IN VITRO INVESTIGATION OF *Fusarium oxysporum* f. sp. *albedinis* UNDER FLAVONIC AGLYCONES ISOLATED FROM DATE PALM LEAVES (*Phoenix dactylifera* L.)

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Abstract. *Fusarium oxysporum* f. sp. *albedinis* (Foa) is one of the major soil-borne pathogens that induces vascular wilt on date palm (*Phoenix dactylifera* L.), known as Bayoud. Although extensive studies have been carried out on the biology of Foa, little is known about the compounds that stimulate or inhibit this fungus. In this study, the effect of flavonic aglycones isolated from date palm leaves on fungal growth, sporulation and fusaric acid (FA) production of Foa was investigated. The flavonic aglycones extract was obtained by acid hydrolysis of the leaves of two date palm cultivars: Takerboucht (TK, a bayoud-resistant cultivar) and Deglet Nour (DN, a susceptible cultivar). The results indicate that the leaflets of TK contain a low amount of flavonic aglycones, estimated at 0.02 $mg.g^{-1}$ equivalent of quercetin, while the leaflets of DN contain a high amount with approximately 0.21 $mg.g^{-1}$ equivalent of quercetin. TLC analysis shows the presence of three flavonols: quercetin, isorhamnetin, kaempferol, and three flavones: luteolin, tricine, chrysoeriol. It is well known that Flavonoids are essential in the resistance of plants against pathogenic fungi. Activities of flavonic aglycone from date palm leaves against Foa were evaluated using agar dilution methods. The experimental results demonstrate that TK flavonic aglycone to Foa culture medium stimulated mycelial growth and inhibited conidiogenesis and FA production. The rate of inhibition of these two last processes on the 4^{th} day of incubation was 91.66% and 87.14%, respectively. In addition, DN flavonic aglycones only affect FA production, the reduction rate being 88.17%. Furthermore, activity of flavonic aglycones in date palm leaves depends on incubation time and cultivar type. In addition, it is very interesting to note that leaf extracts could be applied to control fusariosis vascular (Bayoud) in date palm.

Key words: *Fusarium oxysporum* f. sp. *albedinis*; date palm, bayoud; flavonoïdes; antifungal activity.

INTRODUCTION

Date palm cultivation plays an essential role in human and animal nutrition in desert areas of North Africa. However, date production is reduced year by year due to several diseases. Fusariosis vascular or Bayoud caused by the telluric fungus *Fusarium oxysporum* f. sp. *albedinis* (Foa), is the most devastating disease of date palm. It appeared in Morocco in 1870, was introduced into Algeria from the border oasis of Béni-Ounif in 1898 and Bechar in 1900, and spread to Mauritania in 2003 [36].

Several control methods have been considered; among them: prophylactic measures, cultural control and chemical control, but none has proven effective in limiting the damage and spread of this disease. Also the application of large quantities of chemical fungicides in agriculture (to control these diseases) affects humans and animal health through environmental pollution [20].

For all these reasons, it is desirable to find new natural antifungal products with less toxic effects than chemical fungicids. Several authors have shown the inhibitory effect of various microorganisms on Foa, such as the *Actinomycetes* [4, 34], the fungi (*Penicillium*, *Trichoderma*, *Candida*, *Ulocladium*) [14, 39], the bacteria (*Streptomyces*, *Pseudomonas*, *Bacillus*) [12, 18, 22, 23] and the saprophytic *Fusarium* species [15]. This control strategy is very interesting but remains without large application in field.

The use of natural bioactive compounds from plants offers a choice alternative in the control of pathogens. Several extracts and essential oils of plants have been tested against Foa, of which the majority have antifungal activity. We cite the extracts of *Punica granatum* [24], extracts of *Acacia tortilis* subsp *raddiana* [30], extracts of *Olea europea* subsp *sylvestris* [6]. A few authors have studied the antifungal effect of extracts from the host plant date palm towards Foa. The first work was done by Assef et al. (1986) [3], who tested root extracts of two Moroccan cultivars, one susceptible to bayoud named "Jihel" and an other resistant named "boostammi noir". These extracts contain substances that inhibit the germination of spores and the growth of germ tubes of Foa, thus having an antifungal effect. In Algeria, Gaceb-Terrak (1987) [16] showed that the native phenolic acid extract of Takerboucht palm leaflets, with a concentration of 0.6 $mg{\cdot}L^{-1}$ gallic acid, inhibits the growth of Foa during the first two weeks (up to day 14). Ait Kettout and Rahmania (2008) [1] and Azouaoui-Ait Kettout et al. (2013) [2] showed that aqueous extracts of leaves and flavonic aglycones from roots of susceptible palm known as Deglet Nour stimulate Foa growth, and also sporulation and fusaric acid production. However, flavonic aglycones from the roots of resistant palm named Takerboucht inhibited them.

