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ISOLATION AND PARTIAL PURIFICATION OF TOXINS FROM *EXSEROHILUM TURCICUM,* MAIZE LEAF BLIGHT PATHOGEN

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ABSTRACT

An experiment was conducted at Agricultural College, Bapatla during 2022-2023. Toxins were isolated from 12 maize *E. turcicum* isolates. The partially purified toxin from the isolate BPT-1 having a Rf value of 0.56 was obtained and tested for their role in phytotoxicity using detached leaf bioassay techniques like drop method and leaf injection method. In both methods, leaf bits were examined for symptoms development 48 h after incubation. Yellowing and necrosis of the leaf was observed at all the concentrations tested *i.e.,* 0.5, 4, 5%. In control leaf bit, injected with sterile distilled water no such yellowing or necrosis was observed. This confirmed the phytotoxicity of partially purified toxin obtained from the *E. turcicum* BPT-1 isolate in the present investigation. The partially purified toxin of the isolate BPT-1 was sent to the department of Microbiology, TNAU, Coimbatore for further characterization of toxic compounds using GC-MS analysis. The results from the GC-MS analysis indicated that, about 300 compounds were present in the BPT-1 isolate. Among them, eleven compounds were found associated with *E. turcicum* and about ten compounds were found to have plant pathogenic fungal origin but different from that of *E. turcicum.*

***Keywords*:** *Exserohilum turcicum,* Bioassay, GC-MS analysis, Partial Purification, Phytotoxicity, Toxins.

Introduction

Maize (*Zea mays* L.), a C_4 grass belonging to the family Poaceae, popularly known as "corn" is one of the most versatile emerging cash crops having wider adaptability under varied climatic conditions. Due to its highest genetic yield potential, globally maize is called the "Queen of cereals". It was reported that the present cultivable form of maize is the derivation from the mutation of wild form of pod maize, indigenous to the eastern slopes of Andus in South America which is thought to the place of its origin (Mangelsdorf, 1947).

Turcicum leaf blight of maize (*Zea mays*), also known as northern corn leaf blight caused by *Exserohilum turcicum,* is a widespread disease of maize, which can cause yield losses up to 70% (Yeshitila, 2003). Apart from yield loss, the disease causes qualitative changes in the seed resulting in decreased sugar content, germination capacity and severely infected plants are predisposed to stalk rot (Cardwell *et al*., 1997).

Bashan *et al*. (1996) reported that *E. turcicum* produces E.t toxin which is a phytotoxic peptide and can increase the number of appressoria and the ramification of germinating conidia both on host leaves and on artificial media. They claimed that this toxin plays an important role in infection of corn in northern corn leaf blight. Robeson and Strobel (1982) reported that the toxin produced by *E. turcicum* (syn. *Drechsera turcica*), monocerin, isolated from Johnson grass (*Sorghum halepense*) could inhibit seedling growth of

the Johnson grass, and cucumber to a lesser extent. It was also active against tissues of tomato and Canada thistle. Chauhan *et al.* (2008) studied the toxicity of the compounds in the culture filtrates of *E. turcicum*, which inhibited shoot and root growth, callus growth and reduced the chlorophyll content and cell viability of corn. Results showed that no germination occurred in non-autoclaved extracts at 100% (undiluted) and 50% concentrations, whereas germination was normal at all the concentrations of autoclaved toxic compounds. Zhao and Dong (2000) reported that 18 compounds were used to test their inactive reaction to HT-toxin produced by *E. turcicum*. The results indicated that mancozeb; one out of 18 compounds tested could inactivate the activity of HT toxin.

The main goal of this study is to isolate and partially purify the toxic compounds which are very harmful to maize plants and chromatographic techniques enable to study the toxic compounds produced by pathogen and its role in pathogenicity thus, helping in development of strategies to control the disease.

