F. verticillioides*. Lack of vascular discoloration was due to slow growth of the fungus that evades above ground parts only at favourable conditions (Oren *et al.*, 2003 and Singh *et al.*, 2012) [26, 7]. Hence, the results obtained in present investigations are in accordance with the findings of early researchers.

Table 1: Disease rating scale and Percent Disease Intensity of *F. verticillioides* in different inoculation techniques

Rating scale	Control	Soil inoculation	Toothpick method	Spore injection
1	0	7	0	0
2	0	0	0	0
3	0	0	0	0
4	0	0	0	0
5	0	0	0	0
6	0	0	0	0
7	0	0	4	0
8	0	0	6	0
9	0	0	5	0
Asymptomatic plants	15	8	0	15
Percent Disease Intensity	0	5.18%	89.63%	0



(A) Control



(B) Soil inoculation technique



(C) Toothpick method



(D) Spore injection technique

Fig 5: Plant treated with different inoculation techniques

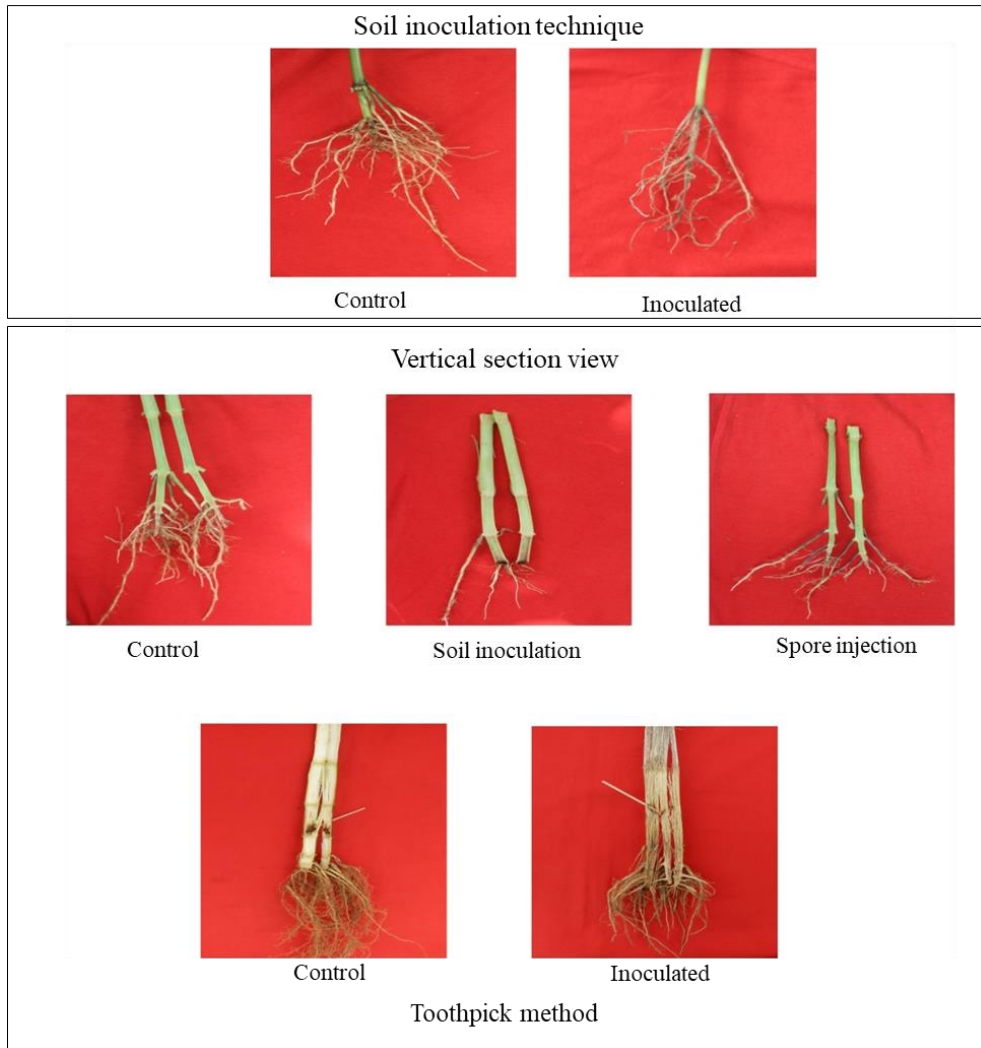


Fig 6: Detailed view of Symptoms in different inoculation techniques

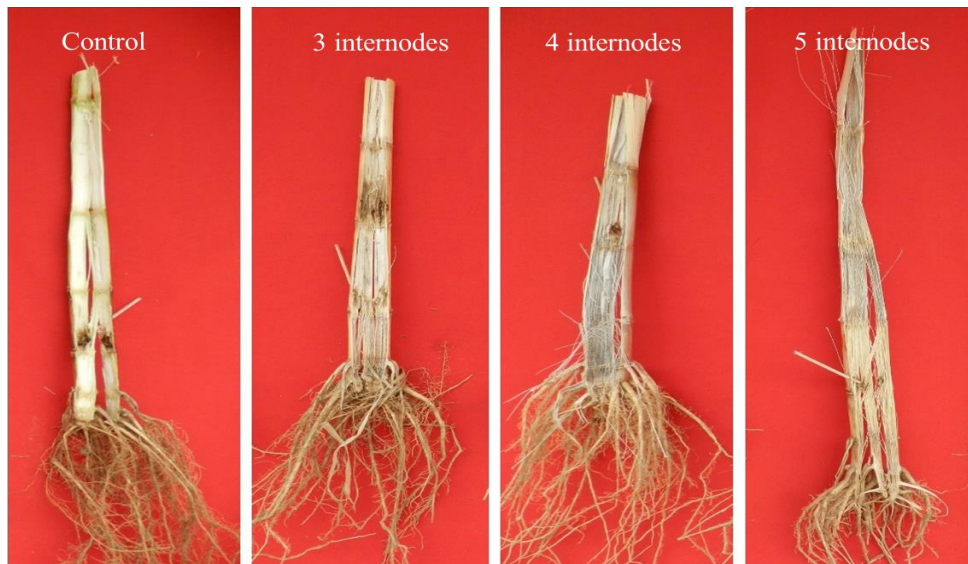


Fig 7: Range of vascular disintegration in toothpick inoculated stalks

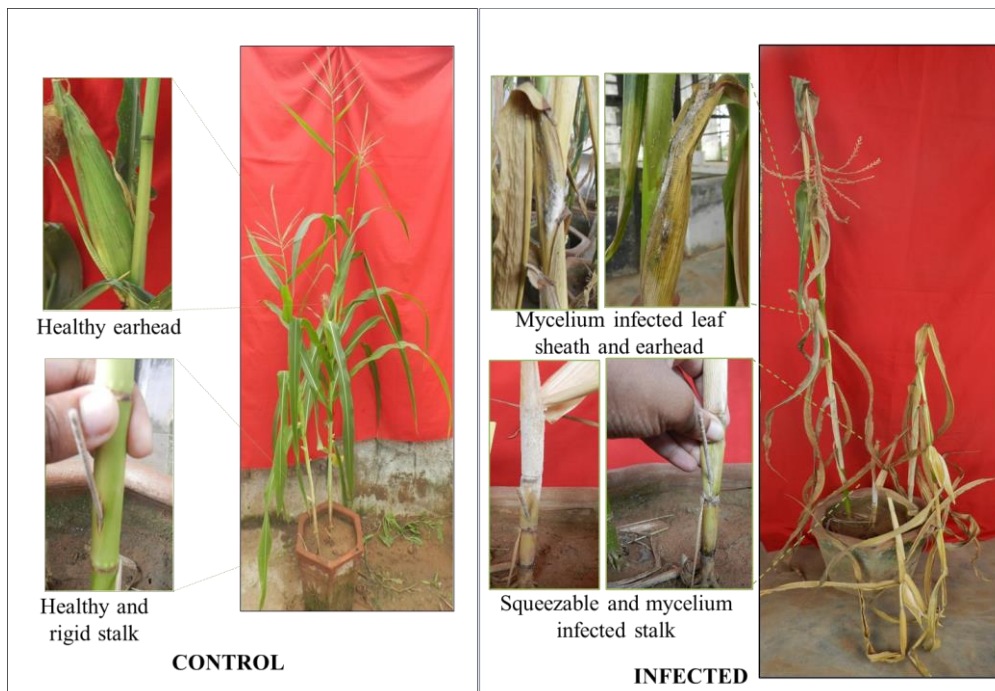


Fig 8: Symptoms in toothpick method of inoculation

Efficacy of different bio-agents against *F. verticillioides* in vitro

Growth inhibition exerted by bio-control agents ranged from 43.70 to 74.81 percent (Table 2). Isolate of *T. harzianum* (Sardarkrushinagar) exhibited highest growth inhibition of 74.81 percent followed by *T. harzianum* (Junagadh) with 70.74 percent. Subsequently, growth inhibition of 64.07 percent was displayed by *T. viride* (Sardarkrushinagar). Growth inhibition by *T. viride* (Junagadh) and *T. viride* (Navasari) were reported to be 59.62 and 51.48 percent. Isolates of *T. harzianum* exhibited faster growth, higher sporulation and overgrowth on the radial colonies of *F. verticillioides*. Drying up of colonies from the border of two fungi was observed in *T. harzianum* co-inoculated medium. Sardarkrushinagar isolates of *T. harzianum* and *T. viride* demonstrated higher growth inhibition than other isolates. Mean inhibition by bacterial antagonists was comparatively lower than *Trichoderma* sp. However, *Bacillus subtilis* recorded an effective inhibition percent of 61.85 which was higher than *T.*

