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Soil Microbial Communities in Corn Fields Treated with Atoxigenic *Aspergillus flavus*

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Abstract: *Aspergillus flavus* refers to a diverse group of saprophytic soil fungi that includes strains producing aflatoxins (toxigenic strains) in the kernels of corn (*Zea mays* L.) and other crops, causing pre-harvest and post-harvest aflatoxin contamination. Some *A. flavus* strains are atoxigenic, and the introduction of such strains into the crop environment helps reduce toxigenic aflatoxin contamination. Corn growers in Texas have used the product FourSure™, which contains four atoxigenic strains of *A. flavus*; however, effects on soil microbial communities associated with these applications are unknown. We compared soil fungal and bacterial communities in corn fields treated with FourSure™ to nearby untreated (control) corn fields in Texas during the summer of 2019. Analysis of soil microbial community structure showed that total fatty acid methyl esters (FAMEs), fungal, and bacterial populations were not significantly different ($p = 0.31$) between the FourSure™-treated and control fields, yet corn fields located in the northern counties had more ($p < 0.05$) Gram—bacteria, actinobacteria, and total bacteria than fields in the southernmost county. The Gram—bacteria and actinobacteria were positively correlated ($p = 0.04$; r = 0.48 and 0.49, respectively) with soil water content. Similar fungal and bacterial abundances between FourSure™-treated and control fields indicated that atoxigenic *A. flavus* had no negative effects on soil microbial communities.

Keywords: aflatoxin-treated corn; *Aspergillus flavus*; atoxigenic aflatoxin; soil health; soil microbial community

1. Introduction

A saprophytic soil fungus, *Aspergillus flavus*, is a diverse species complex of soil fungi that includes strains producing aflatoxin (i.e., toxigenic strains) as well as strains that do not produce aflatoxin (i.e., atoxigenic strains). These fungi can cause aflatoxin contamination in the kernels of corn (*Zea mays* L.) [1], and seeds of cotton (*Gossypium hirsutum* L.) [2] and many other crops both before and after harvest [3]. Aflatoxin contamination on corn negatively affects the yield and profitability of corn [4,5]. *Aspergillus flavus* is an opportunistic pathogen and causes aspergillosis diseases in animals and humans, and is one of several species of *Aspergillus* that cause stonebrood in honey bee (*Apis mellifera* L.) [6]. The life cycle of *A. flavus* consists of two major stages. Sclerotia germinate and form new conidial inoculum in a short period when they are exposed to the soil surface, usually in spring. New inoculum is vectored by insects or wind, then colonizes and infects plant tissues, including grains and seeds [5].

Measures to control the toxigenic strains of *A. flavus* consist of using biological agents such as atoxigenic *A. flavus* strains, modification of cultural practices, and development of host-plant resistance. The use of atoxigenic strains of *A. flavus* is a widely used biocontrol method to reduce the contamination of toxigenic aflatoxin in corn kernels [7]. Atoxigenic *A. flavus* strains were able to alter and displace toxigenic strains after several years of application [8,9]. It is expected that a new product (FourSure™;

Texas Corn Producers Board, Lubbock, TX, USA), which contains four strains of atoxigenic *A. flavus*, would provide control of toxigenic *A. flavus* after several years of application [9]. The stage of corn recommended for FourSure™ application is between the seventh leaf stage and tasseling [10].

Changes in soil microbial communities, including fluctuations in types of fungi and bacteria, can affect soil processes as their enzymes mediate organic matter transformations and biogeochemical processes [11]. Soil fungi constitute a major proportion of the soil microbial biomass and play key roles in C sequestration, organic matter formation, and nutrient cycling. Soil organic matter (SOM) is the single most important soil health indicator [12], but SOM formation is a slow process and its measurement does not provide information about short-term changes in the soil. Instead of SOM, fatty acid methyl ester (FAME) profiling gives a relative abundance of bacteria and fungi, which could provide information regarding short-term changes in soil biogeochemical processes. Characterizing soil microbial communities via the ester-linked fatty acid methyl ester (EL-FAME) method provides an early indicator of short-term changes in fungal and bacterial groups, and the different functions they provide [13].