Materials and Methods

Extraction of Toxin(s) from Representative Isolates

Toxins were extracted from maize *E. turcicum* isolates as suggested by Bashan and Levy (1992) with slight modifications. The pure culture of each isolate was grown in Erlenmeyer conical flasks (250 ml capacity) containing 100 ml of potato dextrose broth, and were incubated at 25 °C ± 1 in an incubator illuminated with fluorescent light in a 16-hrs light and 8 hrs dark cycle. After 21 days of incubation, culture filtrates of each isolate was filtered through three layers of cheesecloth and was taken in to separatory-flasks. Equal amount of ethyl acetate was added and each filtrate was extracted three times by using separating funnel to ensure complete extraction of the samples in the supernatant layer. The samples were then taken into separate round bottom conical flasks. The ethyl acetate extract was concentrated by evaporation in vacuum evaporator at 45 ^{0}C until the toxin(s) containing samples reduced to 5% of the total volume.

Thin Layer Chromatography (TLC)

Toxins were separated from the extracts by thin layer chromatography (TLC). TLC plates were prepared by spreading slurry of silica gel (Silica gel G, 60-120 mesh, E. Merk, Germany) containing 10 per cent gypsum (as binder) in water coated on 5 x 20 cm glass plates, maintaining a uniform thickness of 0.25 mm using a TLC applicator. Prepared plates were then air dried and activated at 120 °C for two hours before use. Each sample solution was spotted on TLC plates with the help of capillary tubes. The plates were developed in benzene: acetone (95:5) solvent system and later dried in air and visualized by spraying ferric chloride solution (0.1%). Rf values of different spots of different samples were recorded as per following formula. The different Rf Value bands on silica gel were separated and put in separate conical flasks. Acetone was added to each flask in order to separate toxic compounds from silica gel.

$$\text{Rf value} = \frac{\textit{Distance travelled by solute on TLC plate}}{\textit{Distance travelled by solvent on TLC plate}}$$

The toxins were further tested for their phytotoxicity on the detached leaf bits of 4 cm using drop method and leaf injection method. The toxins were prepared at three different concentrations *i.e.*, 0.5% (low), 4% and 5% (High).

Characterization of Toxin(s) Using Detached Leaf Bioassay Technique (Drop Method)

Bioassay of different concentrations of toxin was done on detached leaves of 21 days old plants of susceptible maize hybrid Pioneer 3396, following the methods used by Vidyasekaran *et al.* (1986). The leaves were cut into 4-cm pieces. These leaf pieces were then kept inside sterile Petri plates lined with moistened blotting paper in order to maintain humidity. The toxin at different concentrations (0.5%, 4% and 5%) was prepared and a drop of the toxin was placed on the leaf pieces and allowed it for drying. The area was marked where the toxin was applied. Each concentration was replicated three times. The leaf pieces which are dropped with sterile distilled water served as control. The Petri dishes were incubated under favourable condition for disease development at 28 ± 1^{0}C. Observations were recorded after 48 h incubation on appearance of phytotoxic symptoms.

Leaf Injection Method

In order to further confirm the phytotoxicity of partially purified toxin, toxin was injected into leaf bits of 4 cm using insulin syringe by piercing the needle in between upper and lower epidermis and placed in Petri plates of 9 cm diameter moistened with blotting papers. A volume of 2 µl was injected into the leaf. Injected leaves were incubated at 28 ± 1^{0}C. Observations were recorded after 48 h incubation on appearance of phytotoxic symptoms. Control plates were maintained by injecting with sterile distilled water.

Characterization of Toxins Using GC-MS analysis

The partially purified toxin of the isolate BPT-1 was sent to the department of Microbiology, TNAU,

Coimbatore for further characterization of toxic compounds using GC-MS analysis.

Results and Discussion

Toxins from the culture filtrates of the maize *E. turcicum* isolates, namely APK-1, APK-2, BPT-1, BPT-2, CBL-1, CBL-2, LAM-1, LAM-2, PNR-1, PNR-2, TNL-1 and TNL-2 were separated using benzene: acetone solvent system on thin layer chromatographic plates as described in the chapter Materials and Methods (Plate 1). The crude extract of the isolates on TLC plates yielded a Rf values that ranged between 0.56 to 0.71. Nine isolates *i.e.*, APK-1, APK-2, BPT-1, BPT-2, CBL-2, LAM-1, LAM-2, PNR-2 and TNL-2 yielded a Rf value of 0.56 while other isolates *i.e.*, CBL-1, PNR-1 and TNL-1 had a Rf values of 0.59, 0.68 and 0.71 respectively (Table 1; Fig 1 and Plate 2).

