Acta Botanica Brasilica 29(4): 553-560. 2015.
doi: 10.1590/0102-33062015abb0153

Flavonoid profiling and nodulation of some legumes in response to the allelopathic stress of *Sonchus oleraceus* L.

Nasr Hassan Gomaa[1*], Mahmoud Omar Hassan[1], Gamal Mohammad Fahmy[2], Luís González[3], Ola Hammouda[1] and Atteya Mostafa Atteya[1]

Received: June 13, 2015. Accepted: August 21, 2015

ABSTRACT

Annual sowthistle (*Sonchus oleraceus*) has been reported to produce allelopathic effects. Two greenhouse experiments were conducted to estimate the allelopathic potential of both plant residue and root exudates of *S. oleraceus* on flavonoid composition and nodulation in a leguminous crop, *Trifolium alexandrinum*, and in two leguminous weeds, *Melilotus indicu*s and *T. resupinatum*. The results of high performance liquid chromatography-mass spectrometry (HPLC--MS/MS) showed that all three legumes contained six flavonoid aglycones: apigenin, daidzein, kaempferol, luteolin, myricetin and quercetin; and seven flavonoid glycosides: daidzin, genistin, hesperidin, hyperoside, kaempferol-7-*O*--glucoside, naringin and rutin. In general, both plant residue and root exudates had inhibitory effects on the flavonoid composition and nodulation of the target species. However, residue of *S. oleraceus* caused a significant increase in both individual and total detected flavonoids in *T. alexandrinum*. The results suggest that the phytotoxins released from *S. oleraceus* may restrain the biosynthesis of flavonoids in the target species, whereas the accumulated flavonoids in *T. alexandrinum* are allelopathic-induced metabolites and suggest a resistance mode in this crop.

Keywords: allelopathy, flavonoids, legumes, nodulation, *Sonchus oleraceus*

Introduction

Fabaceae is one of the widespread plant families with approximately 19,325 species in 727 genera. (Lewis *et al.* 2005). Legumes receive considerable attention due to their potential as food, forage, fibers, agricultural fuel and fertilizer. In the Mediterranean croplands, leguminous crops are cultivated extensively for local consumption and export. They are also widely used as forage, e.g., Egyptian clover (*Trifolium alexandrinum*) which represents nearly a quarter of all Egyptian crops (Nassib *et al.* 1990). The family includes many excellent sources of bioactive compounds which are used in folk medicine and nutraceutical approaches (Lin & Lai 2006).

The phytochemistry of the Fabaceae has been well explored, and many secondary metabolites –including flavonoids, alkaloids, phenylpropanoids, terpenes, amino acids, anthraquinones and others – have been reported (Wink 1988; 2003). Flavonoids represent one of the most ubiquitous groups of secondary metabolites in the plant kingdom (Aoki *et al.* 2000), and includes some 6000 chemical structures (Hichri *et al.* 2011). The structural diversity of these compounds contributes to their many physiological activities. For example, flavonoids play a key role in the establishment of the root nodules produced in these nitrogen-fixing plants (Stafford 1997), and in inducing the germination of pollen grains (Taylor & Jorgensen 1992; Napoli *et al.* 1999). They are also advantageous to human health, due to their antioxidant properties, in addition to their possible role in preventing certain intractable diseases (Tapas *et al.* 2008; Georgiev *et al.* 2014).

In agricultural ecosystems allelopathic effects occur in the form of phytotoxic compounds either exuded from plants through their root and/or from their decaying residues in soil; these compounds have been found to disrupt seed germination and establishment in various target plants (Gawronska & Golisz 2006). Flavonoid biosynthesis is typically induced in response to both biotic and abiotic stressors (Abdel-Farid *et al.* 2009; Olsen *et al.* 2009), and while much is known, the profiling of flavonoids in response to allelopathy is still largely lacking.

Knowledge of allelopathy as a biotic stress influencing flavonoid biosynthesis in legumes remains preliminary. Flavonoid production has been studied in many legumes (e.g., Shimada *et al.* 2000; Winkel-Shirley 2001; Kim *et al.* 2003; Sreevidya *et al.* 2006; Veitch 2007). However several species,

[1] Department of Botany and Microbiology, Faculty of Science, Beni-Suef University, Beni-Suef, 62511, Egypt
[2] Department of Botany, Faculty of Science, Cairo University, Giza, 12613, Egypt
[3] Universidada de Vigo, Edificio de Ciencias, As Lagoas-Marcosende, Vigo, E- 36310, Spain.
* Corresponding author: nasr.gomaa@science.bsu.edu.eg

including *T. alexandrinum*, *Melilotus indicus* and *Trifolium resupinatum*, need further assessment in this context.

Sonchus oleraceus is one of the most common weeds in Egyptian agricultural old-fields and reclaimed lands (Gomaa *et al*. 2012; Abd El-Gawad 2014; Abd El-Ghani *et al*. 2014). This species was reported to be strongly allelopathic, reducing germination and growth of other crop and weed species due to liberation of several phenolic compounds either from its decaying dry matter and/or from its root exudates (Gomaa *et al*. 2014; Hassan *et al*. 2014a; b). Additionally, during weeding practices in cultivated fields, Egyptian farmers typically uproot this plant and mix it with the soil during ploughing. Thus this weed may affect the incoming crop and the associated weed species via the potential release of phytotoxins from decaying residues.

Not much is known about the allelopathic effect of *S. oleraceus* on flavonoid composition and nodulation in legumes. The main goals of this investigation were to study the patterns of flavonoid production in the common crop, Egyptian clover, *T. alexandrinum*, and two weed species, *Melilotus indicus* and *Trifolium resupinatum*, in response to the influence of plant residue and root exudates of *S. oleraceus*. We also evaluate the allelopathic potential of *S. oleraceus* on nodulation in these legumes and the relationship between flavonoid content and nodulation under allelopathic stress. The results of this study may be helpful for the potential use of these legumes in natural medicine and nutraceutical protocols.