Flavonoïdes play an important role in plant resistance against bacteria and fungi [8, 19, 29]. They are directly involved in the inhibition of the enzymes of the pathogen, in particular hydrolytic enzymes [40], the spore germination, the mycelium growth [9] and the mycotoxins production by fungi such as aflatoxin [31], patulin [35] and trichothecene [8, 10].

Flavonoïdes were found to be the main phenolic compounds in the leaves of date palm. These compounds have only been adopted as cultivar-specific

markers. Indeed, two flavonols (quercetin - isorhamnetin) and three flavones (luteolin - tricin - chrysoeriol) were previously detected in the leaves of some Algerian date palm cultivars by applying acid hydrolysis [32]. Also, flavonoïdes have been identified in the date palm roots by Ziouti et al. (1996) [43] and were correlated to date palm callus defence against Foa. Few studies have been conducted on the use of date palm flavonoïdes against Foa.

The objective of the present work is to test and quantify the effect of natural products flavonic aglycones of palms of two cultivars, that are Takerboucht (TK, a bayoud resistant cultivar) and Deglet Nour (DN, a susceptible cultivar to bayoud), on the growth and production of fusaric acid by Foa, in order to use in control vascular fusariosis (Bayoud) of date palm.

MATERIALS AND METHODS

Plant material

Two Algerian date palm cultivars were considered in this experimental study; Deglet Nour (DN) from the region of Metlili, Ghardaïa (32°16'North, 3°40'East) known to be susceptible to Bayoud and Takerboucht (TK) from Adrar (27°50'45.4"North 0°18'24.2"West), known to be resistant. The median leaves collected in 2011 of adult palms, are dried in the laboratory at room temperature (28 ± 2°C). The dried leaves were ground electric grinder and the powder obtained is used for the extraction of flavonic aglycones.

Extraction and quantification of flavonoïds (flavonic aglycones)

The extraction and quantification of flavonic aglycones were carried out according to the method previously described by Bate Smith (1954) [5] and repeated by Lebreton et al. (1967) [25] and Jay et al. (1975) [21]. Thereby, 3 g of ground dry plant material (DPM) was mixed at room temperature with 240 mL of HCl (2N), and the mixture was then placed in a boiling water bath (100°C) for 40 min. Diethyl ether was added (2 x 90 mL) to the hydrolysate. The ethereal, organic phase was evaporated and the dry residue was recovered in 15 mL of ethanol at 95 %. Two extraction were performed for each cultivar.

The content of flavonic aglycones in the organic phase of our samples was determined by a differential test using the chelation properties with 1% $AlCl_3$. The optical density was measured by Junway 7300 UV/VIS spectrophotometer at 420 nm after 15 min incubation. The differential height of the ΔD0 peak is proportional to the concentration of Quercetin in the sample analysed.

$$Aglycone\ total\ (mg/g) = \frac{A \times M \times V \times f}{\varepsilon \times p}$$

where: A - absorbance of differential peaks; M - molar mass of quercetin (302 g/mol); V - volume of ethanolic solution (mL); f - dilution factor; ε - molar absorption coefficient of the quercetin (23000 $L \cdot mol^{-1} \cdot cm^{-1}$); P - mass of dry vegetable material (g).

Thin-layer chromatography (TLC) analysis

The qualitative analysis of flavonic aglycones was carried out by thin layer chromatography (TLC) on F254 silica gel and was visualized under UV (254 nm), using Acetic acid/chloroform (9-1: v/v) as eluting solvent.

The partial identification of the flavonic aglycones is carried out by comparing the calculated front ratio (FR) of each substance and their physical properties with those available in the literature [21, 25, 32].

