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TIMING ON THE APPLICATION OF AFLASAFETZ01 ON REDUCTION OF TOXIGENIC ASPERGILLUS SPECIES ON MAIZE IN ZANZIBAR

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ABSTRACT

Management of aflatoxin contaminants in food had been a long-term problem until the development of biological control such as toxigenic *Aspergillus* isolates or Aflasafe, and in Tanzania named as Aflasafe TZ01, proved to competitively displace aflatoxin producers and effectively limit aflatoxin contamination in crops such as maize. Experiments were conducted at Zanzibar Agricultural Research Institute aimed at determining the proper time to apply AflasafeTZ01. There was a significant difference ($p<0.001$) on colonies forming unit (c.f.u/g) in log4 measured in soil for different times of AflasafeTz01 application. Treating soil at two weeks before planting significantly reduced *Aspergillus* colony counts c.f.u/g (0.755 c.f.u/g, 0.367 c.f.u/g) in both soil and maize grains, followed by during planting (1.311 c.f.u/g, 0.644 c.f.u/g). The control sample had higher colony counts (3.117 c.f.u/g, 0.522 c.f.u/g). Fungi species isolated from soil and maize grains included; *Fusarium* with highest (3.611 c.f.u/g, 1.567 c.f.u/g), followed by and *Aspergillus flavus* S-strain (2.2942 c.f.u/g, 0.606 c.f.u/g) and *A. spergillus flavus* L-strain (1.783 c.f.u/g,0.372 c.f.u/g). *Aspergillus parasticus* (1.061 c.f.u/g, 0.356 c.f.u/g), *Aspergillus niger* (1.022 c.f.u/g, 0.322 c.f.u/g) and *Penicillium* (0.939 c.f.u/g, 0.250 c.f.u/g). Weather, soil conditions and farming practices affect the ability of Aflasafe TZ01 to compete against fungal toxigenic strains. The study findings suggest that timing of AflasafeTz01 application must be coupled with suitable weather and soil moisture conditions to allow effective control of the toxigenic aspergillus species.

Keywords: Aflasafe Tz01, Timing of Aflasafe application, Colony Forming unit.

1. INTRODUCTION

There is an assorted accumulation of fungi that produce an extensive variety of mycotoxins. According to Kagot et al. (2019), a variety of toxigenic fungal species, including *Aspergillus flavus* and *Aspergillus parasiticus* produce these fungal toxins. According to Miklós *et al.* (2020), there are different types of known aflatoxins that are produced by such fungal species. Nevertheless, the most important ones are aflatoxin B1, aflatoxin B2, aflatoxin G1, and aflatoxin G2, which are commonly found in agricultural crops including maize. Aflatoxins B1 and B2 are produced by *A. flavus*, whereas aflatoxins G1 and G2 are produced by *A. parasiticus (*Ting *et al.*, 2020). Aflatoxins are known to cause cancer, liver diseases, growth retardation and death in humans and domestic animals (Negash, 2018). High absorptions of this toxin are fatal, median absorptions lead to chronic poisoning while incessant exposure to low absorption is what results in hepatic cancer (Negash, 2018).

Aspergillus flavus is the most infectious species in relation to toxin production (Kagot *et al.*, 2019). The fungus displays a high variance within its populations, generally as an outcome of

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sexual reproduction categorized by the formation of ascospore-bearing ascocarps inside sclerotia (Luis *et al.,* 2020). *Aspergillus flavus* is a group of related aflatoxin and non-aflatoxin producing strains, with toxin production varying depending on the isolate (Gell et al., 2020). Large (L) strain produces large sclerotia with an average diameter >400 µm, while Small (S) strain produces numerous small sclerotia with average diameter <400 µm, fewer conidia, and higher quantities of B aflatoxins than the L-strain morphotype (Massomo, 2020).

