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ORIGINAL ARTICLE

Chemical composition and antifungal activity of *Trigonella foenum-graecum* L. varied with plant ploidy level and developmental stage

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Antifungal activity;
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Abstract The efficacy of aerial parts' organic extracts of diploid and mixoploid *Trigonella foenum-graecum* L. plants, harvested at three developmental stages (vegetative, flowering and fruiting) was evaluated for their antifungal activity against *Fusarium oxysporum* f. sp. *radicis-lycopersici* (FORL) and *F. oxysporum* f. sp. *lycopersici* (FOL). All tested extracts inhibited FORL and FOL mycelial growth. The organic extracts of diploid plants were found to be less toxic than mixoploid ones and this toxicity varied with the plant developmental stages. The diploids were most toxic, for the two strains, at the fruiting stage; however, mixoploids were more toxic at the vegetative stage for FOL and at flowering one for FORL. FOL was found to be more sensitive to fenugreek extracts when compared to FORL. LC–MS/MS analysis of methanolic extract of fenugreek aerial parts showed eleven different flavonol glycosides (quercetin, kaempferol and vitexin). Five novel components were identified, for the first time in fenugreek aerial parts, as kaempferol 3-O-β-D-glucopyranoside, kaempferol 7-O-glucoside, kaempferol 3-O-α-L-rhamnosyl (1→2) β-D-xyloside, kaempferol 7-O-β-D-glucopyranosyl (1–4) β-D-glucopyranoside and kaempferol 3-O-β-glucosyl (1→2)

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(6′-O-acetyl)-β-D-galactoside, along with other known compounds of this species. To operate with the maximum efficiency, the allelopathic potential of a given plant, our study showed that it would be advisable to identify the most productive developmental stage of allelochemicals. Similarly, it seems that mixoploidy would be a simple and effective biotechnology tool to improve (in quantity and quality) the allelochemicals' production, since the extracts' toxicity of diploid and mixoploid plants, was different.

1. Introduction

Fungal plant diseases may result in heavy yield losses. Phytopathogenic fungi alone cause nearly 20% reductions in the yield of major food and cash crops (Agrios, 2000). One third global agriculture production has reportedly been destroyed each year by various pests and diseases (Maqbool et al., 1988). *Fusarium oxysporum* is a cosmopolitan fungus that includes pathogenic and non-pathogenic species. The pathogenic ones are best known for causing *Fusarium* wilt and rot diseases of many economically important crops (Tripathi et al., 2009). Pathogenic strains of *F. oxysporum* infect hosts by penetrating through the rhizodermis into the root and ensuing colonization of cortex and endodermis up to the vascular system (Rodríguez-Gálvez and Mendgen, 1995). Control of such diseases mainly depends on genetic resistance, cultural practices and chemical treatments (Rauf, 2000). However, the use of synthetic fungicides may cause hazards to human health and may directly increase environmental pollution (Gnanamangai and Ponmurugan, 2012). In addition, some fungicides may not readily be biodegradable and tend to persist for years in environment (Brady, 1984). Furthermore, some fungi have developed resistance to chemicals. Because of these associated problems, researchers have been trying to use extracts of many allelopathic plants as environmentally safe alternative methods for fungal disease control (Sarovenan and Marimuthu, 2003; Spadaro and Gullino, 2005; Baraka et al., 2006; Abdel-Monaim et al., 2011). Antifungal compounds from plant origins are of the most promise due to their being less toxic and more environmentally compatible by nature (Lee et al., 2007). During the past decades, many plants have been screened for their antifungal activities as is the case of *Citrullus colocynthis* fruit content phytosterol glycoside, albuminoids colocynthis (Oliver, 1986), *Ambrosia maritime* leaf content ambrosin (Mohamed et al., 2006), *Calotropis procera* leaf content glycosides, alkaloids and phenolic glycosides (Yossry et al., 1998).

Trigonella foenum-graecum is a diploid plant with $2n = (2x) = 16$ (Ahmad et al., 2000) that does not exhibit aneuploid forms (Petropoulos, 2002). It has been used as an effective medicinal plant as well as a fodder plant. Polyploidy induction of this plant, to evaluate its morphological and chemical potentialities, was carried out by application of colchicine solution (Marzougui et al., 2009, 2010; Omezzine et al., 2012). Some studies have indicated that fenugreek is rich in active compounds. Also, it was reported that this species showed an insecticidal effects, antifungal activity and a strong allelopathic potential (Evidente et al., 2007; Haouala et al., 2008a,b). All fenugreek plant parts showed antifungal potential and the magnitude of their inhibitory effects was species and plant part dependent (Haouala et al., 2008b). Moreover, it is reported that secondary metabolites are often produced in very small quantities and this production vary (in quantity and in quality) with several factors including the developmental stage (Berger, 2007) and ploidy level (te Beest et al., 2011). Therefore, it is necessary to study the qualitative changes of allelochemicals' production at different developmental stages to identify the most productive one by comparing the toxicity of plant material harvested at different stages and its chemical composition.

The objectives of the present study were to investigate the effect of mixoploidy and the three developmental stages (vegetative, flowering and fruiting) on the antifungal activity and the chemical composition of organic extracts of *T. foenum graecum* L. aerial parts. Antifungal activity was assessed *in vitro* against two phytopathogenic fungi: *F. oxysporum* f. sp. *radicis-lycopersici* (FORL) and *F. oxysporum* f. sp. *lycopersici* (FOL). Qualitative analysis of the chemistry of the plant parts was done by LC–MS/MS.

