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RESEARCH ARTICLE

INJECTION OF MAIZE (*ZEA MAYS*) WITH *FUSARIUM VERTICILLIOIDES* FOR OPTIMAL INFECTION

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ABSTRACT

The optimal spore concentration of *F. verticillioides* that will be just high enough to cause moderate rotting (infective dosage) in the tissues of maize (*Zea mays*) stem was investigated. Two inoculation methods viz., toothpick and injection methods were used for the inoculation. Four spore concentration levels of *F. verticillioides* were employed viz., 1 x 10^6; 5 x 10^5; 3 x 10^5 and 1 x 10^5 spore/ml. Maize seeds (DMR-LSRW) were planted in several pots in the screenhouse. Four weeks after planting, 5 sets each, of the potted plants were injected on the stems at the second internodes with the different concentration levels of *F. verticillioides* using both inoculation methods. Inoculation of sterile distilled water served as control. Experiment was conducted in three replications and experimental design was completely randomized design. The stems were later observed and rated for rot severity within the internodes using modified form of Hooker's scale. Results showed varying extent of rot formation within internodes of stems that received different treatment concentrations of *F. verticillioides*. Concentration levels of 1 x 10^6 and 5 x 10^5 spore/ml of *F. verticillioides*, for both injection and toothpick methods caused total rotting of inoculated internodes in most plants. Rotting was moderate at 3 x10^5 spore/ml concentration for both injection and toothpick methods in most plant stands. Concentration level of 1 x10^5spore/ml had a slightly higher rot formation using toothpick method than using injection method. Rot formation was better with toothpick method than with injection method. 3 x 10^5 spore/ml inoculum concentration of *F. verticillioides* could thus be suggested as the appropriate infective but non-lethal dosage that is just high enough to cause infection in tissues of maize stem without killing the entire stem. Toothpick method could also be said to be better than injection method in the inoculation of maize (*Zea mays*) stem with *F. verticillioides*.

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INTRODUCTION

Fusarium verticillioides is documented to pose a severe threat to human and animal health due to its potent mycotoxigenic and carcinogenic characteristics (Marasas, 1988; Sobowale *et al*., 2005, Marin *et al*., 2013 Jacobsen *et al*., 2014, Yu *et al*., 2017, Blacutt *et al*., 2018, Madege *et al*., 2018). It produces a number of mycotoxins including moniliformins, zearalenones and fumonisins, which have been implicated in animal and human disorders including oesophageal cancers, infertility and kidney disorders (Amoah, 1994; Julian *et al*., 1995; Thiel *et al*., 1991; Munkvold and Desjardins, 1997, Lanubile *et al*., 2017, Silva *et al*., 2017). The pathogen is known to infect maize worldwide, posing one of the greatest threat of fumonisin production (Gelderblom *et al*., 1988; Thiel *et al*., 1991; Mao *et al*., 1997; Munkvold and Desjardins, 1997, Cao *et al*., 2014, Miguel *et al*., 2015, Kamala *et al*., 2015, Kamala *et al*., 2016, Janse van Rensburg *et al*., 2016). The pathogen is also known as able to produce fusaric acid, a toxin that has been implicated in reproductive and birth defects in rats and as mutagenic in plant tissue cultures (Nguen-Khong-Min and Smirnov, 1992; Porter *et al*., 1995). The pathogen has been documented to be a major cause of stalk rot of maize (Christensen and Wilcoxson 1966; Drepper and Renfro 1990, Lanubile *et al*., 2017, Gai *et al*., 2018, Mourice et al., 2018, Czembor *et al*., 2019). There are several methods for inoculation of maize stalks with *F. verticillioides*, some of which are shooting a BB pellet into the stem, the drill/toothpick method, nail punch/sponge method, insertion of infested toothpicks (i.e. punching straight with the infested toothpicks), injection of a spore suspension, wheat kernels, agar disks and insertion of infested pipe cleaners, insertion of infested toothpicks, amongst others (Cappellini, 1959; Drepper and Renfro, 1990; Munkvold and Carlton 1996). Different inoculation methods had been reported to be different in efficiency as regards inoculum delivery (Anonymous, 1986; Fajemisin, 1982). Varying severities in stalk rot caused by artificial inoculation of *F. verticillioides* has also been linked with different inoculation methods (Koehler, 1960; Drepper and Renfro1990). However in artificial inoculation, the CIMMYT's standard infective dosage of the pathogen is documented to be about 1 x 10^6 to 1 x 10^7 spore/ml (Drepper and Renfro, 1990). The aim of the study therefore was to determine the spore concentration of *F. verticillioides,* irrespective of inoculation method, that will be just high enough to cause moderate rotting (40-50% of the inoculated

internode) in the tissues of maize (*Zea mays*) stem, but non-lethal to the stem.