viride isolates of Navasari and Junagadh. Although, reduction in the colony size of the pathogen to 43.70 percent with the presence of an inhibition zone was expressed by *P. fluorescens*. Relatively higher inhibition in *B. subtilis* might be due to its extensive growth. Figueroa-Lopez *et al.*, (2016)^[27] mentioned bio-control potential of *B. subtilis* over *P. fluorescens* against *F. verticillioides*, by the production of certain antibiotics. Santos *et al.*, (2017)^[28] demonstrated the suppression of *F. verticillioides* on co-inoculation with *Trichoderma* sp. by mycoparasitism, competition and antibiosis. Faster growth rate of *T. harzianum* (20.03 mm/day) compared to *T. viride* (16.62 mm/day) at 30°C temperature in PDA was attributed to its relatively higher inhibition potential (Sinha *et al.*, 2018)^[29]. Hernández-Castillo *et al.*, (2020)^[30] reported the superior inhibition of *Fusarium* sp. by *Trichoderma* sp. (54.8 to 62.4%) in comparison to *Bacillus* sp (44.5 to 36.9%). Therefore, results obtained in present investigation also corroborate with the findings of early researchers.

Table 2: Growth inhibition of bio-agents against *F. verticillioides* in vitro

Sr. No.	Name of the bio-agent	Growth inhibition (%)
1	<i>Trichoderma harzianum</i> (Sardarkrushinagar)	59.90 ^a (74.81) *
2	<i>Trichoderma harzianum</i> (Junagadh)	57.26 ^b (70.74)
3	<i>Trichoderma viride</i> (Junagadh)	50.55 ^d (59.62)
4	<i>Bacillus subtilis</i>	51.86 ^{cd} (61.86)
5	<i>Pseudomonas fluorescens</i>	41.38 ^f (43.70)
6	<i>Trichoderma viride</i> (Navasari)	45.85 ^e (51.48)
7	<i>Trichoderma viride</i> (Sardarkrushinagar)	53.18 ^c (64.07)
	Mean	51.43 (60.89)
	C.V.%	2.6

*Figures in parentheses are re-transformed values of arcsine transformed values

Treatment means with common letter(s) are not significant by Duncans' New Multiple Range Test at 5% level of significance.