2. Materials and methods

2.1. Plant material and mixoploidy induction

The mixoploid plants of *T. foenum-graecum* were obtained following seeds' treatment with 0.05% colchicine solution, according to the method of Omezzine et al. (2012). Fenugreek treated and untreated seeds were sown in a field under natural conditions in March 2011. The mixoploidy confirmation was done by flow cytometry, the size of stomata and pollen grain (Omezzine et al., 2012). Aerial parts of diploid (plant from untreated seeds) and mixoploid (plant from colchicine treated seeds) plants were harvested at the vegetative (plants with 8 leaves), flowering (50% of flowers open) and fruiting stages (50% of the pods have reached a typical length). Fresh plants were washed with tap water, then oven-dried at 60 °C for 72 h, powdered and used for extraction.

2.2. Fungal agents

Pure cultures of two *formae speciales* of *F. oxysporum* namely *F. oxysporum* f. sp. *lycopersici* (FOL) and *F. oxysporum* f. sp. *radicis-lycopersici* (FORL) infecting tomato were obtained from the Laboratory of Plant Pathology of the Regional Center of Research on Horticulture and Organic Agriculture, Chott-Mariem, Tunisia. The isolates were obtained from the diseased samples on Potato Dextrose Agar (PDA), purified and maintained at 4 °C until use.

2.3. Extraction

Sequential extraction was done with organic solvents of increasing polarity: hexane, ethyl acetate and methanol. Fifty

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grams of dried powder of each plant stage was soaked in organic solvent for 7 days at room temperature. The organic extracts were evaporated to dryness under reduced pressure at 45–50 °C, using Rotavapor R-114, and used at three concentrations (0.1, 0.3 and 0.6 mg/mL).

2.4. Qualitative analysis by LC–MS/MS

An analytical LC–UV–MS/MS analysis was performed as described by Kite et al. (2011). Briefly, chromatography was performed on a Phenomenex Luna C18 (2) column (150 × 4.6 mm i.d., 5 μm particle size) using a 1 ml/min linear mobile phase gradient of 20–50% aq. MeOH (containing 1% HOAc) for 30 min. Mass spectra were recorded by a Thermo Scientific 'LCQ Classic' ion trap mass spectrometer fitted with an ESI source. Accurate mass measurements were performed on Finnigan MAT900 XLT or Thermo Scientific LTQ Orbitrap XL mass spectrometers in negative and positive ESI modes.

2.5. Antifungal activity assay

Solution of organic extracts (5 mL) prepared as described above at 0.1, 0.3 and 0.6 mg/mL was added to PDA medium. Control received the same quantity (5 mL) of diluted methanol used as control for all bioassays with organic extracts. Ten milliliters of each medium was poured in each sterilized Petri plate (9 cm diameter) and mixed with the medium. After solidification, agar plugs (6 mm diameter) removed from a fast growing fungal colony was plated in Petri dishes amended or not with the extracts tested (three plugs per plate). The colony diameter was noted 5 days after incubation at 25 °C (Omezzine et al., 2011).

Fungal growth was measured by averaging the three diameters taken at right angles for each colony. Percentage growth inhibition (%) of fungal colonies was calculated according to the following formula (Jabeen and Javaid, 2008):

$$\text{Growth inhibition}(\%) = [(\text{Growth in control} - \text{Growth in treatment})/\text{Growth in control}] \times 100$$

2.6. Inhibition index (I)

The *in vitro* effects of *T. foenum-graecum* diploid and mixoploid organic extracts on the two phytopathogenic fungi, FORL and FOL, were assessed by the whole-range assessment method. Inhibition index was calculated by Eq. (1) used by Liu et al. (2007) where concentrations tested ranged from 0 to *Dn*, *Dc* was the threshold dose at which response equaled the value of control and above which the responses were inhibitory, $R(0)$ was the response at 0 extract concentration (control) and $f(D)$ represented the response function. Inhibition of mycelial growth of the different organic extracts of *T. foenum-graecum* (diploid and mixoploid) was used to calculate Inhibition indices (I) using the WESIA (Whole-range Evaluation of the Strength of Inhibition in Allelopathic-bioassay) software (Liu et al., 2007).

$$I = \frac{\int_{Dc}^{Dn}[R(0) - f(D)]dD}{\int_{0}^{Dn} R(0)dD} = 1 - \left[\frac{Dc}{Dn} + \frac{1}{R(0)Dn}\int_{Dc}^{Dn} f(D)dD\right] \quad (1)$$

2.7. Statistical analysis

All data were reported as mean ± standard deviation (S.D.) of three replicates and analyzed using the program PASW Statistics 18. Differences between the means were established using general linear model (GLM) procedure ($P < 0.05$) related to the four variables: extraction type, concentration, fungi tested and developmental stage. Differences at the 5% level ($P < 0.05$) were considered statistically significant.

3. Results

3.1. LC–MS/MS analysis

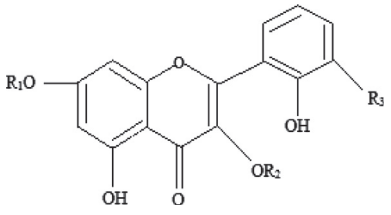
The methanol extract components of fenugreek diploid and mixoploid aerial parts' samples were identified by HPLC and LC–MS. Identifications were confirmed by comparison with standards. A total of eleven peaks were identified as flavonoid compounds in the analysis range time 2–30 min, and they were all quercetin, kaempferol and vitexin. The spectral data of all identified flavonoids are summarized in Fig. 2 and Table 1 and the chemical structures are illustrated in Fig. 1 Their UV spectra are characteristic of flavonol-type structures with two absorption maxima: band I in the 340–350 nm range, with intensities and relative positions reflecting the degree of substitution and hydroxylation, and band II, determined by the benzene moiety, in the 250–270 nm range. Compounds **1**, **7** and **10** corresponded to quercetin derivatives confirmed with MS via the ion at m/z [quercetin-H]$^-$ of 301, while the other compounds (**2**, **3**, **4**, **5**, **6**, **9** and **11**) were kaempferol derivatives defined with MS ion at m/z [kaempferol-H]$^-$ of 285. Finally, compound **8** corresponded to vitexin hexoside (Fig. 2, Table 1).