Efforts to control aflatoxin contamination involved the use of non-aflatoxigenic strains of *A. flavus* as biocontrol agents to outcompete aflatoxigenic strains (Molo *et al.,* 2019). This spectacle has been described as "competitive exclusion", where the non-aflatoxigenic strains effectively compete for space and nutrients, thus excluding their aflatoxigenic counterparts (Mamo *et al.,* 2017). According to Bandyopadhyay *et al.,* (2020) the development of biocontrol agents for commercial application was based on the ability of non-aflatoxigenic strains to reduce aflatoxin contamination in maize, which resulted into products known as Aflasafe. Aflasafe formulation was based on the fact that non-aflatoxigenic strains outcompetes aflatoxigenic strains, through control of germination, growth and colonization leading to their inability to infect crops and synthesize aflatoxin (Bandyopadhyay *et al.,* 2020). Competition between toxigenic and non-toxigenic *Aspergillus* depends on various factors such as weather and soil conditions (Savić *et al.,* 2020). According to Abbas *et al.* (2017) weather and soil factors determine the proper timing of application of such a nontoxigenic strain which results in the successful outcompeting of toxigenic *Aspergillus*. Therefore, the current study intended to generate knowledge on the correct timing of Aflasafe application on maize crops and determine the effectiveness of the ameliorant on fungus contamination.

2. MATERIALS AND METHODS

2.1 Study site and field experimental design

Early maturing (120 days) maize variety (Seedco 403), obtained from Bizred Agro-dealer was planted in April 2022 during rainy season and harvested on August 2022, the experiment was established at Zanzibar Agricultural Research Institute (ZARI) located at Kizimbani Unguja, 6^0 S, 39^0 E, altitude of 50m above sea level and average annual rainfall of 1177 mm, unreliable rainfall conditions observed during the growing season. The field size was about one acre, divided into four blocks of 30.5m length by 30.5m width resulting block area of 930.25 m^2, size experimental unit was 30.5m length by 4m width resulting experimental unit area of 122m^2. Distance from one block to another block was 2m and the distance from one experimental unit to another was 1m, experimental units were designed to be long and narrow to reduce error due to slope observed in the field. Plant spacing was 70cm by 30cm which resulted in a total of 581 plants per experimental unit, only one seed sown per hole. Experiment was conducted under Randomized Complete Block Design (RCBD) with six treatments and four replicates. Treatments were time of application of Aflasafe Tz01 which included; application of Aflasafe Tz01 at 2-weeks before planting (2WBP), during planting (DP), 2- weeks after planting (2WAP), 45-Days after planting (45DAP), 60Days after planting (60DAP) and the control of treatment where Aflasafe TZ01 was not applied.

2.2 Detection of Fungal species on soils before Aflasafe Tz01 application

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Soil samples were collected from the experimental plot before planting. Sub-samples were randomly taken across the field. Three strata were identified based on the slope of the field from which 5 sub-samples were pooled and mixed to represent one sample per strata that is 3-samples from the plot as per Wallenius *et al.* (2011). Fungi isolates were recovered from soil by the dilution plate technique on rose Bengal agar, 1g of soil sample was suspended in 10ml of sterile distilled water, and 100µl aliquots were plated and incubated for 7 days (Masso *et al.*, 2016). The number of fungi isolates was recorded and several colonies forming units (c.f.u/g) obtained. Isolates were assigned to their corresponding species depended on morphological characters, including colony color (Afzal *et al.*, 2013). According to Weledesemayat *et al., (2016),* yellow-brown, brown to black, or shades of green, mostly consisting of a dense felt of erect conidiophores were broadly classified as *A. flavus* species. Isolates that produced numerous small dark green sclerotia were identified as *A. flavus* S-strain, while those with yellow to the bright green colonies without sclerotia were identified as *A. flavus* L-strain (Thathana et al., 2017) Isolates that produce blue spores were Penicillum species, isolates with dark green colonies and rough conidia were considered *A. parasiticus* (Klich, 2002). Isolates that produced carbon black or very dark brown spores were considered to be A. *niger* (Weledesemayat *et al.*, 2016).

Figure 1. Materials and procedures for fungi recovering from maize grains

2.3 Detection of Fungal species on maize at harvesting

Maize samples were collected from standing crops (120 days old) just before harvest at 20 percent moisture content. Samples were collected along 2 meters transect, at each sample experimental unit,10 cobs of maize were then pooled and quartered to obtain one composite sample of about 1 kg for each treatment (Mahuku *et al*., 2019). One kilogram (kg) of composite maize sample was dried grounded to flour using a blender (Waring commercial company, model number 38Bl45, Volt 220-240 and 750 W). 250g of maize flour was collected from each 1kg of composite sample then 10g of maize flour mixed with 100mls of sterile distilled water and serially diluted to 10^{-2} and 100µl aliquots plated to rose Bengal agar (Figure 1) and incubated for 7 days as per Cotty (1994). The number of fungi isolates observed was recorded. Colonies were transferred onto Potato dextrose agar and incubated and isolate were assigned to their corresponding species according to observed morphological characters (Nyongesa *et al.*, 2015).