Fungal material

The strain of Foa (Gh1) used was provided by the Institut National de Protection des Végétaux (INPV) of the regional station of Ghardaïa. It was isolated from infected rachis of date palm collected in the Ghardaïa region in the South of Algeria and maintained on PDA at 4°C.

Mycelial growth measurement

To determine the effect of flavonic aglycones on colony growth of the Foa strain, a 5 mm disc was taken from a 7-day-old PDA (Potato Dextrose Agar) culture and inoculated into the centre of a plate containing PDA medium and 1 mL of flavonic aglycones extract to obtain the final concentration of 10 $\mu g.mL^{-1}$. The control samples were supplemented with an identical volume of water. Petri plates were incubated at 28°C for 10 days following Wu et al. protocol (2009) [41], with minor modifications. Apical growth was measured in four directions on each plate for 2-10 days. Five Petri dishes and three replicates were performed for each cultivar.

Sporulation determination

Sporulation was determined after cultivation of *Fusarium oxysporum* f. sp. *albedinis* in 250 mL flasks filled with 20 mL of potato dextrose broth (PDB) adjusted to pH 4.5, containing a 5 mm agar plug taken from a 7-day-old PDA culture and 1mL of the flavonic aglycones extract of each cultivar. Incubation takes place at 28°C in the dark for 4 and 7 days. The number of conidia was calculated using the Malassez cell following Wu et al. protocol (2009) [41], with minor modifications. Three flasks and three replicates were performed for each cultivar.

Extraction and determination of fusaric acid (FA)

From the 4- and 7-day old inocula (as described above), filtration was carried out using Whatman N° 1 paper to recover the supernatant, which was the filtrate of the Foa culture containing fusaric acid. This latter was extracted by adding 70 mL of ethyl acetate to the supernatant. The organic phase was recovered and evaporated, then the dry extract was resuspended in 3 mL of ethanol at 96%.

The assay was performed using the Eged technique (2005) [13] with a spectrophotometer model 7305 (Jenway) at a wavelength of 270 nm.

The quantification of FAs present in our extracts was done according to the standard fusaric acid curve.

Percent inhibition (PI) of sporulation and fusaric acid production over control was worked out according to this equation:

$$PI(\%) = \left(\frac{C-T}{C}\right) \times 100$$

where, PI = Percent inhibition, C= fusaric acid production in control; T = fusaric acid production in treatment.

Statistical analysis

All data were reported as means ± standard error of the mean (SEM). Differences between the control group and each exposure group were assessed by one-way analysis of variance (ANOVA) followed by Tukey's HSD test at the 5% significance level using XL STAT 2021.2 software.

RESULTS

Quantitative analysis of flavonic aglycones

The flavonic aglycones obtained after complete hydrolysis of all glycosidic bonds present in the extracts correspond to the flavonoïd skeleton. The assay results, expressed as equivalent of quercetin, are presented in Figure 1 and show that the leaflets of the Bayoud-susceptible cultivars (DN) are richer in flavonic aglycones than the resistant cultivar (TK). The content of flavonic aglycones expressed as mg quercetin (flavonol) per g DPM is 0.02 $mg{\cdot}g^{-1}$ ± 0.003 for TK and 0.21 $mg{\cdot}g^{-1}$ ± 0.006 for DN. The ANOVA test revealed no significant effect between DN and TK ($p<0.05$).

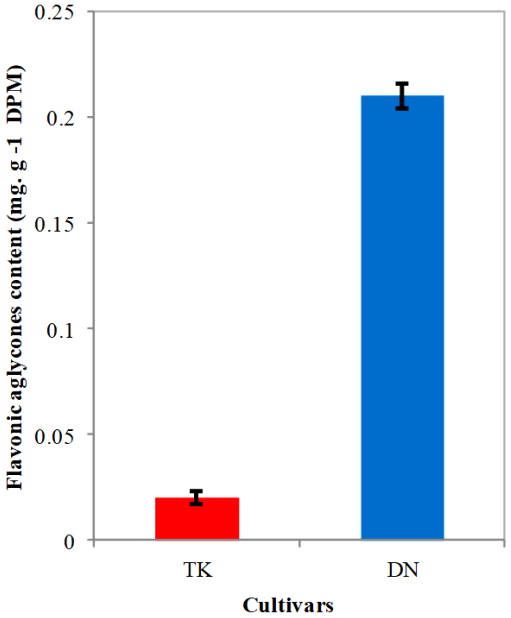

Figure 1. Differential absolute contents of flavonic aglycones expressed in mg quercetin (flavonol) per g DPM of Takerboucht (TK) and DegletNour (DN) palms.