Materials and methods

Collection of plant materials

A large sample of fresh shoots of *S. oleraceus* L. were collected during the fruiting stage from the agroecosystem in Beni-Suef governorate, Egypt. These were air dried in the shade, then pulverized into powder. The dry plant powder was stored in plastic bags in a refrigerator at 2°C until use. The seeds of Egyptian clover (*T. alexandrinum* L. cv. Khadrawy) were obtained from the Agricultural Research Center (ARC) in Giza, while ripe seeds of *M. indicus* (L.) All. and *T. resupinatum* L. were collected from the weed communities in the croplands of Beni-Suef governorate (29°4'N, 31°6'E; 30m a.s.l.), Egypt.

Allelopathic potential of S. oleraceus

In order to examine the allelopathic potential of *S. oleraceus* on the target species, two experiments were conducted. The first was to identify the effect of decaying *S. oleraceus* shoots in soil ('plant residue'), while the second was to clarify the effect of root exudates of *S. oleraceus* on the test legumes. To assess the absolute effect of the allelochemicals derived from *S. oleraceus*, the experimental soil (silty loam in nature) was well-leached throughout by regular flooding with distilled water every day for 20 days. A single set of pots containing leached soils without any treatment was prepared as control for both experiments.

Effect of S. oleraceus residue

Dry shoots of *S. oleraceus* plants were amended with the leached soil at the rate of 10 g tissue per kg soil, whereas the control pots were left without amendment. In each pot (18 × 20 cm each), thirty seeds of one of each of the target species: *T. alexandrinum*, *M. indicus* and *T. resupinatum*, were sown equally spaced at 0.5 cm depth in each pot. Seedlings were later thinned to the most similar six seedlings. All pots were watered regularly by tap water and kept at field capacity.

Effect of root exudates

Five seeds of *S. oleraceus* were sown at 0.5 cm depth in each pot (18 × 20 cm each). After emergence, two healthy individuals of *S. oleraceus* were left to grow in the leached soil for fifty days to trap their root exudates in the test pots. Control pots containing leached soil were kept without *S. oleraceus* seeds but watered regularly in the same way as the tested pots. After fifty days from germination of *S. oleraceus*, thirty seeds of each of the target species were sown equally spaced in each pot at 0.5 cm depth. After the emergence of the target species, the resulting seedlings were thinned to the most similar-sized six. All pots received the same amounts of water and were watered regularly as needed.

Seeds of the target species in both experiments were sown at the same time, in a protected area at Beni-Suef University (latitude 29° 04.934' N and longitude 31° 05.972' E) receiving the prevailing environmental conditions (11 h light and 13 h dark photoperiod, 19.5 to 29°C day temperature, 5.5 to 20°C night temperature, and 54 to 51 % relative humidity). The experiments were maintained in a complete randomized design with three replicates. At harvest, the two-month-old individuals were carefully removed from soil using tap water to maintain root nodules. Both number and fresh weights of the observed root nodules were determined. Shoots of the tested legumes were shade dried at 35°C and stored at 0°C until analysis.

Flavonoid analysis

Flavonoid extraction

Extraction process of flavonoids followed Kenjerić *et al*. (2007) with minor modification. 0.5 g of the dry shoots of the tested legumes were ultrasonicated in 70% methanol at room temperature for 45 minutes, and evaporated under reduced pressure. The dry residues were dissolved in 5 ml of distilled water and partitioned with ethyl ether (3 × 5 ml). The ether extracts were combined and ether removed

under the reduced pressure. At the end of the extraction process, dry residues containing flavonoid fraction were re-dissolved in 0.5 ml of methanol and analyzed using high performance liquid chromatography-mass spectrometry (HPLC-MS/MS).

High performance liquid chromatography (HPLC) system

In the current analysis, the operating system of the instrument consisted of a Supelco Discovery HS C18 column (25 cm × 4.6 mm, 5 lm) at 25°C (HPLC system from Waters Co, Milford, MA, USA). Also included was a binary pump (Waters Model 11525), UV–VIS detector (Waters Model 2487) and Breeze 3.30 SPA software. The mobile phase in the instrument was A: deionized water: acetic acid (98:2) and B: methanol with the flow rate 1 ml min^{-1}. To achieve good separation, a gradient elution timetable was used, starting with 20% methanol which remained isocratic for the first 5 min, and then increased to 40% methanol at 15 min, 50% methanol at 20 min, 60% methanol at 25 min, 80% at 30 min, and then becoming again isocratic to the end of analysis at 40 min. In order to obtain a full absorbance spectrum of flavonoid compounds, detection was monitored over the interval of 200-600 nm, while quantitative determinations were carried out at two wavelengths: 310 and 380 nm.

HPLC–MS/MS analysis

Negative electrospray ionization system (- EIS) was implemented for ionization of the different flavonoids eluted from the column. Nitrogen was used as drying agent with a flow rate of 6 L min^{-1} and as nebulising gas at a pressure of 55 psi. Temperature of the nitrogen gas was 300°C, and the potential applied during the operation was 4000 V. Mass spectra were acquired from m/z 100 to 1000 with an acquisition cycle of 0.5 s. Flavonoid identification was performed throughout the comparison of chromatographic results (retention times and UV spectra) of the tested samples with the available standard compounds, while quantification was obtained through the calibration data with the same compounds. The detected flavonoids separated into aglycones, glycosides and the total detected flavonoids (TDF) are presented as mean values ± standard deviation of three replicates and expressed as μg g^{-1} of dry matter of the studied legumes. Extra peaks were determined by using a Waters Acquity ultrapeformance liquid chromatography in a triple-quadrupole tandem (UPLC-TQD) system (Waters Corp., Milford, Massachusetts, USA), equipped with cooling auto-sampler, column oven and an ACQUITY triple-quadrupole tandem mass spectrometric detection with an electrospray ionization (ESI) interface. An ACQUITY UPLCTM BEH C18 column (50 mm × 2.1 mm, 1.7 μm; Waters Corp, Milford, MA, USA) was used.