2.4 Data analysis

Analysis of variance and comparison of means for colonies forming units under soil and maize grains were performed using GenStat® Executable release 16 Statistical Analysis Software. The

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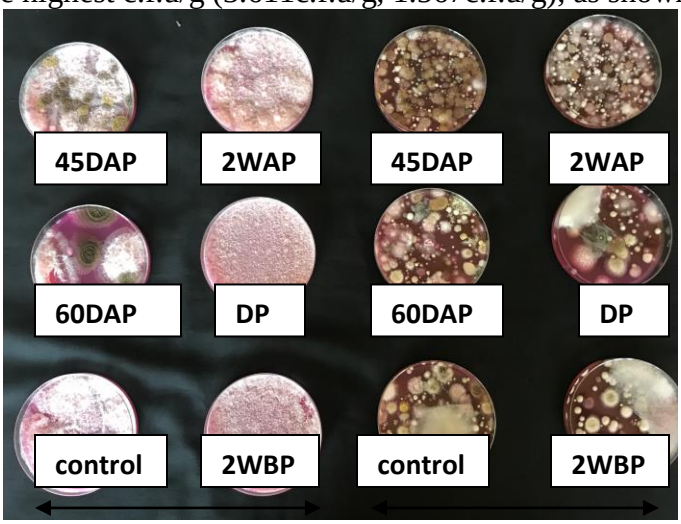
means were compared by the Tukey Honest Significance Difference (THSD) test (DMRT) at 5% probability. Analysis of variance (ANOVA) was used to determine the variability of c.f.u/g among allocated timing of the AflasafeTz01 application. A Regression (R^2) between soil colonies forming units and maize grain colonies forming units was conducted to check their relationships.

3. RESULTS

3.1 Effect of timing of Aflasafe Tz01 on colony-forming unit of fungi isolates

There was a significant difference ($p<0.001$) in colonies forming unit per gram of soil and maize grains for different timing of AflasafeTz01 application (Table 1). Application of AflasafeTz01 two weeks before planting (2WBP) reduced c.f.u/g significantly (0.756 c.f.u/g, 0.367 c.f.u/g) followed by the application at during planting (DP) (1.311 c.f.u/g, 0.644 c.f.u/g) control experiment had significantly high c.f.u/g (3.117 c.u.f/g, 0.522 c.f.u/g). Isolates recovered include *Penicillium* (0.939c.f.u/g, 0.255 c.f.u/g), *A. niger* (1.102 c.f.u/g, 0.322 c.f.u/g), and *A. parasticus* (1.102 c.f.u/g,0.356 c.f.u/g) were significantly had low c.f.u/g, followed by *A. flavus* L-strain (1.783c.f.u/g, 0.372c.f.u/g) and *A. flavus* S-strain (2.229c.f.u/g, 0.606c.f.u/g). *Fusarium* was abundant having the highest c.f.u/g (3.611c.f.u/g, 1.567c.f.u/g), as shown in figure 2.



Key; 2WAP = Two weeks After Planting, 2WBP = Two weeks Before Planting, 45DAP = Forty-five Days after Planting, 60DAP = Sixty Days after Plating, DP = during planting.

Figure 2. Recovered fungi isolate from maize and soil from different timing of Aflasafe.

3.2 Relationship between soil and maize grains colonies forming unit

There was a strong and a positive relationship between soil colonies forming unit and maize grains colonies forming unit measured and recorded from each treatment ($p<0.001$, $r=0.646$, $R2=0.417$) (Figure 3). The relationship implies with a large number of colonies forming unit in soil act as a large source of inoculum which will result an increased colonies forming unit on harvested maize grains.