The first sets of compounds were identified as quercetin glycosides resulting from various glycosylation positions. The different types of glycosylation gave almost identical UV spectral characteristics, while their MS spectrum comparisons were dissimilar. The only common feature was the occurrence of a fragment at m/z 301, corresponding to aglycone quercetin. Peaks **1**, **7** and **10**, generating MS fragments m/z of 787, 625 and 667, were assigned, respectively, as quercetin-3,7-diglycosides, quercetin-3-glycoside galactoside and quercetin-3-glucoside (6′-O-acetyl)-β-D-galactoside (Fig. 2, Table 1). The mass spectrum in negative mode of compounds **4** and **5** exhibited a base peak [M-H]$^-$ at m/z 447 and an aglycone ion at m/z 285. The loss of 162 amu from the intermediate ion is due to the loss of glucose. The λ_{max} of the UV spectrum at 345 and 265 nm for compound **4** and at 255 and 348 for compound **5** suggests that flavonoid compounds **4** and **5** are kaempferol 3-O-hexoside and kaempferol 7-O-hexoside respectively (Fig. 2). The results of the MS and UV spectra combined suggest that compounds **4** and **5** could be kaempferol 3-O-β-D-glucopyranoside and kaempferol 7-O-glucoside, respectively (Table 1). The identification was also confirmed by chromatography with authentic standards such as kaempferol 3-O-glucoside and kaempferol 3-O-β-D-glucopyranoside. A negative ion mass spectrum of compound **2** showed an [M-H]$^-$ ion at m/z 771 (calc. for $C_{33}H_{40}O_{21}$). The appearance of three anomeric proton signals in the LC–MS further confirmed that compound **2** is a kaempferol triglycoside. Three sugar moieties were identified as two glucoses and one galactose from the

Table 1 Flavonoids identified in methanol extract of fenugreek aerial parts. HPLC retention times (t_R), experimental high resolution m/z values of $[M-H]^-$, calculated molecular formulae (of M), UV spectra and means by which the compound was identified (Det.[a]), are listed.

Peak No.	t_R	$[M-H]^-$ (m/z)	UV λ_{max} (nm)	Molecular formula	Identity	Det.[a]
1	4.28	787	256, 267, 357	$C_{33}H_{40}O_{22}$	Quercetin 3,7-diglycosides	UV, MS
2	4.81	771	265, 345	$C_{33}H_{40}O_{21}$	Kaempferol 3-O-β-D-glucosyl (1→2) β-D-galactoside 7-O-β-D glucoside	UV, MS
3	6.68	813	265, 293 (sh), 347	$C_{35}H_{42}O_{22}$	Kaempferol 3-O-β-D-glucosyl (1→2) (6″-O-acetyl)-β-D-galactoside 7-O-β-D glucoside	UV, MS
4	6.9	447	265, 345	$C_{21}H_{20}O_{11}$	Kaempferol 3-O-β-D-glucopyranoside	UV, MS
5	7.19	447	255, 348	$C_{21}H_{20}O_{11}$	Kaempferol 7-O-glucoside	UV, MS
6	8.3	563	266,347	$C_{26}H_{28}O_{14}$	Kaempferol 3-O-α-L-rhamnosyl (1→2) β-D-xyloside	UV, MS
7	8.49	625	256, 356	$C_{27}H_{30}O_{17}$	Quercetin-3-glucoside galactoside	UV, MS
8	8.71	578	268, 337	$C_{21}H_{20}O_{10}$	Vitexin hexoside	UV, MS
9	9.51	609	265, 293 (sh), 347	$C_{27}H_{30}O_{16}$	Kaempferol 7-O-β-D-glucopyranosyl (1–4) β-D-glucopyranoside	UV, MS
10	10.54	667	250, 355	$C_{29}H_{33}O_{18}$	Quercetin-3-glucoside (6′-O-acetyl)-β-D-galactoside	UV, MS
11	11.3	651	265, 292 (sh), 347	$C_{29}H_{32}O_{17}$	Kaempferol 3-O-β-glucosyl (1→2) (6′-O-acetyl)-β-D-galactoside	UV, MS

[a] Interpretation of UV and MS spectra (UV, MS) according to Kite et al. (2011) or by comparison of analytical data with an authentic standard (Std).



Kaempferol

Compounds	R_1	R_2	R_3
2	glc	β-D- glucosyl (1→2) β-D- galactoside	H
3	glc	β-D- glucosyl (1→2) (6"-O-acetyl)- β-D- galactoside	H
4	glc	β-D- glucopyranoside	H
5	glc	H	H
6	H	α-L-rhamnosyl (1→2) β-D- xyloside	H
9	glc	β-D- glucopyranosyl (1-4) β-D- glucopyranoside	H
11	glc	β- glucosyl (1→2) (6'-O-acetyl)- β-D- galactoside	H

Figure 1 Chemical structure of identified *Trigonella foenum-graecum* L. aerial parts' compounds.