No adverse effects are anticipated for nontarget organisms associated with the application of FourSure™ [10]. However, a recent study in Texas corn fields [14] showed a tendency of lower abundances of soil-nesting bees in FourSure™-treated corn fields compared to control, suggesting a need to further investigate nontarget impacts as a result of using atoxigenic *A. flavus*. No literature was found reporting the effect of atoxigenic *A. flavus* on soil microbial communities, therefore, studies are needed to address the impacts of atoxigenic *A. flavus* on nontarget organisms. Our objective was to compare the soil microbial communities between corn fields treated with atoxigenic *A. flavus* and control fields in an assessment of the effects of this biocontrol agent.

2. Materials and Methods

2.1. Description of Field Sites

The research was conducted in corn fields treated with atoxigenic *A. flavus* (applied every year since 2013) and nearby control fields in three counties from north to south Texas (Figure 1). The area of interest representing significant corn-producing zones of Texas included the Blackland Prairie and Cross Timbers ecoregions to the Coastal Prairies ecoregion in southern Texas. Ellis County (32°36′ N, 96°58′ W) near Waxahachie, TX, and Grayson County (33°33′ N, 96°30′ W) near Sherman, TX were the study sites in northern Texas. In the southern site, research was conducted in San Patricio County (28°07′ N, 97°49′ W) near Sinton, TX. In San Patricio County, corn was planted on 14 and 21 February 2019, depending on the cooperating growers, and FourSure™ was applied on 22 April 2019. In Ellis County, corn planting and FourSure™ applications were performed on 8 March and 19 May 2019, respectively. Corn was planted on 22 March 2019, and FourSure™ was applied on 6 June 2019 in Grayson County. FourSure™ application was performed using an all-terrain vehicle-mounted spreader at the rate of 11.3 kg h^{-1} in all three counties. Further details about the research sites and management practices on corn fields in each county are described elsewhere [14].

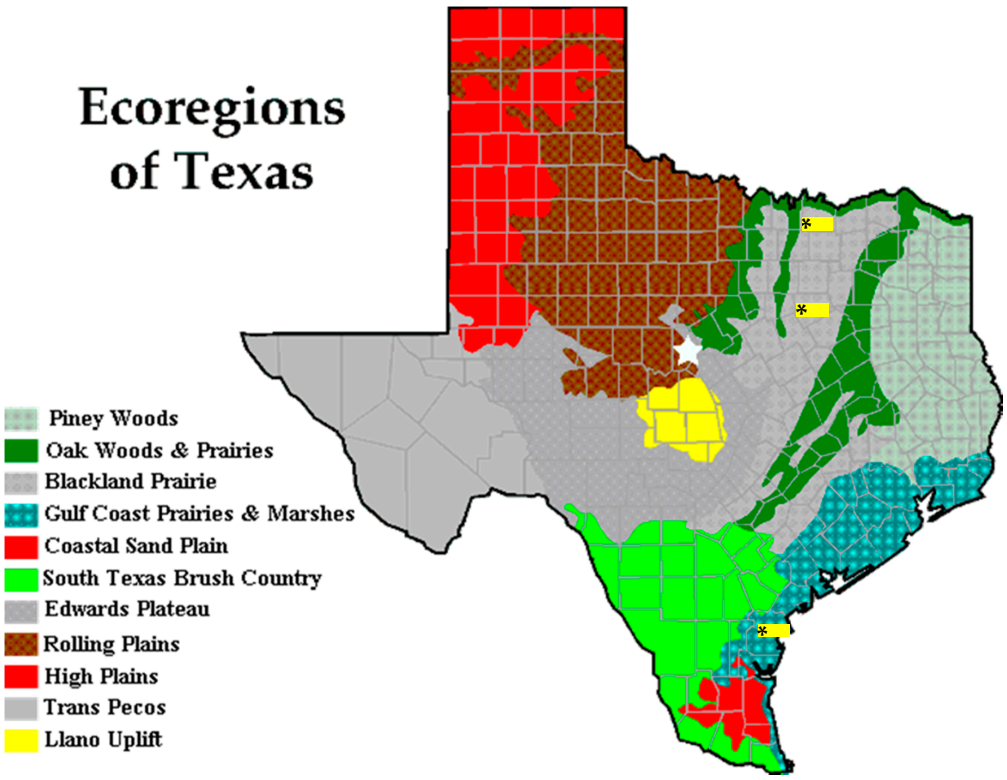

Figure 1. USEPA level three ecoregions of Texas with counties delineated. Asterisk (*) mark shows the counties sampled for soil microbial community analysis (Source: https://texasbeyondhistory.net/bowie/images/ecoregions-tpwd.html).