Qualitative analysis of flavonic aglycones

Flavonoïd aglycones separations were performed on silica gel TLC plates, containing fluorescence indicator and Acetic acid/chloroform (9-1:v/v) as eluting solvent. This technique has allowed to separate 26 compounds (Table 1). The analyses revealed under corresponding UV fluorescence different flavonols (yellow), flavones (purple), phenolic acids (blue) and traces of pink-red chlorophyll. The results show three flavonols (Quercetin, Isorhamnetin, Kaempferol) and

Table 1. Main flavonic aglycones identified by TLC in leaflets of date palm TK (a Bayoud-resistant cultivar) & DN (a Bayoud-susceptible cultivar).

Spot	FR	Fluorescence	Identification
Tr1	0.03	Colourless	Not identified
Tr2	0.06	Colourless	Not identified
1	0.11	Purple	Not identified
2	0.14	Yellow	Kaempferol
3	0.20	Yellow	Quercetin
4	0.22	Purple	Luteolin
5	0.25	Blue	Phenolic acid
6	0.31	Yellow	Isorhamnetine
7	0.33	Purple	Chrysoeriol
8	0.38	Dark purple	Not identified
9	0.44	Blue	Phenolic acid
10	0.49	Dark purple	Tricine
11	0.57	Pink	Traces of chlorophyll
12	0.61	Blue	Phenolic acid
13	0.64	Pink	Traces of chlorophyll
14	0.66	Blue	Phenolic acid
15	0.71	Yellow	Flavonol not identified
16	0.75	Light purple	Not identified
17	0.78	Purple	Not identified
18	0.80	Dark purple	Flavone not identified
19	0.82	Red	Traces of chlorophyll
20	0.87	Dark purple	Flavone / Flavanonol
21	0.95	Dark blue	Phenolic acid
22	0.95	Red	Chlorophyll *a*
23	0.98	Blue	Phenolic acid
24	0.98	Red	Chlorophyll *b*

FR: Frontal Reference

three flavones (Luteolin, Tricin, Chrysoeriol). The other yellow and purple spots were not identified.

Stimulation of mycelial growth by flavonic aglycones

The mycelial growth of *Fusarium oxysporum* f. sp. *albedinis* (Foa) in the presence of natural products flavonic aglycones of DN and TK was followed for 0, 2, 4, 6, 8 and 10 days, as shown in Figure 2. The application of these compounds significantly increased the growth of Foa from the 4th day up to the 8th day as compared to the untreated control ($P<0.05$).

On the 7th day of growth on PDA medium, flavonic aglycones of DN and TK cause an increase of 23% and 9.85%, respectively, compared to the control.

Inhibition of sporulation

The inhibitory activity of the flavonic aglycones of DN and TK on the number of microconidia was studied on the 4th and 7th days. Flavonic aglycones from leaves of the resistant cultivar TK exhibited a significant effect on conidia production. The number of conidia was reduced compared to the control by 91.66% on the 4th day and 95.5% on the 7th day of incubation on the PDB medium. However, sporulation was increased with leaves of the susceptible cultivar DN on the 4th day and reduced was 17.22% on the 7th day ($P<0.05$) (Fig. 3).

Inhibition of fusaric acid production

The effect of flavonic aglycones on mycotoxin production was evaluated by quantification of fusaric acid in treated versus non-treated solution (PDB + Foa).

The production of fusaric acid by *Fusarium oxysporum* f. sp. *albedinis* in PDB medium was significantly inhibited by flavonic aglycones from TK and DN leaflets. On the 4th day, the inhibition rate was above 85% ($P<0.05$); this inhibition decreases by half on the 7th day, it is about 30% (Fig. 4).