Statistical analysis

Two experiments were conducted, one to evaluate the effect of plant residue, the other to test the effect of the root exudates of *S. oleraceus* on the test plants. The experimental design was completely randomized with three replications. Kolmogorov-Smirnov and Levene's tests were applied in order to ensure both normality and homogeneity of variances, respectively. One-way ANOVA with Duncan's test ($P \leq 0.05$) was implemented to compare the means between study groups due to HPLC-MS/MS analysis and nodulation of the studied legumes. Application of Duncan's as well as Tukey's test are better for a study like the present one since it gives more accurate results for small and large proportions than the most commonly used formulae (Zar 1999). Pearson's correlation coefficient at $P \leq 0.01$ and $P \leq 0.05$ was used to determine the relationship between the values of different flavonoids and nodulation in order to assess if there is a relationship between both parameters under the potential allelopathic stress of *S. oleraceus*. All statistical analyses were executed using the SPSS Statistics software package, version 19.0 (IBM Corporation, Armonk, NY, USA).

Results

A total of thirteen flavonoid compounds were identified and quantified by HPLC in the shoots of the tested legumes: six flavonoid aglycones –apigenin, daidzein, kaempferol, luteolin, myricetin and quercetin; and seven flavonoid glycosides –daidzin, genistin, hesperidin, hyperoside, kaempherol-7-*O*-glucoside, naringin and rutin, which were recorded in significant concentrations among the test legumes (Tab. 1). The changes occurred in the concentrations of the detected flavonoids of the different species grown under the influence of the plant residue and root exudates of *S. oleraceus* are also clarified through the multiple comparison Tukey's test ($P \leq 0.05$). The response of the flavonoid pattern of the studied legumes varied depending on the target species and the type of treatment as well. For *T. alexandrinum*, the total concentrations of flavonoid aglycones, glycosides and the estimates of TDF (total detected flavonoids) were significantly stimulated ($P \leq 0.05$) in response to application of *S. oleraceus* residue, while the opposite holds true for treatment with root exudates.

For *M. indicus* and *T. resupinatum*, both flavonoid types decreased significantly ($P \leq 0.05$) due to the incorporation of the plant residue. Root exudates of *S. oleraceus* had no effect on the total aglycones of *M. indicus* ($P = 0.215$, $F = 10.2$, d. f. = 2), but significantly ($P \leq 0.05$) reduced the TDF of the *M. indicus* and concentrations of both flavonoid types as well as TDF of *T. resupinatum* compared with control (Tab. 1). Considering the changes occurred in the levels of the TDF of the target species, the reduction observed in flavonoid content of *M. indicus* was more pronounced under the influence of plant residue than due to root exudates of *S.*

Table 1. Contents of the different flavonoid compounds: aglycones, glycosides and the total detected flavonoids (TDF) (μg g^{-1} dry weight) (Mean±SD, n = 3), of the target legumes in response to the plant residue and root exudates of *S. oleraceus*.