2.2. Environmental Parameters

A summary of temperature and rainfall data for the sampling sites is described by Bhandari et al. [14]. In brief, minimum and maximum temperatures on the sampling date (21 May) in San Patricio County were 26.7 and 34.4 °C (mean 30.6 °C). Mean minimum and maximum temperatures for the previous week of sampling in San Patricio were 23.8 and 31.3 °C (overall mean 27.6 °C). Similarly, 16.1 °C and 26.1 °C were the minimum and maximum temperatures on the sampling date (11 June) in Ellis County (mean 21.1 °C). Minimum and maximum temperatures for the previous week of sampling in Ellis County were 20.5 and 29.8 °C (overall mean 25.2 °C). In Grayson County, 21.1 °C and 31.4 °C were the minimum and maximum temperatures on the sampling date (20 June) with a mean temperature of 26.3 °C. Weekly minimum and maximum temperatures for the previous week were 20.7 °C and 30.9 °C (overall mean 25.8 °C).

No rainfall occurred during the week prior to sampling in San Patricio County. In Ellis County, the total rainfall in the week prior to the sampling date (11 June) was 34 mm with rainfall events of 0.3 mm, 33.4 mm, and 0.3 mm occurred on 5 June, 6 June, and 9 June, respectively. Similarly, total rainfall in the week prior to the sampling date (21 June) in Grayson County was 34.8 mm, with rainfall events of 14.5 mm, 16.0 mm, and 4.3 mm occurring on 16 June, 17 June, and 19 June, respectively.

2.3. Soil Sampling

In treated and control fields, soil samples were collected at 0–10 cm depth in the late spring of 2019 in San Patricio, Ellis, and Grayson counties. In San Patricio County, samplings were performed on 22 May of 2019. Similarly, in Ellis and Grayson counties, soil samples were collected on 12 June and 21 June of 2019. In each county, soil samples were collected from three treated and three nearby control corn fields. A total of nine treated and nine control corn fields were sampled in three counties.

From each treated and control field replicate in each county, soil samples were collected from five sites within each field. Soil sampling locations were separated by at least 30 m. The five soil samples collected from each field were composited prior to analysis. Composite soil samples were immediately transferred into air-tight storage bags and placed on ice for transport to the laboratory. Within 48 h of soil sampling, composite samples were sieved using a 4.75 mm sieve and stored at 4 °C prior to analysis.

2.4. Soil Water Content Determination and EL-FAME Analysis for Soil Microbial Community Structure

Gravimetric soil water content was determined by drying for 48 h at 105 °C. The EL-FAME method as described by Schutter and Dick [15] was used to characterize soil microbial community structure (details in Bhandari et al. [16]). During methylation, 3.5-g of field-moist soil in 20 × 150-mm screw-cap test tubes with 15 mL of 0.2 M KOH in methanol was heated in a 37 °C water bath for 60 min. Then test tubes were cooled for 5 min. For neutralization, each test tube was vortexed for 5 s after 3 mL of 1.0 M acetic acid was added and then cooled at ambient temperature for 5 min to complete the reaction. During extraction, 3 mL of 100% hexane was added to each tube followed by centrifugation at 2200 rpm for 8 min. The organic phase from the top of the sample was dried at 37 °C for 20 min, standard hexane was added, and again dried under N_2. FAME fractions were transferred to gas chromatography vials.