UV, MS spectral data. Therefore, compound **2** was kaempferol 3-O-β-D-glucosyl (1→2) β-D-galactoside 7-O-β-D-glucoside. Compound **3** was also confirmed as a kaempferol triglycoside on the basis of its fragmentation behavior in the LC–MS spectrum and the appearance of three anomeric proton signals in the MS spectrum. Spectral data indicated the presence of an additional acetyl group in **3**. The UV, MS data of the sugar moieties of **3** showed great similarity to those of **2**. Thus, these data suggested the attachment of the acetyl group to C-6″ hydroxyl group of the galactosyl moiety. Hence, compound **3** was characterized as kaempferol 3-O-β-D-glucosyl (1→2) (6″-O-acetyl)-β-D-galactoside 7-O-β-D-glucoside, an acetylated derivative of **2** (Fig. 2, Table 1). Flavonoid **6** showed an $[M-H]^-$ ion at m/z 563. The loss of 146 amu from the pseudo-molecular ion represents the sugar rhamnose, and the loss of 162 amu from the intermediate ion is due to the loss of glucose. The UV spectra (λ_{max} 266, 347 nm) and MS results combined of compound **6** suggested that it could be a kaempferol 3-O-α-L-rhamnosyl (1→2) β-D-xyloside. The LC–MS analysis of compounds **9** and **11** (Table 1) produced a negative ion of m/z 609 and 651, corresponding to the $[M-H]^-$ precursor ion. This identification was confirmed after acid hydrolysis of methanolic extract followed by HPLC analysis of the reaction products, indicated that kaempferol was present in its aglycone form. In a similar way, compounds **9** and **11** were identified as kaempferol 7-O-β-D-glucopyranosyl (1–4) β-D-glucopyranoside and kaempferol 3-O-β-glucosyl (1→2) (6′-O-acetyl)-β-D-galactoside by comparing its HPLC retention time, UV spectra and mass spectra with the data obtained from standards. Compound **8** was detected as vitexin hexoside.

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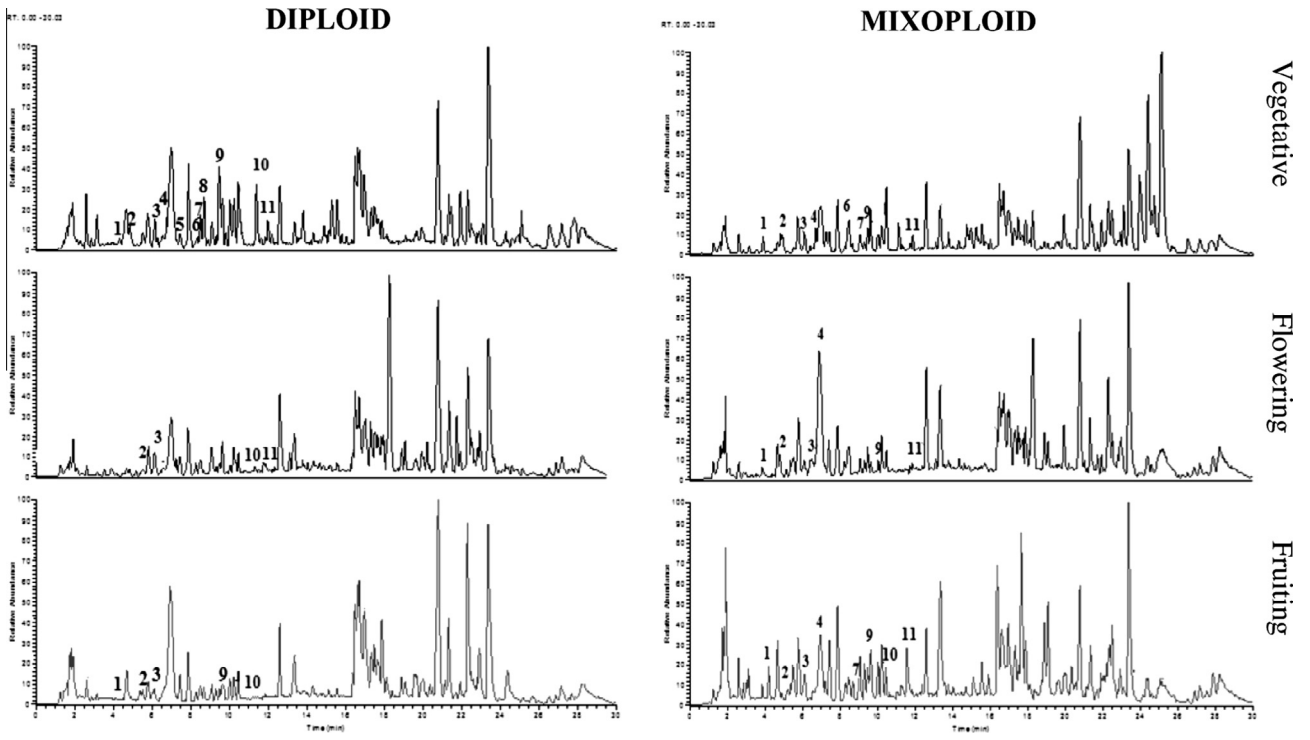

Figure 2 Chromatograms of methanolic extracts of aerial parts of *Trigonella foenum-graecum* diploid and mixoploid plants, harvested at the vegetative, flowering and fruiting stages analyzed by LC–MS/MS in negative ion mode. Peaks assignment listed in Table 1.