Compound	*T. alexandrinum*			*M. indicus*			*T. resupinatum*		
	Control	Plant Residue	Root Exudates	Control	Plant Residue	Root Exudates	Control	Plant Residue	Root Exudates
Flavonoid aglycones									
Apigenin	$405.2^{b} \pm 20.1$	$585.8^{a} \pm 21.5$	$2.6^{c} \pm 0.36$	$4.6^{a} \pm 0.61$	$1.1^{b} \pm 0.14$	$4.2^{a} \pm 0.45$	$572.8^{a} \pm 28.1$	$46.2^{b} \pm 5.6$	$24.3^{b} \pm 2.7$
Daidzein	$87.5^{a} \pm 6.1$	$96.8^{a} \pm 9.0$	$10.6^{b} \pm 1.58$	$44.9^{a} \pm 4.16$	$34.2^{b} \pm 3.2$	$40.6^{ab} \pm 5.2$	$582.3^{a} \pm 28.7$	$140.3^{b} \pm 14.2$	$170.2^{b} \pm 17.5$
Kaempherol	$69.7^{b} \pm 3.93$	$84.8^{a} \pm 4.2$	$6.3^{c} \pm 0.75$	$2.3^{a} \pm 0.22$	$2.1^{a} \pm 0.2$	$2.5^{a} \pm 0.25$	$14.7^{c} \pm 1.36$	$34.4^{a} \pm 1.5$	$23.3^{b} \pm 2.1$
Luteolin	$617.1^{b} \pm 19.3$	$776.0^{a} \pm 18.5$	$42.02^{c} \pm 5.6$	$2.4^{a} \pm 0.29$	$1.5^{b} \pm 0.14$	$2.2^{a} \pm 0.26$	$27.13^{b} \pm 0.6$	$22.7^{ab} \pm 1.3$	$73.92^{a} \pm 3.7$
Myricetin	$649.1^{b} \pm 10.0$	$773.7^{a} \pm 9.9$	$32.8^{c} \pm 2.4$	$0.76^{a} \pm 0.04$	$0.78^{a} \pm 0.05$	$0.66^{b} \pm 0.04$	$183.8^{a} \pm 8.4$	$73.2^{c} \pm 9.5$	$109.8^{b} \pm 5.8$
Quercetin	$28.3^{b} \pm 2.87$	$36.7^{a} \pm 3.2$	$2.1^{c} \pm 0.21$	$1.3^{a} \pm 0.13$	$0.80^{b} \pm 0.07$	$1.14^{a} \pm 0.06$	$11.9^{c} \pm 0.94$	$54.4^{a} \pm 1.8$	$36.7^{b} \pm 1.5$
Total aglycones	$1856.9^{b} \pm 62.3$	$2353.8^{a} \pm 66.3$	$96.42^{c} \pm 10.9$	$56.26^{a} \pm 5.45$	$40.48^{b} \pm 3.8$	$51.3^{a} \pm 6.26$	$1392.63^{a} \pm 68.1$	$371.2^{b} \pm 33.9$	$438.22^{b} \pm 33.3$
Flavonoid glycosides									
Daidzin	$649.1^{b} \pm 25.4$	$773.7^{a} \pm 28.4$	$33.4^{c} \pm 2.5$	$0.79^{a} \pm 0.07$	$0.73^{ab} \pm 0.05$	$0.62^{b} \pm 0.06$	$182.7^{a} \pm 18.3$	$73.08^{c} \pm 7.0$	$108.4^{b} \pm 9.5$
Genistin	$486.2^{b} \pm 25.6$	$703.0^{a} \pm 23.6$	$3.1^{c} \pm 0.36$	$5.52^{a} \pm 0.45$	$1.4^{b} \pm 0.26$	$5.04^{b} \pm 0.5$	$687.0^{a} \pm 29.5$	$92.3^{b} \pm 6.6$	$29.14^{c} \pm 2.96$
Hesperidin	$129.3^{b} \pm 8.5$	$154.7^{a} \pm 11.1$	$6.61^{c} \pm 0.9$	$0.16^{a} \pm 0.02$	$0.15^{a} \pm 0.02$	$0.12^{a} \pm 0.02$	$3727.1^{a} \pm 25.3$	$14.6^{b} \pm 0.9$	$21.6^{b} \pm 2.3$
Hyperoside	$8440.4^{b} \pm 108.2$	$9099.5^{a} \pm 276.9$	$442.6^{c} \pm 30.3$	$42.3^{b} \pm 5.1$	$77.1^{a} \pm 7.55$	$51.3^{b} \pm 3.7$	$2943.3^{a} \pm 269.9$	$1779.8^{b} \pm 45.9$	$242.02^{c} \pm 30.1$
Kaempherol-7-*O*-glucoside	$649.1^{b} \pm 20.1$	$773.7^{a} \pm 19.4$	$33.62^{c} \pm 3.4$	$0.79^{a} \pm 0.05$	$0.73^{ab} \pm 0.05$	$0.62^{b} \pm 0.062$	$182.7^{a} \pm 17.4$	$73.08^{c} \pm 6.1$	$108.4^{b} \pm 9.5$
Naringin	$649.1^{b} \pm 31.4$	$773.7^{a} \pm 24.9$	$32.9^{c} \pm 2.7$	$0.79^{a} \pm 0.06$	$0.73^{a} \pm 0.05$	$0.62^{b} \pm 0.062$	$182.7^{a} \pm 14.49$	$73.08^{c} \pm 5.6$	$108.4^{b} \pm 9.5$
Rutin	$35.5^{b} \pm 3.7$	$127.4^{a} \pm 12.4$	$12.2^{c} \pm 1.97$	$826.6^{a} \pm 21.1$	$7.2^{c} \pm 0.5$	$594.2^{b} \pm 14.7$	$291.9^{b} \pm 23.22$	$267.6^{b} \pm 20.3$	$372.2^{a} \pm 25.2$
Total glycosides	$11038.7^{b} \pm 222.9$	$12405.7^{a} \pm 396.7$	$564.43^{c} \pm 42.1$	$876.95^{a} \pm 26.9$	$88.04^{b} \pm 8.5$	$652.5^{a} \pm 19.1$	$8197.4^{a} \pm 398.1$	$2373.54^{b} \pm 92.4$	$990.16^{c} \pm 89.1$
TDF	$12895.6^{b} \pm 285.2$	$14759.5^{a} \pm 463.0$	$660.85^{c} \pm 53.03$	$933.21^{a} \pm 32.3$	$128.52^{c} \pm 12.3$	$703.8^{b} \pm 25.4$	$9590.03^{a} \pm 466.2$	$2744.74^{b} \pm 126.3$	$1428.38^{c} \pm 102.4$

Different superscripts in the row within the same species represent significant difference among treatments at $P \leq 0.05$ according to Duncan's test.

oleraceus, while the opposite was true for *T. alexandrinum* and *T. resupinatum* (Tab. 1).

The individual flavonoid compounds also showed different responses among the target species as well as by treatment. For *T. alexandrinum*, with the exception of daidzein ($P = 0.25$, F = 166.37, d. f. = 2), all the detected compounds increased significantly ($P \leq 0.05$) in the residue-amended soils. By contrast, all of them were reduced significantly ($P \leq 0.05$) in response to the root exudates (Tab. 1).

For *M. indicus*, the plant residue significantly ($P \leq 0.05$) reduced the accumulation of apigenin, daidzein, luteolin, quercetin, genistin and rutin, while stimulation was established only for hyperoside content by 82.3 % from control. Root exudates had no effect on the individual aglycones except for myricetin which was reduced by about 13.2 % from control. By contrast, they mostly suppressed the accumulation of the individual glycosides in the same species (Tab. 1).

For *T. resupinatum*, both treatments had a general inhibitory effect on the flavonoids. Nevertheless, the contents of flavonols kaempferol and quercetin were significantly increased ($P \leq 0.05$) in response to both treatments, while luteolin and rutin significantly ($P \leq 0.05$) accumulated in individuals exposed to root exudates, compared with control (Tab. 1).

Root nodulation was severely reduced in the test species growing in soils treated with the plant residue and root exudates of *S. oleraceus* (Fig. 1). Application of *S. oleraceus* residue significantly ($P \leq 0.05$) decreased the number and fresh weight of nodules in the three legumes investigated. For *M. indicus* and *T. resupinatum*, nodule formation was completely inhibited in response to root exudate. For Egyptian clover, the same treatment was only slightly less effective, reducing the numbers and fresh weight of nodules by about 90.3 and 94.6 %, respectively (Fig. 1).

Results of Pearson correlation analysis (Tab. 2) indicate that individual flavonoids as well as the total showed non-significant correlations with nodule formation in *T. alexandrinum* and *M. indicus*. For *T. resupinatum*, the individual flavonoids and TDF showed significant correlations with both the number of nodules and their fresh weights per each individual. Kaempferol and quercetin both showed significant negative correlations with nodulation, whereas the remaining compounds were mostly positively correlated with nodulation.