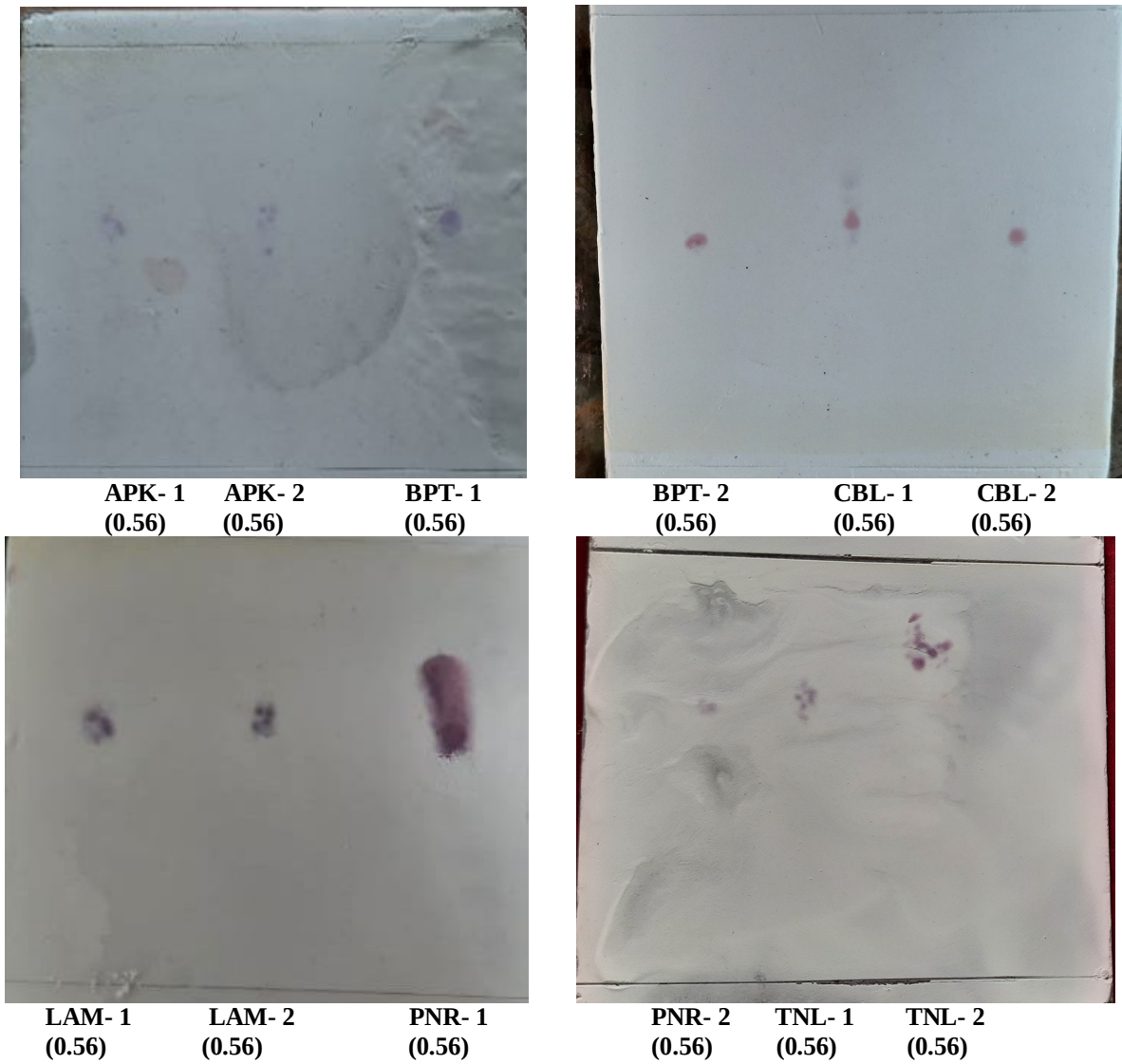

Plate 2 : Observation of colour for the different maize *E. turcicum* isolates on thin layer chromatographic plate