3.2. Variation of the chemical composition with developmental stage and ploidy level of T. foenum-graecum plants

The flavonoid compounds in *T. foenum-graecum* aerial parts' methanolic extract varied qualitatively with developmental stage and ploidy level (Table 2 and Fig. 2). Concerning the effect of developmental stages, the diversity of chemical composition of methanolic extracts decreased significantly in diploid plants. Thus, at the vegetative stage, analysis revealed the presence of 11 compounds as compared to only 4 and 5 compounds detected, with UV, MS, at the flowering and fruiting stages, respectively. For example, compound **1** (quercetin 3,7-diglycosides) was detected at the vegetative and fruiting stages but not at flowering one. Indeed, quercetin-3-glucoside galactoside (compound **7**) and vitexin hexoside (compound **8**) were detected at the vegetative stage and not for the others (Table 2). Kaempferol compounds were generally absent at the fruiting and flowering stages except for compounds **3**, **9** and **11**.

Similarly, for mixoploid plants, 8 constituents were detected at the vegetative and fruiting stages and 6 compounds at the flowering stage (Table 2, Fig. 2). For example, compounds **6**

Table 2 Variation in content of flavonoid compounds between diploid and mixoploid fenugreek plants and three developmental stages (vegetative, flowering and fruiting).

Peak No.	Compounds	DIPLOID			MIXOPLOID		
		Vegetative	Flowering	Fruiting	Vegetative	Flowering	Fruiting
1	Quercetin 3,7-diglycosides	+	-	+	+	+	+
2	Kaempferol 3-O-β-D-glucosyl (1→2) β-D-galactoside 7-O-β-D-glucoside	+	+	+	+	+	+
3	Kaempferol 3-O-β-D-glucosyl (1→2) (6″-O-acetyl)-β-D-galactoside 7-O-β-D-glucoside	+	+	+	+	+	+
4	Kaempferol 3-O-β-D-glucopyranoside	+	–	–	+	+	+
5	Kaempferol 7-O-glucoside	+	–	–	–	–	–
6	Kaempferol 3-O-α-L-rhamnosyl (1→2) β-D-xyloside	+	–	–	+	–	–
7	Quercetin-3-glucoside galactoside	+	–	–	+	–	+
8	Vitexin hexoside	+	–	–	–	–	–
9	Kaempferol 7-O-β-D-glucopyranosyl (1–4) β-D-glucopyranoside	+	–	+	+	+	+
10	Quercetin-3-glucoside (6′-O-acetyl)-β-D-galactoside	+	+	+	–	–	+
11	Kaempferol 3-O-β-glucosyl (1→2) (6′-O-acetyl)-β-D-galactoside	+	+	–	+	+	+

(+) detected by LC–MS; (–) not detected by LC–MS.

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(kaempferol 3-O-α-L-rhamnosyl (1→2) β-D-xyloside) and **10** (quercetin-3-glucoside (6′-O-acetyl)-β-D-galactoside) were detected at the vegetative and fruiting stages, respectively. As regards the effect of colchicine on the chemical composition, the results showed a qualitative variation between diploid and mixoploid plants. In fact, compounds **5** (kaempferol 7-O-glucoside) and **8** (vitexin hexoside) were identified in diploid plants at the vegetative stage but they were absent in mixoploid plants. Furthermore, compound **11** was identified at the vegetative and flowering stages in diploid extracts but was missed in mixoploids at the same developmental stages. However, compound **4** (kaempferol 3-O-β-D-glucopyranoside) was detected at the flowering and fruiting stages in mixoploid plants but they have not existed in diploids at the two stages. Similarly, compounds **1** (quercetin 3,7-diglycosides) and **9** (kaempferol 7-O-β-D-glucopyranosyl (1–4) β-D-glucopyranoside) were detected in mixoploids at the flowering stage but they were absent in diploid plants at this same stage. Likewise for compounds **7** (quercetin-3-glucoside galactoside) and **11** (kaempferol 3-O-β-glucosyl (1→2) (6′-O-acetyl)-β-D-galactoside) which were identified in extracts from mixoploid plants harvested at the fruiting stage; and were not detected in diploid ones (Table 2).

3.3. Organic extracts' effect on fungal mycelial growth

The results obtained from bioassays of fenugreek aerial parts' organic extracts against the target agents are presented in Fig. 3. A variable effect of various extracts and concentrations was recorded for the test fungal species. Moreover, the response of the fungal agents to the tested extracts seems to be different depending on the developmental stage and the ploidy level of *T. foenum-graecum*. Generally, organic extracts of diploids were found to be less toxic than mixoploid ones. For hexane fractions, mixoploid vegetable material harvested at the vegetative and fruiting stages was more toxic to FOL, inducing a mean inhibition of 55% and 51%, respectively, compared to 26% and 47% recorded with diploid extracts (Fig. 3). For FORL, these are the hexane extracts obtained at the flowering and fruiting stages of mixoploids which showed the highest toxicity with an average reduction in radial growth of 31% and 37%, respectively, against 17% and 16% for extracts of diploid plants. In the presence of the ethyl acetate fractions, once more, FORL and FOL seemed to be more sensitive to extracts of mixoploids, and no significant differences were recorded between developmental stages. Indeed, all ethyl acetate extracts of mixoploids induced an average inhibition of 39.23% for FOL and 39.47% for FORL. These values were 18.49% and 20.42% in the presence of extracts of diploids. Finally, the methanol extract exhibited the highest antifungal activity when the plant material was harvested at the flowering stage, as well, for both fungal agents in both types of plants. FOL was mostly inhibited by the extract of diploid than mixoploid plants with an average inhibition of 70% and 45%, respectively. However, FORL seemed to be more sensitive to mixoploid extracts than to diploid ones where the average reduction in the radial growth noted was of about 41% and 24%, respectively (Fig. 3).