MATERIALS AND METHODS

Four different spore concentration levels of *F. verticillioides* were employed viz. 1 x 10^6; 5 x 10^5; 3 x 10^5 and 1 x 10^5 spore/ml. Two methods of inoculation were employed i.e. toothpick and injection method.

Injection method

The injection method of Drepper and Renfro (1990) was employed, but here rather than punching first with the nail before injecting the spore suspension, the punching was made straight with the injecting needle. This was done in order to avoid wounding as much as possible; also rather than using the 2 ml spore suspension, 1 ml spore suspension was used, the wound size being too small to contain the former spore suspension. Maize seeds (DMR-LSRW) were planted in seventy-five pots (four seeds per pot, later thinned down to two) and watering was done on a regular basis. Four weeks after planting, 5 sets of potted plants were injected on the stems at the second internodes (i.e. the first elongated internode) with 1ml of 1 x 10^6 spore/ml concentration of *F verticillioides* using sterile syringe. This was repeated for 5x10^5, 3x10^5 and 1 x 10^5 spore/ml concentration of *F verticillioides*. For control, another set of 5 potted plants were injected at the same position with equal volume (1ml) of sterile water, making 25 potted plants with each pot having two plant stands. There were three replications while experimental design was completely randomized design.

Toothpick method

The nail punch method consisting of 2.3-mm- diameter nail protruding 1.5 cm from a wooden handle (Drepper and Renfro 1990) were employed. Maize seeds (DMR-LSRW) were planted in pots as done in the injection method with three replications and experimental design being completely randomized design. Toothpicks were boiled in distilled water on a hot plate for 4-5 hours and then rinsed with sterile distilled water for several times to remove the ceresin in the toothpicks. Several toothpicks were put separately into five 250 ml conical flasks and 10 ml of melted prepared PDA were dispensed into each of them. The mouth of the conical flasks were plugged with cotton wool and then wrapped with aluminium foil before being autoclaved at 121^{0}C (1.1 kg/cm^2) for 15 minutes. After cooling, 3-5ml of 1 x 10^6, 5x10^5, 3x10^5 and 1 x 10^5 spore/ml concentration of *F. verticillioides* was dispensed into each of the conical flasks while 3-5ml sterile distilled water was dispensed into the other conical flask. Just before the agar gelled, all the flasks were shaken vigorously so that the toothpicks were incrusted with agar mixed with the inoculum suspension. The flasks were incubated, just to sporulation point on the toothpicks, at 28^{0}C for 5 days. After four weeks of planting in the screen house, sterilized nails on wooden handles were used to puncture the maize (*Zea mays*) stems at the second internodes, which in most cases were the first elongated internode as suggested by Drepper and Renfro (1990). Sporulating toothpicks from each conical flask were inserted into the holes on the stems of 5 sets each of the potted plants using sterile forceps. The toothpicks from the flask containing ordinary water were also inserted into the holes on the stems of another set of 5 potted plants using sterile forceps, this serving as control.

Data collection

Six weeks after inoculation (10 weeks after planting), the maize stems were observed for rot formation. This disease rating (incidence and severity) was done using a modified form of the Hooker's (1956) scale which indicates the percentage of infection in the inoculated internode. This was done by centrally splitting the stalks of the inoculated plants open lengthwise thereafter recording degree of rot formation according the scale below:

1 = 0-4% of internode rotten.
2 = 5-25% of internode rotten.
3 = 26-50% of internode rotten.
4 = 51- 75% of internode rotten.
5 = 76-100% of internode rotten.

Attention was given to the inoculation method (toothpick or injection) and the spore concentration level at which appropriate (40-50% of inoculated internode) rot formation occurred and was taken as the infective but non-lethal dose of *F. verticillioides*.

RESULTS

Table 1 shows summary of the mean percentage rot formation and the rot rating for the replicates in each concentration for the two inoculation methods (i.e. injection and toothpick methods). Plate 1 shows the varying extent of rot formation within the internodes of stems that received treatments of different concentrations of *F. verticillioides*. The 1 x 10^6 spore/ml of *F. verticillioides* (injection method), the 5 x 10^5 spore/ml (injection method), the 1 x 10^6 spore/ml (toothpick method) and the 5 x 10^5 spore/ml (toothpick method) caused total rotting of inoculated internodes in all the plant stands for all replications except in two or three plant stands in the case of 5 x 10^5 spore/ml (injection method) where the rotting was slight (Plate 1).