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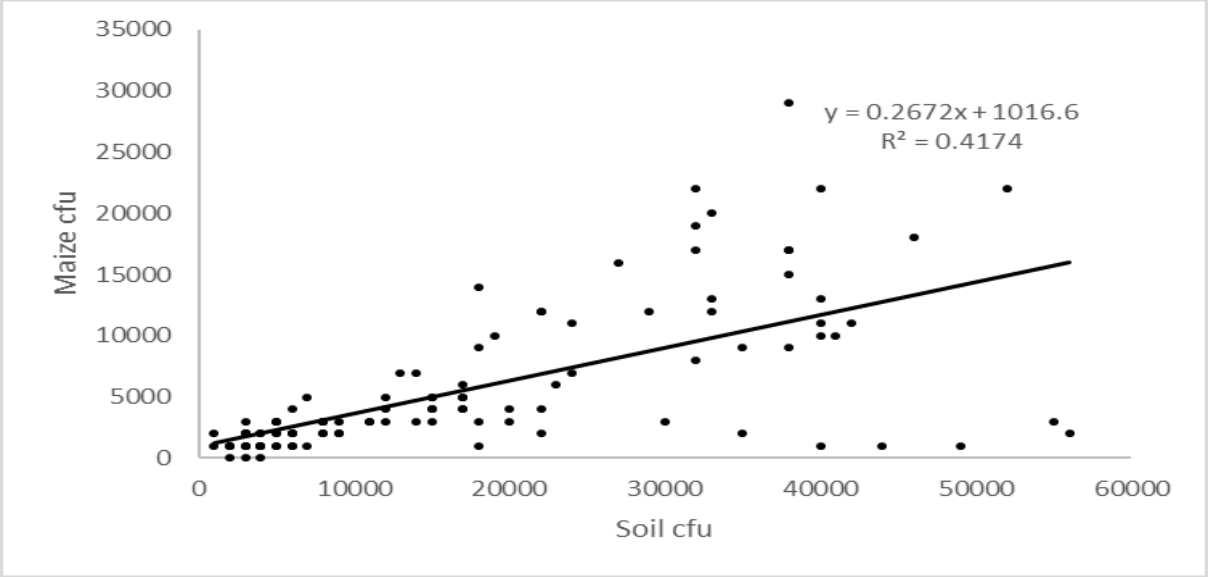

Figure 3. Regression relationship between c.f.u of soil and maize grain

Table 1. Effect of timing of application of AflasafeTz01on soil and maize grains c.f.u

Timing of AflasafeTz01 application	***Soil c.f.u/g***	***Maize c.f.u/g***
2WAP	16333**bc**	4833**ab**
2WBP	7556**a**	3667**a**
45DAP	20222**c**	9667**c**
60DAP	18722c	4889**ab**
DP	13111**b**	6444**b**
No application	31167**d**	5222**ab**
p-value	<0.001	<0.001

Key; 2WAP = Two weeks After Planting, 2WBP = Two weeks Before Planting, 45DAP = Forty-five Days After Planting, 60DAP = Sixty Days After Plating, DP = During planting. Means with the same letters along the same column are not significantly different ($p<0.05$)

Table 2. Recovered Isolates c.f.u in soil and maize grains

Recovered fungi isolates	***Soil c.f.u/g***	***Maize c.f.u/g***
Aspergillus flavus L-strain	17833**b**	3722**a**
Aspergillus flavus S-strain	22944**c**	6056**b**
Aspergillus niger	10222**a**	3222**a**
Aspergillus parasticus	10611**a**	3556**a**
Fusarium specie	36111**d**	15667**c**

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Penicillium specie	9389**a**	2500**a**
p-value	<0.001	<0.001

Key; Means with the same letters along the same column are not significantly different (p<0.05)

Recovered Isolates c.f.u/g in soil and maize grains (Figures 4 & 5) were A. flavus (L-strain) (1.7 c.f.u/g, 0.3 c.f.u/g), A. flavus *(S-strain*) (2.2 c.f.u/g, 0.6 c.f.u/g), A. niger (1.0 c.f.u/g, 0.3 c.f.u/g), A. parasticus (1.0 c.f.u/g, 0.3 c.f.u/g), Fusarium specie 3.6 c.f.u/g, 1.5 c.f.u/g) and Penicillium specie (0.9 c.f.u/g, 0.2 c.f.u/g)