Discussion

To our knowledge, this study is the first attempt to investigate flavonoid composition in *T. alexandrinum*, *M. indicus* and *T. resupinatum*. The flavonoid profile of these legumes is similar to that reported by Carlsen *et al.* (2012) for *T. repens*. However, some compounds deviated from the profile of this species such as the aglycone myricetin and the glycosides hesperidin, kaempferol-7-*O*-glucoside and naringin. This discrepancy in flavonoid constituents of the legumes could

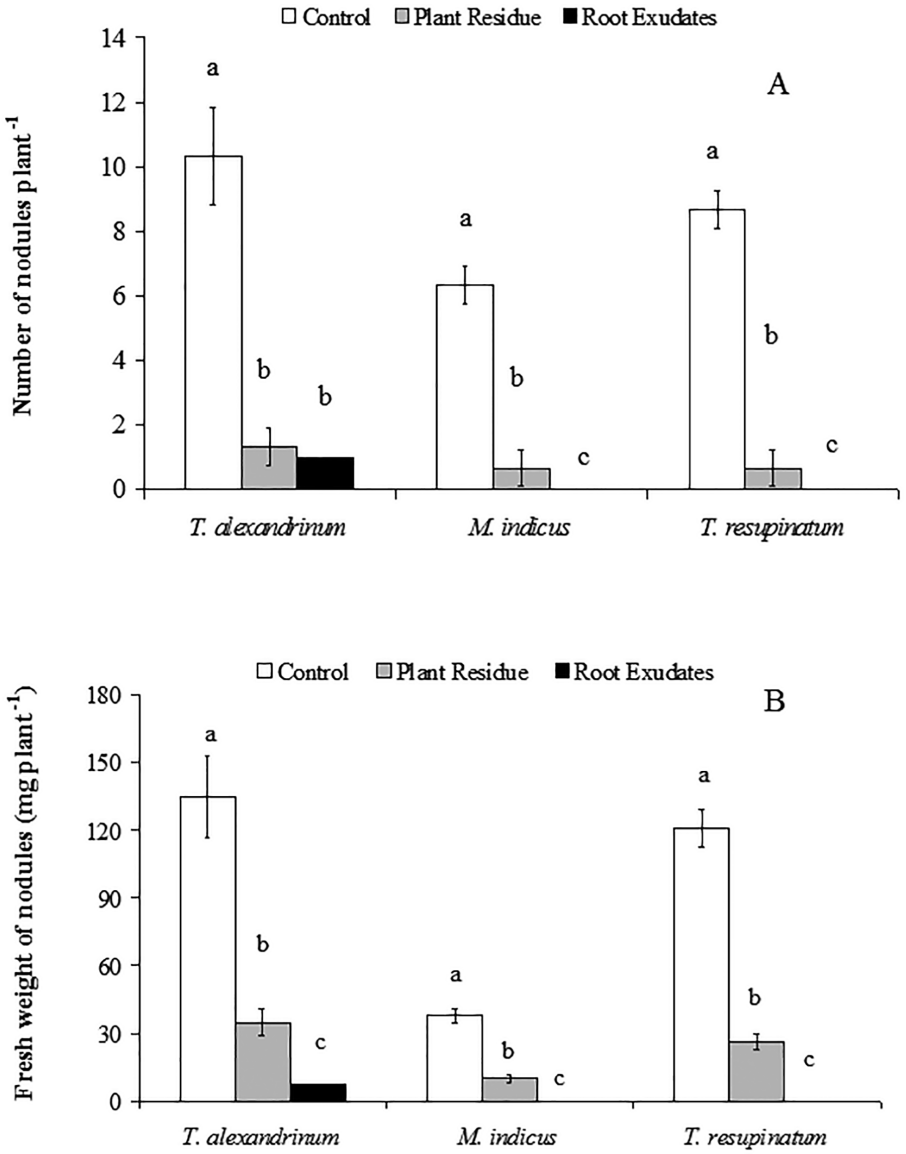

Figure 1. Effect of plant residue and root exudates of *S. oleraceus* on (A) number and (B) fresh weights of the root nodules of the investigated leguminous species. The bars on each column show the standard deviation. Different letters on columns indicate significant differences among treatments at $P \leq 0.05$ according to Duncan's test.

be attributed to physiological and/or genetic characteristics of the species examined. This notion was supported by Miean & Mohamed (2010) who reported variations in specific and total flavonoids of 62 edible tropical plants. In addition, biosynthesis of plant flavonoids is strongly correlated with enzymatic pathways and genetic characteristics (Petrussa *et al.* 2013) which vary among species.

In general, this study has confirmed that both plant residue and root exudates of *S. oleraceus* had a common inhibitory effect on the flavonoid concentrations in the target legumes. This influence could be attributed to the phenolics such as ferulic-, caffeic-, syringic- and p-hydroxybenzoic acids, which were previously detected in plant residue and root exudates of *S. oleraceus* (Gomaa *et al.* 2014; Hassan *et al.* 2014a; b). Moreover, the plant tissues of *S. oleraceus* were also reported to contain considerable contents of alkaloids, tannins and saponins (Gomaa *et al.* 2014). These were suggested as possible allelopathic candidates which interact with several enzymes causing deviations from the standard biosynthetic pathways of secondary metabolites (Einhellig 2004).

Abdel-Farid *et al.* (2009) indicated that the decrease of secondary metabolites under biotic stress refers to the flux of these compounds towards their precursors. The inhibitory effect was more pronounced for the flavonoid glycosides compared with aglycones. This finding suggests the phenolic allelochemicals may inactivate glycosyl transferase, an enzyme responsible for the transfer of a sugar moiety to the aglycones.

Table 2. Correlation values (*r*) between the concentration of flavonoid compounds and the number of nodules as well as their fresh weights (mg) per each individual of the target species.