Conclusion

Post flowering stalk rot of maize caused by *F. verticillioides* is a highly destructible disease with its systemic nature. Fungus also exhibited a versatile host - pathogen interaction with maize due to its necrotrophic action in the toothpick inoculation method, soil inhabitation and root infecting nature in soil inoculation technique as well symptomless endophytic nature in spore injection technique. Pith disintegration in toothpick method appeared to be augmented by the wound created to pierce the mycelium infected toothpick, susceptible basal stalk region due to less carbohydrate concentration. In nature, injury in the susceptible stalk region could be attributed by pests like corn borer infestation. Mycelium infected toothpick served an effective source of inoculum with adequate mycelium and spore load for infestation. Apparently, toothpick method of inoculation indicated ideal for the laboratory analysis post flowering stalk rot symptoms. Three-week-old plants with sufficient carbohydrate assimilation and absence of wound in spore injection method suppress the stalk rot due to the absence of stress. Inoculum in the soil possess the potential to cause disease with root rot symptoms which can eventually lead to stunted growth and death of the plant. Soil inhabiting *F. verticillioides* exhibited reduced infection on above ground parts of the plant due to its slow growing nature. However, several fungal and bacterial isolates of *Trichoderma*, *Bacillus* and *Pseudomonas* with its adaptation in soil and foliar environment demonstrated excellent potential in controlling *F. verticillioides*. Future thrust of the present study extends its scope for the analysis of *F. verticillioides* in the production of degradative enzymes and its utility in the production of biofuel. Infection of the pathogen through inoculated soil can be evaluated further for its longevity in soil and thus recommend an ideal duration of crop rotation cycle.

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