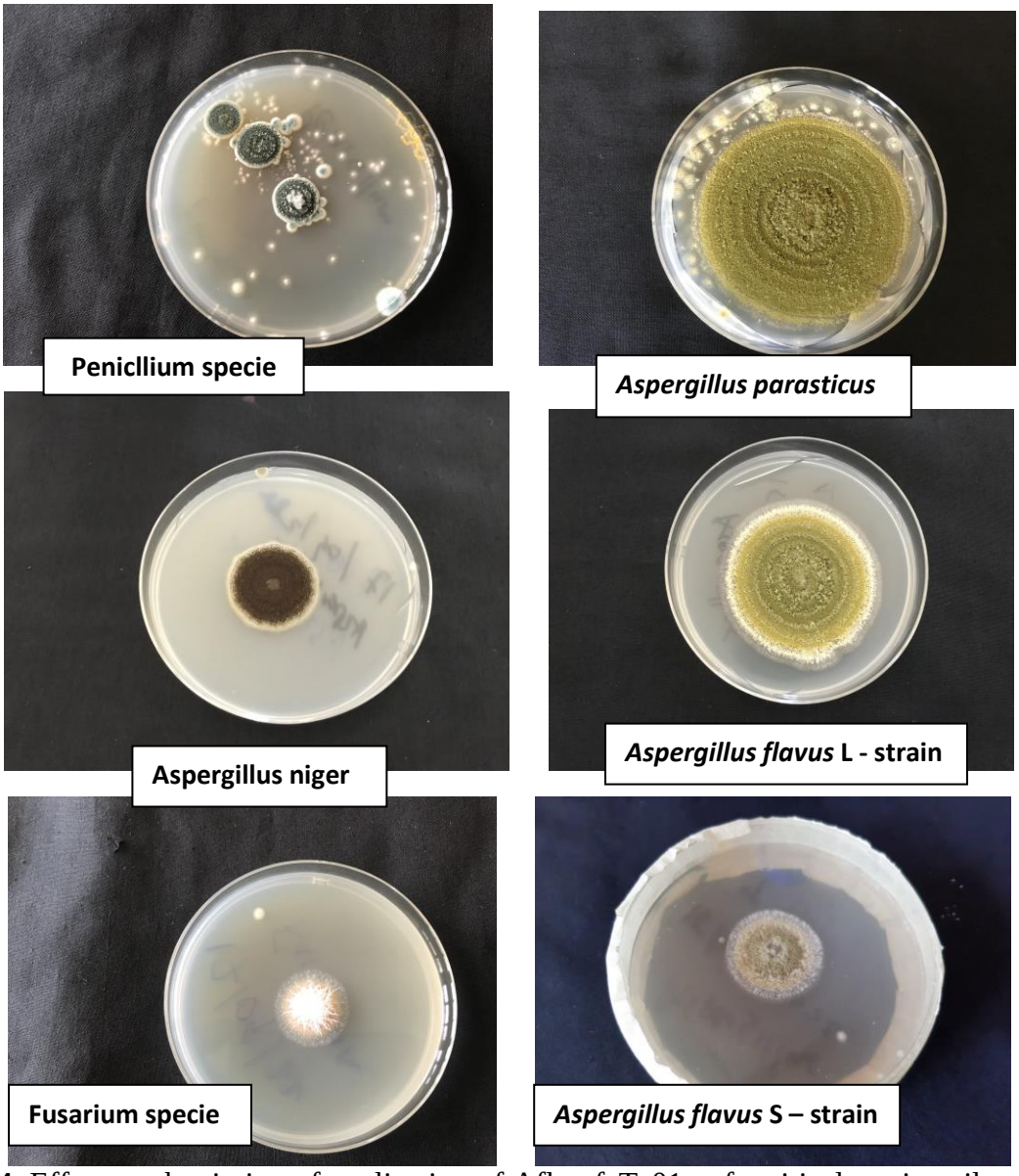

Figure 4. Effect on the timing of application of AflasafeTz01on fungi isolates in soil and maize grains c.f.u