3.4. T. foenum-graecum antifungal activity assessed by the inhibition index (I)

The experimental results revealed that the inhibition of mycelial growth of both pathogens tested were higher in the presence of organic extracts of *T. foenum-graecum* mixoploid aerial parts than those of diploids. Data re-analyzed using the inhibition index, calculated by WESIA, revealed that these extracts did not only increase the inhibition index, but also affected the details of the grouping and ranking order, i.e. this analysis allowed to group and to identify the most toxic extracts (or the developmental stages producing the most toxic compounds). Furthermore, the whole-range assessment can display a visual comparison between different fungi tested while the conventional analysis cannot provide any context concerning this aspect (Table 3).

The results shown in Table 3 indicate that the mycelial growth of FOL was more inhibited by the extracts tested than FORL. Furthermore, for FOL, the methanolic extract of plant material harvested at the fruiting stage of diploid plants was the most toxic (I = 31.49%) followed by hexane fraction of mixoploid plants at the vegetative stage (I = 23.25%) then methanol fraction of mixoploid plants harvested at the flowering stage (I = 13.26%). However, mycelial growth of FORL was affected especially by hexane extract of mixoploid plant material harvested at the flowering stage (I = 11.28%) followed by ethyl acetate fraction of diploids harvested at the fruiting stage (I = 8.38%) (Table 3).

4. Discussion

Due to the increased use of pesticides in agricultural areas and their negative effects on environment and human health, the recent researches are more focused on more safety alternatives based on the use of eco-friendly products (Akin et al., 2010; Rongai et al., 2012). Unfortunately, eco-friendly products, corresponding to allelochemicals, are characterized by very low levels within a plant and these levels are influenced by many factors, including, developmental stage and ploidy level. Thus it is important to identify the developmental stage with the greatest level of allelochemicals. In the present study, fenugreek is used as a potential source of bioactive molecules. To accomplish this purpose, the chemical composition and the antifungal activity of the aerial parts were followed depending on plant ploidy level and developmental stages.

In recent years, liquid chromatography coupled with mass spectrometry (LC–MS) has been increasingly applied for research in natural products (Liang et al., 2002; Li et al., 2006). Most studies on the chemical composition of fenugreek were realized on seeds (Liu et al., 2012; Belguith et al., 2013; Kang et al., 2013). Among the phenolic compounds identified, kaempferol-3-O-α-L-rhamnoside, kaempferol 3,7-O-α-L-dirhamnoside, kaempferol 3,7-O-α-L-rhamnoside and 3-O-α-L-rhamnosyl quercetin were reported in fenugreek seeds for the first time (Chatterjee et al., 2009). LC–MS coupled to MS was utilized to further separate and evaluate chemical constituents in the fenugreek aerial part fractions and to assess their antifungal activity. In the current study, a total of eleven peaks were identified as flavonoid compounds in the analysis and they were all quercetin, kaempferol glycosides and vitexin hexoside. The first sets of compounds were identified as quercetin glycosides resulting from various glycosylation positions. These findings are in agreement with those of Han et al. (2001) who identified the quercetin glycoside (quercetin 3-O-β-D-glucosyl (1→2) β-D-galactoside 7-O-β-D-glucoside), along with a known kaempferol glycoside lilyn (kaempferol

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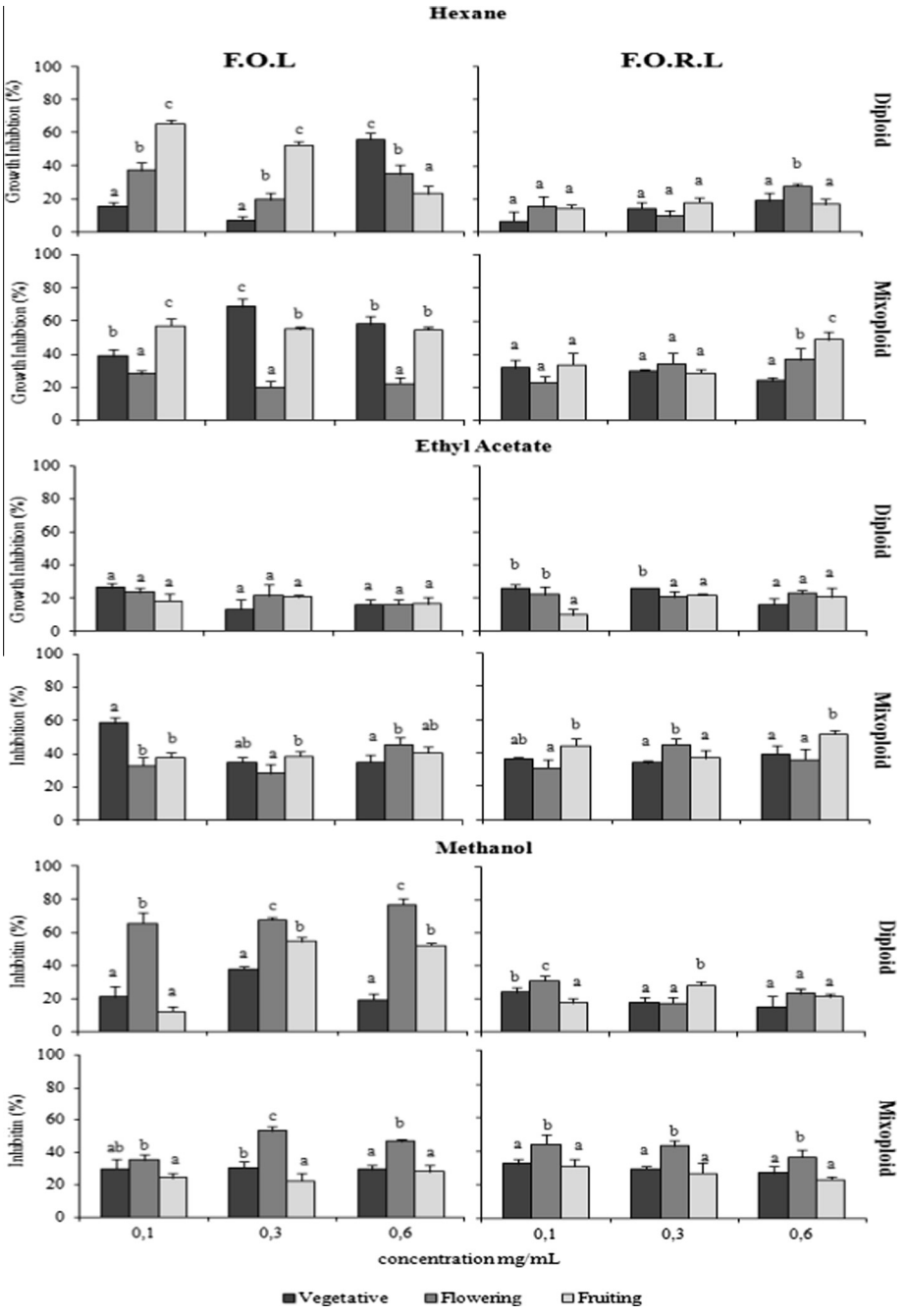