Compound	*T. alexandrinum*		*M. indicus*		*T. resupinatum*	
	Number of nodules	Weight of nodules (mg)	Number of nodules	Weight of nodules (mg)	Number of nodules	Weight of nodules (mg)
Apigenin	0.4	0.47	0.39	0.28	0.96**	0.96**
Daizein	0.57	0.64	0.46	0.41	0.94**	0.92**
Kaempherol	0.51	0.579	-0.096	-0.14	-0.76*	-0.79*
Luteolin	0.49	0.56	0.48	0.41	-0.52	-0.47
Myrcetin	0.53	0.6	0.389	0.44	0.83**	0.79*
Quercetin	0.47	0.51	0.48	0.40	-0.78*	-0.72*
Daidzin	0.53	0.59	0.53	0.62	0.80**	0.80**
Genistin	0.41	0.47	0.39	0.29	0.98**	0.96**
Hesperidin	0.53	0.60	0.53	0.61	0.96**	0.94**
Hyperoside	0.61	0.66	-0.53	-0.43	0.91**	0.96**
Kaempherol-7-*O*- glucoside	0.53	0.6	0.54	0.63	0.80**	0.79**
Naringin	0.53	0.59	0.53	0.63	0.81**	0.8**
Rutin	-0.104	-0.04	0.56	0.47	-0.38	-0.30
TDF	0.564	0.623	0.516	0.47	0.982**	0.997**

* Correlation is significant at $P \leq 0.05$.
**Correlation is significant at $P \leq 0.01$.

The *S. oleraceus*-amended soils caused significant induction for all flavonoids detected in *T. alexandrinum*. In general, stress conditions introduced by the release of allelochemicals in the donor plant, could have synergistic effects on flavonoid biosynthesis (Balakumar *et al.* 1993). Furthermore Kenjerić *et al.* (2007) found significant increments in flavonoid concentrations of *Robinia* honeys when the plants were raised under stressful conditions, and concluded that many severe physicochemical conditions act as flavonoid inducers.

In response to biotic stresses other than allelopathy, significant increase of some flavonoid compounds (kaempferol and quercetin analogues) occurred in *Brassica rapa* cultivars when infected with the pathogenic fungus *Fusarium oxysporium* (Abdel-Farid *et al.* 2009). The induction of flavonoids in the residue-treated *T. alexandrinum* may be due to enzymatic and/or genetic changes. This speculation is supported by results of Li *et al.* (2008) who indicated that allelopathic stress could induce the genes encoding the enzymes involved in flavonoid synthesis. Moreover, Olsen *et al.* (2009) showed that the activity of the phenylalanine ammonialyase enzyme and transcript levels of regulators in flavonoid pathway were all promoted under stress conditions. Hence flavonoids may be considered as allelopathic-induced metabolites.

On the other hand, some of the induced flavonoids in *T. alexandrinum* –such as kaempferol, quercetin, myricetin and rutin –have been reported as allelochemicals themselves (Einhellig 2004; Weston & Mathesius 2013). Induction of these compounds is often regulated by the plants via signal perception, the phenomenon of allelopathy-signalling which occurs when acceptor plants are exposed to allelochemicals (Gawronska & Golisz 2006).

The present results indicating elevated flavonoid contents in *T. alexandrinum* in response to *S. oleraceus* residue differ from those observed in *M. indicus* and *T. resupinatum*. Generally, crop plants may be more resistant to allelochemicals, whereas weeds are more susceptible (Al-Sherif *et al.* 2013; Gomaa *et al.* 2014; Hassan *et al.* 2014b). Possibly *T. alexandrinum* has an ability to detoxify the phytotoxic compounds in soil via release of sugars or enzymes which, in turn, alters the metabolic activities. The increase in flavonoids of the residue-treated *T. alexandrinum* may be due to the resistance of this crop. This result suggests also that the enzymatic pathways of flavonoids in this crop species were up regulated, whereas most of these pathways in weeds were disintegrated or, at least, redirected.

For *T. resupinatum*, certain flavonols (kaempferol, quercetin and rutin) increased in response to the allelopathic potential of *S. oleraceus*. This observation seemed to be a partial resistance for this legume and may be related to activation of the flavonol synthase (FLS) enzyme for biosynthesis of kaempferol and quercetin. In addition, the induced rutin may be fluxed from quercetin by rutin synthase enzyme, which activates this conversion under stress conditions (Lucci & Mazzafera, 2009). Similarly, accumulation of luteolin may originate from the decreasing apigenin through the flavonoid-3'-hydroxylase (F3'H) enzyme, which activates hydroxylation of apigenin into luteolin (Nakamura *et al.* 2010).

The present study revealed that both plant residue and root exudates of *S. oleraceus* suppressed the formation of root nodules in the test species. This observation is in agreement with Batish *et al.* (2006), who noted the aggressive effect of *Ageratum conyzoides* residue on nodulation in chickpea. Batish *et al.* (2007) showed extreme inhibition in numbers and fresh weights of nodules in chickpea and pea in response to residue of *Chenopodium murale*. This result suggests that *S. oleraceus* releases various toxic compounds which not only suppress germination and growth of the target species (Gomaa *et al.* 2014), but also inhibit nodulation of the studied legumes.

Flavonoids of many leguminous plants have been considered as chemo-attractants for rhizobia, activating the *Rhizobium* genes responsible for nodulation (Bais *et al.* 2004; Zhuang *et al.* 2013). This characterization is apparently true under normal conditions, but may change under the allelopathic stress. Although a stimulatory effect was found in the flavonoids from *T. alexandrinum*, treated with *S. oleraceus* residue, nodulation was significantly inhibited. Moreover nodulation was not correlated with flavonoid concentrations in this species and *M. indicus* under the allelopathic effect. This finding emphasizes that the inhibition of nodulation in the target legumes may be attributed to allelopathic compounds released from both dry residue and root exudates of *S. oleraceus*, and not to the reduction in flavonoid contents of the legume.