A 6890 GC series II (Hewlett Packard, Wilmington, DE, USA) was used for GC analysis. The carrier gas used was ultrahigh purity H_2 [17]. The temperature was increased from 170 to 250 °C at 5 °C min^{-1}. Retention times and peak areas were compared with components of MIDI standards (Microbial ID, Inc., Newark, DE, USA) for fatty acid identification and quantification. The relative peak areas (percentage) of FAMEs were determined by using the Aerobe method of the MIDI system [18]. The conversion of peak data to molar percentages was according to Liebig et al. [18]. The absolute amounts of FAMEs were calculated according to a 19:0 internal standard as described by Zelles [19], which in turn were used to calculate mole percent. The FAME nomenclature includes the number of C atoms followed by a colon, the number of double bonds, and the position of the first double bond from the methyl ($\acute{\omega}$) end. Notation such as a suffix c was used to indicate cis isomers. Similarly, prefixes a and i indicated anteiso- and isobranched FAMEs, respectively. Methyl, hydroxy, and cyclopropane groups were indicated by Me, OH, and cy, respectively.

The protozoan marker was indicated by 20:4ω6c. The marker 16:1ω5c described arbuscular mycorrhizal fungi (AMF). Saprophytic fungi were indicated by 18:3ω6c, 18:4ω3c, 18:1ω9c, 18:2ω6c, 18:1ω7c, 18:1ω5c, and i18:0 [19–21]. Markers such as a14:0, i14:0, a15:0, i15:0, a16:0, i16:0, a17:0, i17:0, and i19:0 described Gram+ bacteria and 17:0 3OH, cy17:0, and cy19:0 indicated Gram− [22]. The FAME markers 10Me 16:0, 10Me 17:0, 10Me 18:0, 10Me 17:1ω7c, 10Me 18:1ω7c, and 10Me 19:1ω7c described the actinobacteria. Fungal, bacterial, and protozoa FAMEs were added to calculate the total FAME.

2.5. Statistical Analyses

Analysis of variance was used to compare the means of two treatments and three replications for within-county tests. To test for the main effects of treatment and treatment × county interactions, counties were combined using Proc Mixed in SAS 9.4 [23]. For the combined analysis, a split-plot design was used with treatments nested within counties. FourSure™-treated and nearby control fields were set as fixed effects, and replicate and county were set as random effects in the ANOVA. Differences were considered significant at $p \leq 0.05$. Pearson correlation coefficients (n = 18) were calculated across three counties to show the relationships between soil water content and total FAMEs, fungal, and bacterial groups.

3. Results

3.1. Soil Water Content, Protozoal Abundances, and Total FAMEs

FourSure™-treated and control corn fields in San Patricio, Ellis, and Grayson counties and all counties combined were not significantly different ($p \geq 0.09$) in soil water content (Table 1). There was a county effect in soil moisture content in which Ellis and Grayson counties had significantly greater ($p < 0.001$) soil water content compared to San Patricio County, but the treatment × county interactions were not significant ($p = 0.53$). The FAME marker for protozoa was not significantly different between the treatments both within and across the counties. These trends were similar for total fatty acid methyl esters (FAMEs). There was no treatment x county effect for soil water content, protozoa, and total FAME ($p \geq 0.27$).

Table 1. Soil water content, protozoa, and total fatty acid methyl esters (FAMEs) averaged over three field replicates in each county.

Variables	Treatment	County			Mean
		San Patricio	Ellis	Grayson	
Soil water (g g^{-1})	FourSure™	0.17	0.24	0.25	0.22
	Control	0.13	0.25	0.23	0.20
	Treatment effect	$p = 0.09$	$p = 0.75$	$p = 0.66$	$p = 0.46$
	County effect		$p < 0.001$		
Protozoa (nmol g^{-1})	FourSure™	1.1	1.7	1.6	1.5
	Control	1.4	1.3	1.3	1.3
	Treatment effect	$p = 0.48$	$p = 0.10$	$p = 0.53$	$p = 0.46$
	County effect		$p = 0.59$		
Total FAME (nmol g^{-1})	FourSure™	116	192	220	176
	Control	155	161	192	169
	Treatment effect	$p = 0.55$	$p = 0.31$	$p = 0.64$	$p = 0.82$
	County effect		$p = 0.17$		

Protozoa is indicated by 20:4ω6c.