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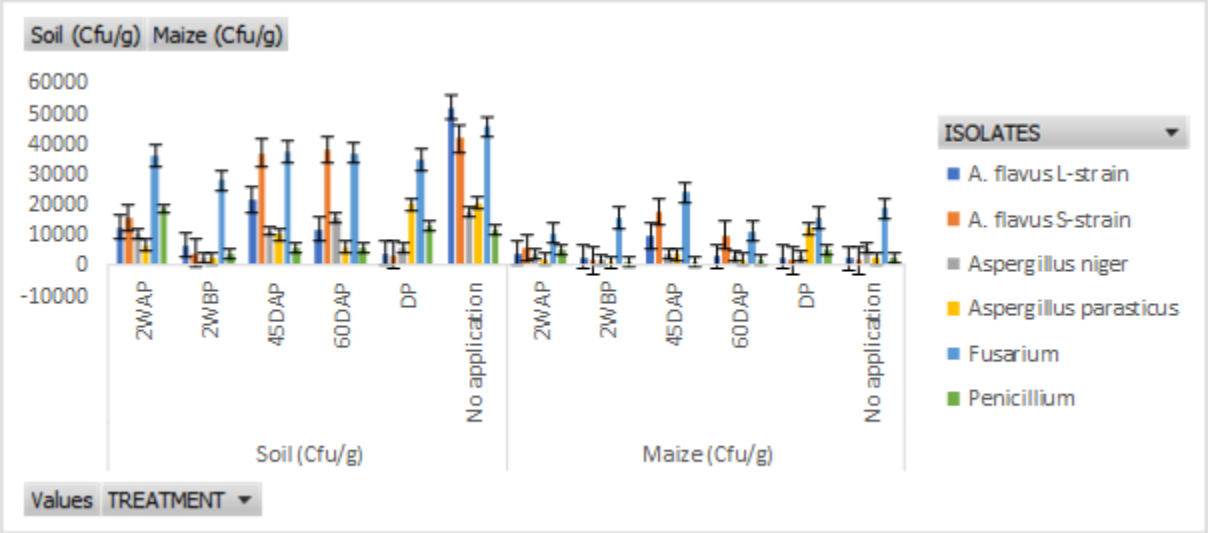

Figure 5: Relationship between timing of AflasafeTZ01 application and Aspergillus colonies
Key; 2WAP = Two weeks After Planting, 2WBP = Two weeks Before Planting, 45DAP = Forty-five Days After Planting, 60DAP = Sixty Days After Plating, DP = During planting.

4. DISCUSSION

The study shows that application of Afllasafe Tz01 as biocontrol for toxigenic *Aspergillus*, at 2 weeks before planting and during planting significantly reduces the ability of toxigenic fungi to infect maize crops when applied in the field, at the right crop phenological stage and under suitable weather conditions, Alfasafe TZ01 (atoxigenic biocontrol isolates) establish and displace aflatoxin producers resulting in lowered infection rates for toxigenic isolates (Senghor *et al.*, 2020). An evaluation of this type provides evidence of the greatest ability of Aflasafe Tz01 to create a founding population in the soil and then successfully move to the grains in the treated fields (Agbetiameh *et al.*, 2019). When a biocontrol product is applied at a time when there is no conducive environment for it to establish in the soil, it does not completely protect the crop from contamination by toxigenic *Aspergillus* as observed when it is applied 45 days and 60 days after planting.

The competitiveness of potential biocontrol agents is influenced by environmental factors, especially water availability and temperature (Abdel-Hadi *et al.*, 2012). It is essential, when considering the efficacy of biocontrol agents, to ensure that control can occur over the relevant environmental ranges in which *A. flavus* can colonize different ecological niches effectively (Mwakinyali et al., 2019). In addition, weather can play an unpredictable role in the success of the biocontrol process, if heavy rain occurs during the week following application it will wash away the biocontrol, certainly leading to poor results (Medina *et al.*, 2017). Also, if conditions dry rapidly after application the soil surface may crust, reducing water activity in the inoculum so that the fungus sporulates poorly or not at all. Again, this will fail in the process (Pitt *et al.*, 2015). Worse than that, if dry, hot weather persists for more than a few days, the exposed nontoxigenic spores on the substrate will die (Pitt, 2019). When subsequent rainfall occurs, raising the substrate water activity, existing toxigenic spores in the soil may invade, greatly increasing the toxigenic spore load in the soil (Pitt, 2019).

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5. CONCLUSION

Proper weather monitoring information should be provided to farmers to allow proper time to apply AflasafeTz01 thus influence effectiveness of biocontrol agent that successful changes the composition of the fungal population in favor of atoxigenic species adapted to the introduced environment.

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