With regard to possible applications of the flavonoids recorded in these test species, it has been reported that various flavonols including quercetin, kaempferol, myricetin and rutin exhibited a wide range of beneficial effects, such as anti-allergy, anti-inflammation, anti-viral activity and anti-cancer effects. These flavonoids also play a protective role in liver and cardiovascular diseases (Tapas *et al.* 2008). Hesperidin, the most common flavonone in *T. resupinatum*, possesses significant anti-inflammation, analgesic properties (Galati *et al.* 1994; Farmica & Regelson 1995) and anti-cancer activity, in combination with quercetin (Tanwar & Modgil 2012; Li *et al.* 2014). On the other hand, many leguminous species were reported to be associated with prevention or reduction of some otherwise intractable diseases, due to their flavonoids (Filho 2009; Al-Sayed *et al.* 2014; Santos *et al.* 2014). The results of this study may warrant possible use of these legumes in food supplements and folk medicine.

The flavonoid responses of the target legumes in the present study to the allelopathic potential of *S. oleraceus* was species-and treatment-dependent. The allelopathic effects of *S. oleraceus* produced a common inhibitory effect on the accumulation of flavonoids and on nodulation in these legumes. The flavonoid content of *T. alexandrinum* increased in response to *S. oleraceus* residue, suggesting a possible resistance mechanism. Flavonoids do not appear likely to affect nodulation under the allelopathic stress.

Acknowledgement

We thank Dr. H. R. AbdElgawad, Department of Biology, University of Antwerp, Belgium, for his help in flavonoid analysis. The authors also appreciate Prof. Jon Lovett-Doust, University of Windsor, Canada, for his valuable comments and improving the English.

References

Abd El-Gawad AM. 2014. Ecology and allelopathic control of *Brassica tournefortii* in reclaimed areas of the Nile Delta, Egypt. Turkish Journal of Botany 38: 347-357.

Abd El-Ghani M, Soliman A, Hamdy R, Bennoba E. 2014.Weed flora in the reclaimed lands along the northern sector of the Nile Valley in Egypt. Turkish Journal of Botany 37: 464-488.

Abdel-Farid IB, Jahangir M, Hondel CAMJJ, Kim HK, Choi YH, Verpoorte R. 2009. Fungal infection-induced metabolites in *Brassica rapa*. Plant Science 176: 608-615.

Al-Sayed E, Martiskainen O, Seif el-Din SH, *et al.* 2014. Hepato-protective and anti-oxidant effects of *Bauhinia hookeri* extract against carbon tetrachloride-induced hepatotoxicity in mice and characterization of its bioactive compounds by HPLC-PDA-ESI-MS/MS. BioMed Research International 2014: 1-9.

Al-Sherif E, Hegazy AK, Gomaa NH, Hassan MO. 2013. Allelopathic effect of black mustard tissues and root exudates on some crops and weeds. Planta Daninha 31: 11-19.

Aoki T, Akashi T, Ayabe S. 2000. Flavonoids of leguminous plants: structure, biological activity, and biosynthesis. Journal of Plant Research 113: 475-488.

Bais HP, Park SW, Weir TL, Callaway RM, Vivanco JM. 2004. How plants communicate using the underground information superhighway. Trends in Plant Science 9: 26-32.

Balakumar T, Vincent HB, Paliwal K. 1993. On the interaction of UV-B radiation (280–315 nm) with water stress in crop plants. Physiologia Plantarum 87: 217-222.

Batish DR, Singh HP, Kaur S, Kohli RK. 2006. Phytotoxicity of *Ageratum conyzoides* towards growth and nodulation of *Cicer arietinum*. Agriculture, Ecosystem and Environment 113: 399-401.

Batish DR, Lavanya K, Singh HP, Kohli PK. 2007. Phenolic allelochemicals released by *Chenopodium murale* affect growth, nodulation and macromolecule content in ckickpea and pea. Plant Growth Regulation 51: 119-128.

Carlsen SCK, Pedersen HA, Spliid NH, Fomsgaard IS. 2012. Fate in soil of flavonoids released from white clover (*Trifolium repens* L.). Applied and Environmental Soil Science 2012: 1-10.

Einhellig FA. 2004. Mode of Allelochemical Action of Phenolic Compounds. In: Macías FA, Galindo JCG, Molinillo JMG, Cutler HG. (eds.) Allelopathy Chemistry and Mode of Action of Allelochemicals. Boca Raton, CRC Press LLC. p. 217-238.

Farmica JV, Regelson W. 1995. Review of the biology of quercetin and related bioflavonoids. Food and Chemical Toxicology 33: 1061-1080.

Filho VC. 2009. Chemical composition and biological potential of plants from the genus *Bauhinia*. Phytotherapy Researech 23: 1347-1354.

Galati EM, Monforte MT, Kirjavainen S, Forestieri AM, Trovato A, Tripodo MM. 1994. Biological effects of hesperidin, a citrus flavonoids (Note I): antiinflammatory and analgesic activity. Farmaco 40: 709-712.

Gawronska H, Golisz A. 2006. Allelopathy and biotic stresses. In: Reigosa MJ, Pedrol N, González L. (eds.) Allelopathy: A physiological Process with Ecological Implications. Netherlands, Springer. p. 211-227.

Georgiev V, Ananga A, Tsolova V. 2014. Recent advances and uses of grape flavonoids as nutraceuticals. Nutrients 6: 391-415.

Gomaa NH, Al Sherif EA, Hegazy AK, Hassan MO. 2012. Floristic diversity and vegetation analysis of *Brassica nigra* (L.) Koch communities. Egyptian Journal of Biology 14: 63-72.

Gomaa NH, Hassan MO, Fahmy GM, González L, Hammouda O, Atteya AM. 2014. Allelopathic potential of *Sonchus oleraceus* L. on germination and seedling growth of crop and weed species. Acta Botanica Brasilica 28: 408-416.