Table 1. Mean percentage rot formation and rating within inoculated internodes of maize (*Zea mays)* stems that received different treatments of *F. verticillioides.*

Spore concentration (spore/ml)	Mean rot formation (%)	Scale for rot formation
1 x 10^6 (Injection method)	91	5
5 x 10^5 (Injection method)	78	5
3 x 10^5 (Injection mehod)	43	3
1 x 10^5 (Injection method)	5	1
Water (Injection method)	0	1
1 x 10^6 (Toothpick method)	98	5
5 x 10^5 (Toothpick method)	88	5
3 x 10^5 (Toothpick method)	49	3
1 x 10^5 (Toothpick method)	10	2
Water	44	3

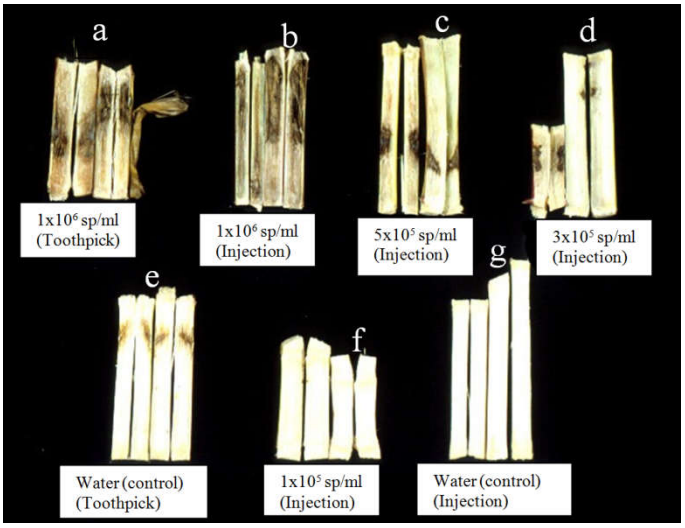

Plate 1. Rot formed within maize stem tissues by different spore concentrations of *F. verticillioides* and different inoculation methods (injection and toothpick). (a & b)-: total damage of the entire internodes by $1x10^6$ spore/ml; (c & d)-: moderate rot caused by $5x10^5$ and $3x10^5$ spore/ml; (e, f, & g)-: little or no rot caused by $1x10^5$ spore/ml and in the controls.

It was however moderate for 3 x 10^5 spore/ml for both injection and toothpick methods in almost all the plant stands for all the replicates. Some plant stands that received 3 x 10^5 spore/ml (injection method) had no rot formed within the inoculated internodes. The 1 x 10^5 spore/ml (injection method) and the control (injection method) caused little or no rot in the tissues of all the plant stands for all the replicates. Stems that received 1 x 10^5 spore/ml (toothpick method) however had a slightly higher rot formation than stems that received the same concentration in injection method (Table 1) in all the plant stands for all the replicates. The same was also true for the control of toothpick method compared with that of injection method.

DISCUSSION

The better results obtained with regards to rot formation in toothpick method compared to injection method underscores the preference of the former method over the latter in the discharge of inoculum of *F. verticillioides* and probably other fungi whatsoever into the tissues of maize (*Zea mays*) stem. The contrast between injection and toothpick methods as regards rot formation might be because the needle could not easily discharge its inoculum content into the stem tissues. This could be due to the higher likelihood of blockage of the eye of the needle by the stem tissues. It could also explain the absence of rot formation in some stems that received treatments of *F. verticillioides* by injection method. This thus agreed with the conclusion of Drepper and Renfro (1990) as regards the efficiency of nail punch method of inoculation, which aids injection of higher amount of inoculum into the stem tissues. Good results have been obtained with drill/toothpick method in different experiments in the introduction of microorganisms into plant tissues (Fajemisin, 1982; Anonymous, 1986; Sobowale *et al*., 2007). However, the slightly higher rot formation obtained in the control for toothpick method compared to the control for injection method could be due to secondary infections aided by wounding due to nail punch. Generally, corn plants in the field are known to become infected with different fungi through wounds caused by mechanical wounding, amongst other factors (Kucharek and Kommedahl, 1966; Christensen and Wilcoxson, 1966). This could be said to be a limitation in the use of drill/toothpick method, as efficient as it had proven to be. The mean rot formation obtained for concentrations of 1 x 10^6 spore/ml and 5 x 10^5 spore/ml of *F. verticillioides* for both injection and toothpick methods, for all plant stands in all the replicates, showed that the two concentration levels could be considered as lethal, causing total rotting of the inoculated internodes. However, the mean rot formation obtained for the concentration 3 x 10^5 spore/ml in both toothpick and injection methods showed that this concentration level could be regarded as a non-lethal but infective dosage of *F. verticillioides* in the maize stem tissue. Conclusively then, considering the moderate rot formation caused by this concentration (i.e. 3 x 10^5 spore/ml), it could be suggested as the appropriate dosage of inoculum of *F. verticillioides* that is just high enough to cause infection without killing the entire tissues of the stem. It could be also said that toothpick method is better than injection method in the inoculation of maize (*Zea mays*) stem with *F. verticillioides*.

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