Table 1 : Rf values of the different maize *E. turcicum* isolates

S. No.	Name of the isolate	Rf value	S. No.	Name of the isolate	Rf value
1.	APK-1	0.56	**7.**	LAM-1	0.56
2.	APK-2	0.56	**8.**	LAM-2	0.56
3.	BPT-1	0.56	**9.**	PNR-1	0.68
4.	BPT-2	0.56	**10.**	PNR-2	0.56
5.	CBL-1	0.59	**11.**	TNL-1	0.56
6.	CBL-2	0.56	**12.**	TNL-2	0.71

Partial Purification of Toxin(s) from *E. turcicum* Isolate

The toxin produced by local isolate BPT-1 and obtained through thin layer chromatography was further purified and concentrated for its use in phytotoxicity studies by challenge inoculating via drop method and leaf injection method through detached leaf technique.

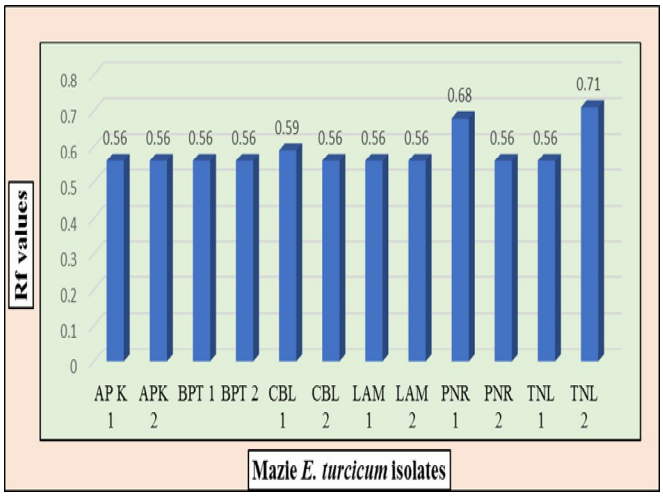

Fig. 1 : Rf values of different maize *E. turcicum* isolates

Detached Leaf Bioassay Technique (Drop Method and Leaf Injection Method)

The partially purified compounds pertaining to BPT-1 isolate was tested for its role in phytotoxicity using detached leaf technique. The toxin was prepared at different concentrations *i.e.,* 0.5% (low concentration), 4.0 and 5.0% (high concentration). A drop of the toxin (2 µl) was applied on marked area (0.7 cm) of 4 cm leaf bits taken from 30 days old plant and was allowed for incubation. After 24 hours of inoculation yellowing of the leaves was observed in all the test samples at all the concentrations. Small minute and round yellow spots were observed at 0.5 % concentration while medium round to oval yellow spots of about 60 mm diameter were noticed at 4.0 % concentration. However, at 5.0 % concentration elongated yellow spots of 80 mm diameter were observed where majority of leaf area turned to yellow colour. Leaf bits in control plates with sterile distilled water remined unchanged.

At 48 hours after inoculation yellowing dominated the cut leaf bits with characteristic necrotic symptoms at 0.5% concentration while at 4.0% and 5.0% concentration necrotic area was dominated and more than 75% of the leaf area was found to be necrotic at both concentrations. Absence of symptoms in control further confirmed the phytotoxicity of partially purified toxin obtained from the *E. turcicum* BPT-1 isolate (Plate 3 and 4).

The results were in accordance with the earlier reports given by Robeson and Strobel (1982), who reported the phytotoxicity after performing leaf dip assay using monocerin at a concentration of 0.097 mM (0.3 mg/ml) to a punctured creeping thistle leaf. A necrotic spot of 7 mm in diameter together with necrotic spots along the main vein was reported 16 hours after incubation, while at 40 hours of incubation, more than 50% of the leaf area was necrotic and brittle. The maize *E. turcicum* strains from France, produced a lipophilic phytotoxin that has been structurally characterized as monocerin. This molecule was reported to cause brown necrotic lesions on punctured leaves (Cuq *et al*., 1993). Eight fractions from the pathogenic plant fungus *Setosphaeria turcica* were separated and collected using high performance liquid chromatography and their toxic activity were assayed through leaf puncturing on corn differentials. All these fractions were found to be toxic and cause necrotic symptoms on the leaves (Zhang *et al*., 2007).