Figure 3 Growth inhibition of *Fusarium oxysporum* f. sp. *radicis-lycopersici* (FORL) and *Fusarium oxysporum* f. sp. *lycopersici* (FOL) induced by organic extracts (at 0.1, 0.3 and 0.6 mg/mL) of *Trigonella foenum graecum* aerial parts of diploid and mixoploid plants harvested at the vegetative, flowering and fruiting stages, recorded 5 days after incubation at 25 °C. The bars on each column show standard error. Value ($N = 3 \pm$ S.E.). Different letters on columns indicate significant differences among concentrations at $P < 0.05$.

3-O-β-D-glucosyl (1→2) β-D-galactoside) from the stems of *T. foenum-graecum*. Khurana et al. (1982) had isolated the trimethylcoumarin 3,4,7-trimethylcoumarin from *T. foenum-graecum* stems. In fact, compounds **2** and **3** were already identified and described in buthanolic extract of fenugreek stems (Han et al., 2001). Therefore, compounds **4**, **5**, **6**, **9**, **8** and **11** were reported in fenugreek aerial part for the first time.

Furthermore, the results indicate a high qualitative variability in the identified compounds depending on the developmental stage of *T. foenum-graecum* and its ploidy level. te Beest et al. (2011) suggested that the polyploidization can change the quality and quantity of secondary metabolites. The chromosome doubling may also alter the secondary chemical profile of a plant in a qualitative manner. The diversity of chemical composition of fenugreek methanolic extracts decreased significantly with the increase of plant growth. Levy (1976) found such differences in the glycoflavone profiles of 14 and 15 synthetic autotetraploids of *Phlox drummondii* populations as compared to their diploid prototypes. This included 14 instances of flavonoids present in the polyploidy

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Table 3 Toxicity of organic extracts of *Trigonella foenum-graecum* aerial parts of diploid and mixoploid plants harvested at the vegetative, flowering and fruiting stages, on phytopathogenic fungi: *Fusarium oxysporum* f. sp. *radicis-lycopersici* (FORL) and *Fusarium OXYSPORUM* f. sp. *lycopersici* (FOL), assessed by inhibition index (*I*) estimated with WESIA (Whole-range Evaluation of the Strength of Inhibition in Allelopathic-bioassay).

	Phytopathogenic fungi	Developmental stage / Extract	Inhibition index (I) (%)	Toxicity
DIPLOID	FOL	Fruiting / Methanol	31.49	More toxic (+)
		Flowering / Methanol	9.84	
		Vegetative / Hexane	7.28	
		Flowering / Hexane	0	
		Flowering/Ethyl Acetate	0	
		Vegetative/Ethyl Acetate	0	
		Fruiting / Hexane	0	
		Fruiting /Ethyl Acetate	0	
		Vegetative/ Methanol	0	Less toxic (-)
	FORL	Fruiting/ Ethyl Acetate	8.38	More toxic (+)
		Vegetative / Hexane	7.30	
		Fruiting / Methanol	3.74	
		Fruiting/ Hexane	2.06	
		Flowering/Hexane	1.61	
		Flowering/Ethyl acetate	0.05	
		Flowering /Methanol	0	
		Vegetative/Ethyl Acetate	0	
		Vegetative / Methanol	0	Less toxic (-)
MIXOPLOID	FOL	Vegetative / Hexane	23.25	More toxic (+)
		Flowering / Methanol	13.26	
		Flowering/Ethyl Acetate	2.19	
		Fruiting / Ethyl Acetate	1.56	
		Fruiting / Methanol	0.50	
		Vegetative/Ethyl Acetate	0	
		Fruiting / Hexane	0	
		Flowering / Hexane	0	
		Vegetative / Methanol	0	Less toxic (-)
	FORL	Flowering / Hexane	11.28	More toxic (+)
		Flowering/Ethyl Acetate	6.00	
		Fruiting / Hexane	2.82	
		Fruiting / Ethyl Acetate	0.81	
		Vegetative/Ethyl Acetate	0.37	
		Flowering / Methanol	0	
		Vegetative / Methanol	0	
		Fruiting / Ethyl Acetate	0	
		Fruiting / Methanol	0	Less toxic (-)