Hassan MO, Gomaa NH, Fahmy GM, González L, Hammouda O, Atteya AM. 2014a. Interaction between *Sonchus oleraceus* L. and some weeds in agroecosystems in Egypt. Annals of Agricultural Science 59: 221-228.

Hassan MO, Gomaa NH, Fahmy GM, González L, Hammouda O, Atteya AM. 2014b. Influence of *Sonchus oleraceus* L. residue on soil properties and growth of some plants. Philippine Agricultural Scientist 97: 368-376.

Hichri I, Barrieu F, Bogs J, Kappel C, Delrot S, Lauvergeat V. 2011. Recent advances in the transcriptional regulation of the flavonoid biosynthetic pathway. Journal of Experimental Botany 62: 2465-2483.

Kenjerić D, Mandić ML, Primorac L, Bubalo D, Perl A. 2007. Flavonoid profile of *Robinia* honeys produced in Croatia. Food Chemistry 102: 683-690.

Kim BG, Kim SY, Song HS, *et al*. 2003. Cloning and expression of the isoflavone synthase gene (*IFS-Tp*) from *Trifolium pretense*. Molecules Cells 15: 301-306.

Lewis G., Schrire B., Mackinder B, Lock M., 2005. Legumes of the World. London, Royal Botanic Gardens, Kew.

Li W, Liu M, Xu YF, Feng Y, Che JP, Wang GC, Zheng JH. 2014. Combination of quercetin and hyperoside has anticancer effects on renal cancer cells through inhibition of oncogenic microRNA-27a. Oncology Reports 31: 117-124.

Li Z, Zhang Z, Xiong J, Chen H, Lin W. 2008. Allelopathic effect of continuously cropped soils under the chinese medicinal plant *Achyranthes bidentata* Blume and its molecular mechanism. New York, 5th World Congress on Allelopathy.

Lin PY, Lai HM. 2006. Bioactive compounds in legumes and their germinated products. Journal of Agriculture and Food Chemistry 54: 3807-3814.

Lucci N, Mazzafera P. 2009. Rutin synthase in fava d'anta: Purification and influence of stressors. Canadian Journal of Plant Science 89: 895-902.

Miean KH, Mohamed S. 2001. Flavonoid (myricetin, quercetin, kaempferol, luteolin, and apigenin) content of edible tropical plants. Journal of Agriculture and Food Chemistry 49: 3106-3112.

Nakamura N, Fukuchi-Mizutani M, Fukui Y, *et al*. 2010. Generation of pink flower varieties from blue *Torenia hybrida* by redirecting the flavonoid biosynthetic pathway from delphinidin to pelargonidin. Plant Biotechnology 27: 375-383.

Napoli CA, Fahy D, Wang HY, Taylor LP. 1999. White anther: A petunia mutant that abolishes pollen flavonol accumulation, induces male sterility, and is complemented by a 20 chalcone synthase transgene. Plant Physiology 120: 615-622.

Nassib AM, Rammah A, Hussein AHA. 1990. The role of legumes in the farming systems of Egypt. In: Osman AE, Ibrahim MH, Jones MA. (eds.) The role of legumes in the farming systems of the Mediterranean areas. London, Kluwer academic publisher. p. 51-61.

Olsen KM, Slimestad R, Lea US, *et al*. 2009. Temperature and nitrogen effects on regulators and products of the flavonoid pathway: experimental and kinetic model studies. Plant, Cell and Environment 32: 286-299.

Petrussa E, Braidot E, Zancani M, *et al*. 2013. Plant flavonoids-biosynthesis, transport and involvement in stress responses. International Journal of Molecular Science 14: 14950-14973.

Santos AE, Kuster RM, Yamamoto KA, *et al*. 2014. Quercetin and quercetin 3-O-glycosides from *Bauhinia longifolia* (Bong.) Steud. show anti-Mayaro virus activity. Parasites Vectors 2014: 1-7.

Shimada N, Akashi T, Aoki T, Ayabe S. 2000. Induction of isoflavonoid pathway in the model legume *Lotus japonicus*: molecular characterization of enzymes involved in phytoalexin biosynthesis. Plant Science 160: 37-47.

Sreevidya VS, Rao CS, Sullia SB, Ladha JK, Reddy PM. 2006. Metabolic engineering of rice with soybean isoflavone synthase for promoting nodulation gene expression in rhizobia. Journal of Experimental Botany 57: 1957-1969.

Stafford HA. 1997. Roles of flavonoids in symbiotic and defense functions in legume roots. Botanical Reviews 63: 27-39.

Tanwar B, Modgil R. 2012. Flavonoids: Dietary occurrence and health benefits. Spatula DD 2: 59-68.

Tapas AR, Sakarkar DM, Kakde RB. 2008. Flavonoids as nutraceuticals: A Review. Tropical Journal of Pharmaceutical Research 7: 1089-1099.

Taylor LP, Jorgeinsen R. 1992. Conditional male fertility in chalcone synthase-deficient petunia. Journal of Heredity 83: 11-17.

Veitch NC. 2007. Isoflavonoids of the Leguminosae. Natural Product Reports 24: 417-464.

Weston LA, Mathesius U. 2013. Flavonoids: Their structure, biosynthesis and role in the rhizosphere, including allelopathy. Journal of Chemical Ecology 39: 283-297.

Wink M. 1988. Plant breeding: Importance of plant secondary metabolites for protection against pathogens and herbivores. Theoretical Applied Genetics 75: 225-233.

Wink M. 2003. Evolution of secondary metabolites from an ecological and molecular phylogenetic perspective. Phytochemistry 64: 3-19.

Winkel-Shirley B. 2001. Flavonoid biosynthesis. A colorful model for genetics, biochemistry, cell biology, and biotechnology. Plant Physiology 126: 485-493.

Zar JH. 1999. Biostatistical analysis. Prentice Hall, Upper Saddle River.

Zhuang X, Gao J, Ma A, Fu S, Zhuang G. 2013. Bioactive molecules in soil ecosystems: masters of the underground. International Journal of Molecular Science 14: 8841-8868.