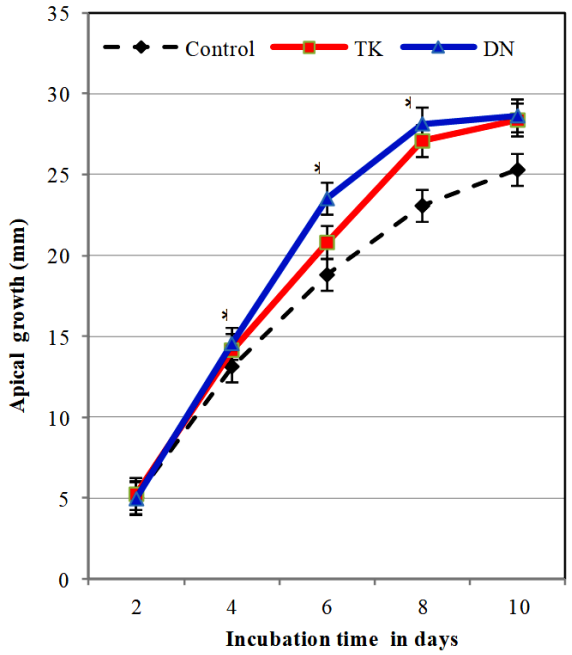

Figure 2. Effect of flavonic aglycones of leaves of two date palm cultivars on mycelial growth of Foa after 10 days incubation on PDA plates inoculated without flavonic aglycones were treated as controls. **C:** control, **TK:** Takerboucht, **DN:** Deglet Nour. Values are represented as means of four replicates. The symbols represent statistical significance using the HSD test for $p<0.05$ compared to the control.

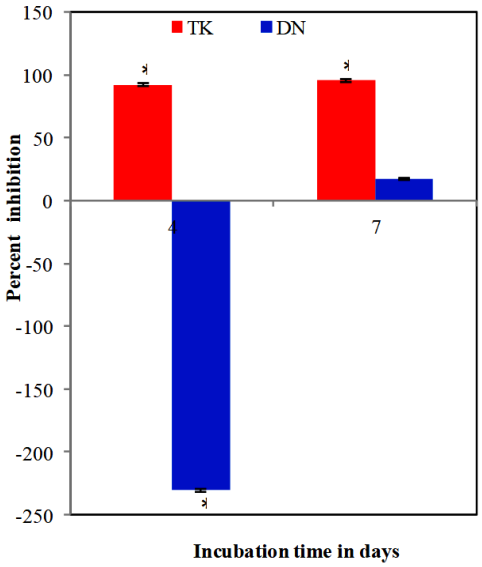

Figure 3. Effect of flavonic aglycones from leaflets of two date palm cultivars on sporulation of Foa after 4 and 7 days of liquid culture. PDB flasks inoculated without flavonic aglycones were treated as controls. **TK:** Takerboucht, **DN:** Deglet Nour. Values are represented as means of three replicates. The symbols represent statistical significance using the HSD test for $p<0.05$ compared to the control.

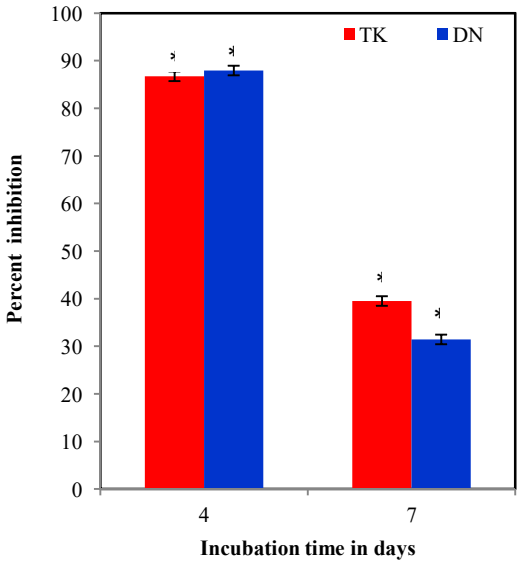

Figure 4. Effect of flavonic aglycones from leaflets of two date palm cultivars on fusaric acid production by Foa after 4 and 7 days of liquid culture. PDB flasks inoculated without flavonic aglycones were treated as controls. **TK:** Takerboucht, **DN:** Deglet Nour. Values are represented as means of three replicates. Symbols represent statistical significance using the HSD test for $p<0.05$ compared to the control.