which were absent in diploid populations and 8 instances of the flavonoids were present in the diploid and absent in the polyploidy plants. Qualitative differences in the chemical composition depending on ploidy levels were also noted in *Briza* (Murray and Williams, 1976). Variations of flavonoid compounds during fenugreek growth indicated the influence of the three developmental stages on production and release of these metabolites. In the same way, the aerial parts of *Crithmum maritimum* collected before flowering and at the beginning of the flowering stage were the richest in total polyphenols (Males et al., 2003). Nevertheless, Ayan et al. (2007) reported that total phenol content reached the highest level at floral budding in *Hypericum hyssopifolium* and *Hypericum scabrum* and at full-flowering in *Hypericum pruinatum*.

In this study, organic extracts of *T. foenum-graecum* diploid aerial parts were found to be less toxic than mixoploid extracts and this toxicity varied with developmental stages, types of extract and concentrations used. The *in vitro* efficacy of fenugreek aerial part against some fungal agents has been investigated by Haouala et al. (2008a,b); they reported that all three organic fractions of fenugreek aerial parts (petroleum ether, ethyl acetate and methanol) showed antifungal potential and that the magnitude of their inhibitory effects was species dependent. Inderjit and Dakshini (1995) discussed the importance of a critical age for donor plant, i.e., the particular age attained after which release of allelopathic compound starts. Plant tissues' maturity also affects the content and the quantity of allelochemicals. Thereby, the quantity and content of allelochemicals in soybean stubs were different in different decomposing times and growth stages (Wang et al., 2001; Hu and Kong, 2002) and the allelopathic activity of *Populus deltoids* increased by the age (Sharma et al., 2000). The differences in

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the toxicity of different extracts could be attributed to the presence of the active principles that are extracted by different solvents, which may be influenced by several factors such as method of extraction, type of extracting solvent and time of harvesting of plant materials (Qasem and Abu-Blan, 1996). Nidhi and Trivedi (2002) found that plant leaf extracts of *Datura stramonium* and *C. procera* were highly effective in reducing radial growth of *F. oxysporum* isolated from cumin. Baraka et al. (2006) reported that plant extracts of rosemary leaves and *C. colocynthis* fruits, followed by leucarna seeds and alfalfa roots reduced significantly lupine root infection by *F. oxysporum*, *Fusarium solani*, *Rhizoctonia solani* and *Macrophomina phaseolina*. In fact, the methanol extract was more toxic when the plant material was harvested at the flowering stage for both fungi and both plant types. At the same time, the different types of extracts of the same plant exhibited variation in the importance of antifungal activity. It may be concluded that each type of the compounds extracted by certain solvents resulted in variable activity of the different extracts of the same plant (Javaid et al., 2008). In fact, FOL was found to be more sensitive to methanolic extracts of diploids than mixoploids whereas FORL was more affected by extracts of mixoploids. This variability in the antifungal activity of organic extracts of fenugreek aerial parts depending on its ploidy level was more attributed to their differences in chemical composition mainly flavonoid compounds. Hence, flavones are involved in various interactions with other organisms, microbes as well as insects or other plants. For the host plant, these interactions can be both, beneficial or harmful (Martens and Mithofer, 2005). Based on spectral analyses, the compounds detected in fenugreek aerial part were identified as flavonol glucosides. This finding is in agreement with those reported by Picman et al. (1995) on the antifungal activity of flavonoid compounds against the fungus *Verticillium albo-atrum*. The obtained data indicated that carnation is able to synthesize compounds with antifungal activity against one of its major pests, *F. oxysporum* (Curir et al., 2001, 2003, 2005). Moreover, the flavonoid compounds, quercetin and kaempferol (Santas et al., 2010), quercetin, rutin and apigenin (Basile et al., 2000) appeared to have antifungal activity.

5. Conclusion

Biological control with natural products is becoming an important component of plant disease management. Our results showed that the methanolic extracts of *T. foenum-graecum* aerial parts were highly active against FOL and FORL and could be considered as one of the sources of natural products active against fungal plant pathogens. In addition, the fractioning of methanolic extract permitted to isolate eleven flavonoid compounds, including compounds **4**, **5**, **6**, **9**, **11** and **8** identified as, respectively, kaempferol 3-O-β-D-glucopyranoside, kaempferol 7-O-glucoside, kaempferol 3-O-α-L-rhamnosyl (1→2) β-D-xyloside, kaempferol 7-O-β-D-glucopyranosyl (1–4) β-D-glucopyranoside, kaempferol 3-O-β-glucosyl (1→2) (6′-O-acetyl)-β-D-galactoside and vitexin hexoside, which are isolated for the first time from *T. foenum-graecum* aerial parts. The high fungitoxic properties recorded could be attributed to these compounds. Finally, our study showed that it would be advisable to identify the most productive developmental stage of allelochemicals, to operate, with the maximum efficiency, the allelopathic potential of a given plant. Similarly, it seems that mixoploidy would be a simple and effective biotechnology tool to improve (in quantity and quality) the allelochemicals' production, since the extracts' toxicity of diploid and mixoploid plants, was different.

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