3.2. Fungal and Bacterial Abundances, and Their Ratios

The arbuscular mycorrhizal fungi (AMF) were not significantly different ($p \geq 0.13$) between FourSure™-treated and control fields within the county and means across the counties (Table 2). There was no county effect for AMF. A similar trend was found for saprophytic fungi and total fungi. Treatments did not differ for bacterial numbers, but there was a county effect ($p \leq 0.047$) for Gram− bacteria, actinobacteria, and total bacteria, such that these groups were more abundant in Ellis and Grayson counties than in San Patricio County. The trend was similar for the fungi–bacteria ratio. The total fungal population abundances in both FourSure™-treated and control corn fields in San Patricio County exceeded the abundance of its bacterial populations, resulting in a greater than 1.0 fungi–bacteria ratio, whereas the ratio was less than 1.0 in Ellis and Grayson counties. There were no treatment × county interactions for all parameters ($p \geq 0.20$).

Soil water content was not significantly correlated ($p = 0.20$) with total FAMEs nor with the different fungal groups ($p = 0.18$–0.38) (Table 3). Soil water content was not significantly correlated ($p = 0.09$) with Gram+ bacteria, but was positively correlated ($p \leq 0.05$) with Gram− bacteria, actinobacteria, and total bacteria.

Table 2. Fungal and bacterial abundances (nmol g^{-1}), and their ratios (average number over three replicated fields) between FourSure™-treated and control corn fields in three counties in Texas.

Variables	Treatment	County			Mean
		San Patricio	Ellis	Grayson	
		(nmol g^{-1})			
AMF fungi	FourSure™	3.5	7.4	9.6	6.8
	Control	4.4	5.5	3.6	4.5
	Treatment effect	$p = 0.50$	$p = 0.59$	$p = 0.23$	$p = 0.13$
	County effect		$p = 0.30$		
Saprophytic fungi	FourSure™	29.6	44.1	50.7	41.5
	Control	41.7	35.5	39.3	38.8
	Treatment effect	$p = 0.58$	$p = 0.42$	$p = 0.45$	$p = 0.73$
	County effect		$p = 0.61$		
Total fungi	FourSure™	33.2	51.5	60.3	48.3
	Control	46.1	40.9	42.8	43.3
	Treatment effect	$p = 0.57$	$p = 0.45$	$p = 0.38$	$p = 0.57$
	County effect		$p = 0.54$		
Gram+ bacteria	FourSure™	15.1	27.9	29.2	24.1
	Control	20.9	24.9	29.2	25.0
	Treatment effect	$p = 0.48$	$p = 0.40$	$p = 0.99$	$p = 0.81$
	County effect		$p = 0.08$		
Gram− bacteria	FourSure™	6.5	10.2	12.8	9.8
	Control	6.7	8.6	9.5	8.3
	Treatment effect	$p = 0.82$	$p = 0.34$	$p = 0.17$	$p = 0.14$
	County effect		$p < 0.01$		
Actinobacteria	FourSure™	11.3	21.0	22.5	18.3
	Control	13.9	19.6	18.7	17.4
	Treatment effect	$p = 0.49$	$p = 0.69$	$p = 0.36$	$p = 0.72$
	County effect		$p = 0.047$		
Total bacteria	FourSure™	32.9	59.1	64.5	52.2
	Control	41.5	53.1	57.4	50.7
	Treatment effect	$p = 0.50$	$p = 0.49$	$p = 0.54$	$p = 0.84$
	County effect		$p = 0.047$		
Fungi–bacteria ratio	FourSure™	1.0	0.9	0.9	0.9
	Control	1.1	0.8	0.8	0.9
	Treatment effect	$p = 0.81$	$p = 0.46$	$p = 0.19$	$p = 0.28$
	County effect		$p = 0.05$		

Total fungi: AMF (16:1ω5c), saprophytic fungi (18:3ω6c, 18:4ω3c, 18:1ω9c, 18:2ω6c, 18:1ω7c, 18:1ω5c, i18:0); total bacteria: Gram+ (a14:0, i14:0, a15:0, i15:0, a16:0, i16:0, a17:0, i17:0, i19:0), Gram− (17:0 3OH, cy17:0, cy19:0) and actinomycetes (10Me 16:0, 10Me 17:0, 10Me 18:0, 10Me 17:1ω7c, 10Me 18:1ω7c, 10Me 19:1ω7c).

Table 3. Pearson correlation coefficients between soil water content and total FAMEs and selected soil microbial groups; n = 18.