DISCUSSION

Flavonoïds and other phenolic compounds have been found to inhibit a range of root pathogens and parasites, from bacteria to fungi and insects [28]. Pathogen challenge can lead to the *de novo* synthesis of flavonoid phytoalexins that exhibit antifungal activities. These molecules can also be stored in an inactive form for broad-spectrum phytoanticipins to provide a rapid defence against future attacks [26].

In the present work, two date palm cultivars, resistant and susceptible to Bayoud disease, were used to confirm the importance of flavonoïds in the date palm-Foa interaction.

Flavonoïds are the main phenolic compounds present in date palm leaves [43]. There are four flavonic families: proanthocyanidins, flavonols, flavones and phenolic acids [33]. According to the results obtained, leaflets of fusarium-susceptible (DN) and resistant (TK) cultivars contain very few flavone aglycones; the rate is 0.21 and 0.02 $mg \cdot g^{-1}$ DPM, respectively. Similar results [17] were obtained.

TLC analysis detected six flavonic aglycones in date palm: three flavonols (quercetin, isorhamnetin and kaempferol) and three flavones: luteolin, chrysoerol and tricine. These compounds are known in date palms [16, 32]. They accumulate during infection and contribute to resistance against pathogenic fungi [11].

This study shows that flavonic aglycones in date palm leaves increase the mycelial growth of Foa from the 4th day. Comparable results were obtained by Azouaoui - Ait Kettout et al. (2013) [2] under the effect of root flavonic aglycones of cultivars TK and DN. In contrast, research on flavonic aglycones from DN and TK seeds do not affect the mycelial growth of Foa [7]. Individual concentrations of flavones (apigenin, luteolin) and flavonols (kaempferol and quercitin) resulted in a slight increase in fungal growth of the tomato pathogen *Fusarium oxysporum* f. sp. *lycopersici*, but the lowest flavonoïd concentrations showed an inhibitory effect on mycelial growth for all flavonoïds tested [37]. The lowest dose of quercetin tested (0.001 mM) resulted in a slight increase in the mycelial volume of *Fusarium oxysporum* f. sp. *nicotianae* [38]. We hypothesize that this pathogen can degrade flavonic aglycones and reduce oxidative stress while enhancing mycelial growth. Flavonoïds are known to be potent antioxidants [42]. That flavonic aglycone of TK and DN leaves inhibit *in vitro* sporulation of Foa. This inhibition is more important in the presence of flavonic aglycones of the resistant cultivar; moreover, it is not dependent on the concentration of flavonic aglycones as the contents are higher in DN than in TK. Similar results were obtained on date palm roots [2]. Some studies [41] have been shown sporulation inibition after exogenously applied of 800 and 1600 $mg.L^{-1}$ of cinnamic acid precursor of flavenoïds on *Fusarium oxysporum* f. sp. *niveum*.

It should also be noted that flavonic aglycones inhibit the synthesis of fusaric acid (even in DNs known to be susceptible to vascular fusarium). This inhibition is more important on the 4th day than on the 7th day and is therefore dependent on the incubation time. The strong reduction in mycotoxin content by these compounds is mainly due to toxicity. The initial stage of fungal incubation seems critical for the total FA accumulation in the medium due to the early activation and high expression of genes in the first days of fungal incubation.

Bollina and Kushalappa (2011) [10], Lee et al. (2014) [27] and Bilskaet al. (2018) [8], speculate that the reduction in trichothecene content could result from an early conversion of naringenin to luteolin, kaempferol and quercetin by *Fusarium graminearum* and *Fusarium colorum,* which interferes with mycotoxin production.

This experimental investigation shows the flavonic aglycones the leaflets of date palm stimulate mycelial growth and inhibit virulence factors (conidia and fusaric acid) of *Fusarium oxysporum* f. sp. *albedinis* (Foa). On the 4th day of incubation, the inhibition rate of conidia and FA in presence of TK flavonic aglycone, was 91.66% and 87.14%, respectively. DN flavonic aglycones only affect FA production, of which the reduction rate being 88.17%. This is the first report that flavonoid aglycone from date palm is active against Foa. It could be due to the stimulation of primary metabolism (mycelial growth) and the simultaneous reduction of secondary metabolism [8]. The different types of flavonic aglycones of date palm, alone or in combination, could be used as biopesticides; an environmentally safe alternative to fungicides to help control vascular fusariosis (Bayoud) of date palm.

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