Wathaneeyawech *et al*. (2015) treated two weeks old susceptible sweet corn plants with the toxin of *E. turcicum* isolate, and found long oval shaped brownish black lesions with a yellow halo along the length of the leaf.

Plate 1 : View of the thin layer chromatographic plate

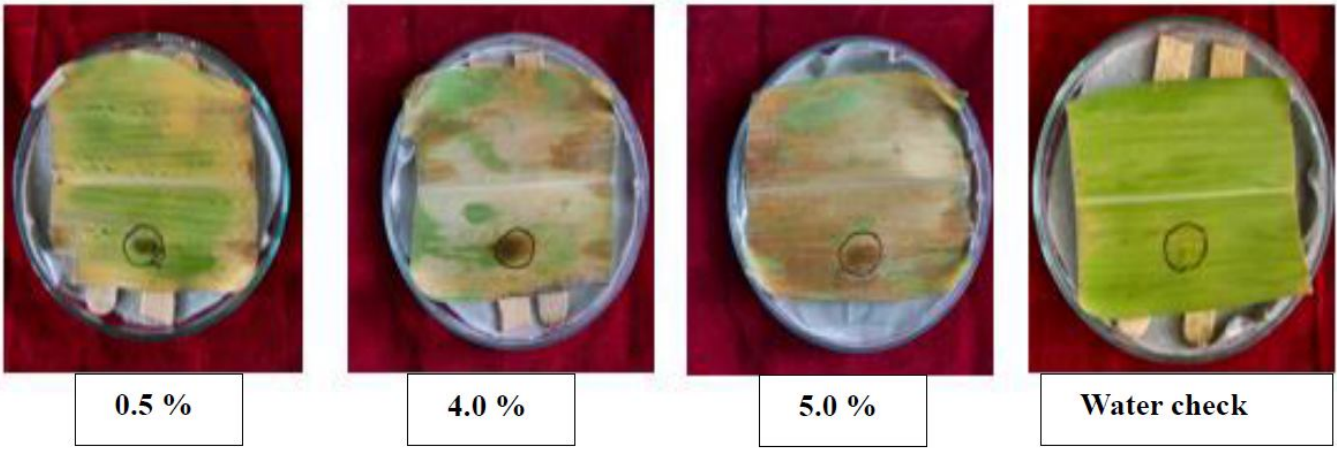

Plate 3 : Yellowing and necrotic symptoms on the leaf bits inoculated with toxin at different concentrations (Drop method)

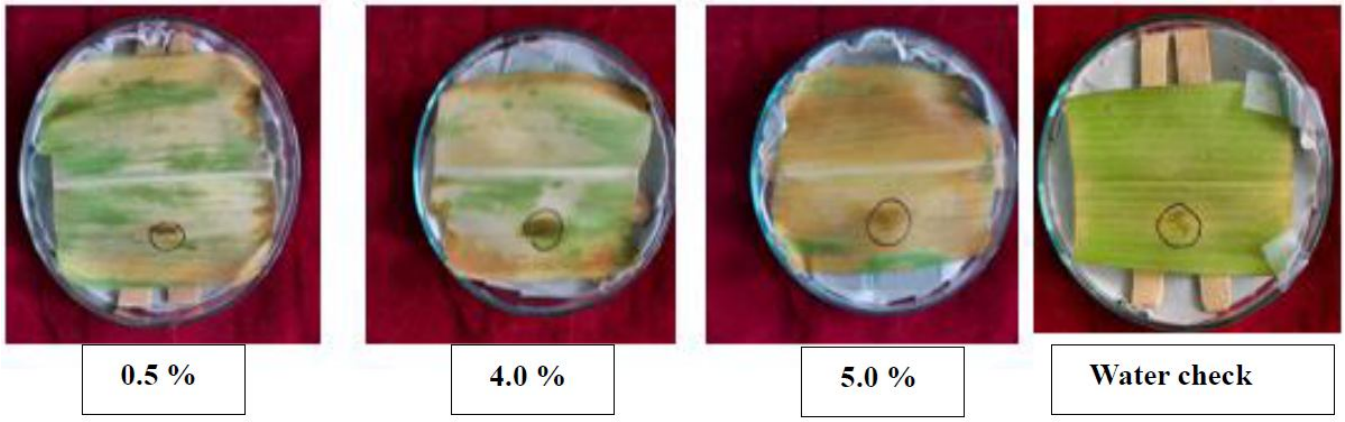

Plate 4 : Yellowing and necrotic symptoms on the leaf bits inoculated with toxin at different concentrations (Injection method)