	Total FAMEs †	AMF †	Saprophytic Fungi	Total Fungi	Gram+ Bacteria	Gram−Bacteria	Actino-Bacteria	Total Bacteria
Soil Water Content	0.32	0.33	0.22	0.25	0.41	0.48	0.49	0.46
	$p = 0.20$	$p = 0.18$	$p = 0.38$	$p = 0.31$	$p = 0.09$	$p = 0.04$	$p = 0.04$	$p = 0.05$

† FAME = fatty acid methyl ester; AMF = arbuscular mycorrhizal fungi.

4. Discussion

We documented soil microbial communities in corn fields treated with atoxigenic *Aspergillus flavus* and compared these to nearby control fields. While another recent study using the same corn fields reported a trend of lower abundances of soil-nesting native bees in *A. flavus*-treated corn fields than in control fields [14], we found similar abundances of soil microbial communities across *A. flavus*-treated and control fields, indicating that the soil microbial component was not affected by atoxigenic *A. flavus* (FourSure™) applications. More extensive multiyear and multilocation research could ascertain possible long-term effects of atoxigenic *A. flavus* on soil microbial communities. Interestingly, a similar trend of greater soil moisture and bacterial populations (particularly Gram−bacteria and actinobacteria) in Ellis and Grayson counties compared to San Patricio County suggests that greater abundances of bacteria in Ellis and Grayson counties in this study can be explained in part by greater soil moisture.

The reasons for differences in bacterial populations between San Patricio and Ellis/Grayson are not clear, but differences in soil water content may be associated with observed patterns in soil microbes. This pattern is supported by positive correlations between bacterial populations and soil water content (Table 3). There was no rainfall in San Patricio County corn fields, whereas three rain events occurred in Ellis and Grayson counties prior to sampling. In addition, the temperature in San Patricio County was higher than in Ellis and Grayson counties before and during the soil sampling. Thus, the soil water content in Ellis and Grayson counties was greater than in San Patricio County. The lower abundances of bacteria (particularly Gram− bacteria and actinobacteria) in San Patricio County than in Elis and Grayson counties likely occurred as a consequence of higher temperatures and lower precipitation, and thus lower soil moisture. This indicates that increased soil moisture has a stimulating effect on the soil bacterial community. These results are in line with a previous study in which greater soil moisture favored soil microbial communities in Texas pastures [16]. Less fluctuation in soil moisture in combination with greater available substrates in the soil stimulated growth and activity of soil microbial communities compared to greater fluctuation in soil moisture with lesser available substrates [17].

This study observed two trends related to soil microbial communities in atoxigenic *A. flavus*-treated corn fields and nearby control fields across north to south Texas. Similar abundances of soil microbial communities were found in atoxigenic *A. flavus* treated and nearby control corn fields within the county, suggesting that soil microbes were not affected by atoxigenic *A. flavus*. Greater abundances of specific soil bacterial communities, especially Gram− bacteria and actinobacteria, were recorded in the fields of northern Ellis and Grayson counties than those of southern San Patricio County, indicating that soil moisture had a stimulating effect on the soil bacterial community.

5. Conclusions

This is the first attempt to document soil microbial communities in corn fields treated with atoxigenic *Aspergillus flavus* in Texas. Consistently lower numbers of soil-nesting bees in corn fields treated with atoxigenic *A. flavus* at the same sites (although not significantly) [14] prompted this study to assess potential nontarget effects of atoxigenic *A. flavus* on the soil microbial component. This study clearly showed similar abundances of soil fungal and bacterial communities between FourSure™-treated and control corn fields. Differences in bacterial communities between south and north Texas corn fields remain unclear, but the results of a significant positive correlation between bacterial populations and soil moisture and consistent trends of greater soil bacterial communities in northern counties could be explained in part by higher soil moisture content. Further investigations of the soil bacterial communities from multiple sites with a range of soil moisture conditions would further elucidate relationships of soil moisture on soil bacterial communities in corn production fields. Nonetheless, this study found that soil microbial communities including fungal and bacterial populations were not affected by the application of atoxigenic *A. flavus* (FourSure™), suggesting that ecosystem services provided by soil microbes involved in nutrient transformations would not be mitigated by FourSure™ applications.

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Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

AMF	arbuscular mycorrhizal fungi
EPA	Environmental Protection Agency
FAME	fatty acid methyl ester

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