Characterization of the *E. turcicum* Toxin Using GC-MS Analysis

Partially purified toxin of the *E. turcicum* BPT-1 isolate was further characterized using GC-MS analysis at the Department of Microbiology, TNAU, Coimbatore. The results indicated that, about 300 compounds were present in the given sample. Among them, eleven compounds were found associated with *E. turcicum* and are in accordance as reported by Yong *et al.* (2009). About ten compounds were found to have plant pathogenic fungal origin but different from that of *E. turcicum* and corroborated with that as mentioned by Zhang *et al.* (2000), Tamam *et al.* (2004) and Wathaneeyawech *et al.* (2015). The list of compounds their molecular formulae, retention time and relative content were presented in the Table 2 and 3.

Table 2 : Chemical compounds of the toxin (*E. turcicum* BPT-1 isolate) identified by the GC-MS analysis

S. No	Retention time (min)	Compounds	Molecular formula	Relative content (%)
1.	4.219	2-o-methyl PAF	$C_{26}H_{54}NO_7P$	91.8
2.	9.516	Phthalic anahydrate	$C_8H_4O_3$	70.9
3.	14.683	Phthalamic acid	$C_8H_6O_4$	60.2
4.	10.011	1- isobenzofuranone	$C_8H_6O_2$	38.9
5.	14.683	Diethyl phthalate	$C_{12}H_{14}O_4$	25.5
6.	3.023	Tetradecanoic acid	$C_{14}H_{28}O_2$	02.6
7.	3.023	Oleic acid	$C_{18}H_{34}O_2$	04.5
8.	4.944	16- Octadecanol	$C_{18}H_{38}O$	03.7
9.	6.135	Cyclohexamethanol	$C_7H_{14}O$	11.5
10.	4.219	Fumaric acid	$C_4H_4O_4$	04.0
11.	6.501	4-1,2,4-Triazole-3-thiol	$C_9H_{10}N_4S$	70.5
12.	7.450	Phenol	C_6H_6O	30.9

Table 3 : Chemical ingredients of the toxins (BPT-1) identified by the GC- MS analysis

S. No.	Retention time (Min)	Compounds	Molecular formula	Relative content (%)
1.	3.023	Hexadecanol	$C_{16}H_{34}O$	4.0
2.	3.233	Hexadecanoic acid	$C_{16}H_{32}O_2$	7.9
3.	4.219	Octadecane	$C_{18}H_{38}$	0.6
4.	3.379	Benzothiazole	$C_{17}H_4NS$	4.7
5.	3.799	Benzyl benazoate	$C_{14}H_{12}O_2$	10.8
6.	4.009	Tetradecanol	$C_{14}H_{30}O$	7.7
7.	4.154	Anthracene	$C_{14}H_{10}$	3.7
8.	4.219	Methyl 4,7-trimehyl-4,7 dihydroindan 6- carboxylate	$C_{14}H_{20}O_2$	0.5
9.	4.509	N- Dodecanol	$C_{12}H_{26}O$	2.7
10.	4.219	2 methyl PAF	$C_{25}H_{54}NO_6P$	91.8
11.	3.499	2 - Ethanol	$C_{14}H_{30}O_2$	72.0

The toxin of the BPT-1 isolate which was characterized by the GC-MS analysis was able to produce characteristic disease symptoms on detached leaf of maize plant with typical brown lesions. It was concluded that variable toxic compounds are responsible for the symptom development as emphasized by different Rf value fractions.

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Conflicts of Interest: All the authors declared that there is no conflict of interest and Authors have seen, read and approved the manuscript being submitted.

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