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Original Article

Allelopathy Effects of *Trifolium alexandrium* L. on Germination and Nutrient Uptake in Medicinal Plant *Peganum harmala* L.

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Abstract

The present study focused on effects of *Trifolium alexandrium* L. extract (0.002, 0.004 and control) which is a fast-growing plant on germination, growth and nutrients uptake of medicinal plant *Peganum harmala* L. Results revealed that *T. alexandrium* extract had significant (P<0.01).effect on seed germination of *P. harmala* The maximum (54%) and minimum (28%) seed germination were observed at 0.002 and 0.004 treatments, respectively. The highest radical (11.27 mm) and pedicle (19.10 mm) lengths were obtained in the 0.002 treatment, and the minimum radical (4.05 mm) and pedicle (9.40 mm) length were related to 0.004 treatment. With increased extract concentration, dry weight of *P. harmala* decreased. Also, the maximum (15.79 mg g^{-1} fresh weight) and minimum (6.94 mg g^{-1} fresh weight) chlorophyll contents of *P. harmala* were observed at 0.002 and control treatments, respectively. The highest (6.08 mg g^{-1} fresh weight) and lowest (4.34 mg g^{-1} fresh weight) chlorophyll b and also the highest (4.89 mg g^{-1} fresh weight) and lowest (2.69 mg g^{-1} fresh weight) carotenoids contents were obtained in 0.002 and 0.004 treatments, respectively. Furthermore, *T. alexandrinum* extract had significant effects on the uptake of N, P, K, Zn and Mn. The maximum amount of N uptake (3.77%) was observed in the extract concentration of 0.002. The maximum (0.3%) and minimum (0.21%) P uptake was observed in the control and 0.004 treatments, respectively. With increasing the extract concentration K uptake was decreased. The maximum (0.22 and 0.19%, respectively) and minimum (0.14 and 0.15%, respectively) Zn and Mn uptake were observed in the control and 0.004 treatments, respectively. In general, results showed that high concentration of *T. alexandrium* extract has deterrent effects on seed germination and growth of *P. harmala*, so that lower concentration of extract, showed positive effect on seed germination plant growth.

Keywords*:* Plant growth, Photosynthesis, *Peganum harmala* L., *Trifolium alexandrium* L.

Introduction

Medicinal plants have been used extensively in both pharmaceutical and food industries, with consumers showing increasing interests in these products [1]. These plants are an accessible, affordable and culturally appropriate source of primary health care for more than 80% of world's population [2]. One of the well-known medicinal plant species in Iran is *Peganum harmala* L., The plant is a perennial species from Zygophyllaceae family which grows in arid and semi-arid areas (steppe areas and sandy soils). It is a shrub with 0.3-0.8 m tall and short creeping roots, white flowers and round seed capsules having more than 50 seeds. The plant is widely used as a medicinal plant in central Asia, north Africa and Middle East [3,4]. Some parts of the plant including seeds, fruits and roots, have been used as traditional medicine in Iran and other countries. Many studies have

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reported different effects of *P. harmala* and/or its active alkaloids (particularly harmaline) [5,6]. It has also been shown in various pharmacological studies that *P. harmala* extract and its main active alkaloids, harmine and harmaline have different cardiovascular effects such as bradycardia, decreasing systemic arterial blood pressure and total peripheral vascular resistance, increasing pulse pressure, peak aortic flow and cardiac contractile force [7], Vasorelaxant [8].

However, the quality and quantity of the medicinal plants are influenced by a multitude of factors, chief among them and environmental [1]. Environmental conditions are important factors affecting the yield of the plants. Beside climatic condition and light [9], drought [10], temperature [11], mineral nutrients [12] and edaphic factors [13] at the growing site, others are biochemicals/ allelochemicals [14,15]. Allelopathy is one of the most important interference methods of the plants that can help to know plants interaction [15]. Allelopathy is a biological phenomenon by which an organism produces one or more biochemicals (allelochemicals) that influence the germination, growth, survival, and reproduction of other organisms [16]. Recently allelopathy is considered as a method for reducing environmental contaminations and increasing agricultural products in sustainable agriculture [17]. There are various organic compounds in plants that have an impact on the plants power, seed care and plant production [18,19]. These compounds are classified as secondary plant materials or sub-materials of metabolic pathways of plants, and include terpenes, tannins, alkaloids, flavonoids, phenylpropanoids, quinones, coumarin, phenolic, glycosides [18]. These materials are derived from their branches and leaves or are secreted by the roots to the environment [20].

Nevertheless, biochemicals undoubtedly influence the yield of the plants, their role affecting the growth of plants remains unclear. The aims of the present study were to investigate the effects of *Trifolium alexandrium* L. extract which is fast growing plant species, on seed germination, morphological characteristics and nutrient uptake of *Peganum harmala* L., which is ecologically one of the most important medicinal plant species in southern Iran.

Material and Methods

Pot Preparation and Plant Extract

The present study was carried out in a greenhouse, with 23±5 °C, 60% relative humidity and 70% water-holding capacity of soil. The experimental design was completely randomized with four replications. Soil samples were selected from Deging village, which located in Khash city (Sistan and Baloochestan province in south-east of Iran). Soil samples were obtained from the depth of 0–30 cm with a 5.5 cm diameter hand driven corer and mixed. All soil samples were sieved to 4 mm and moisture contents were adjusted to 70% water–holding capacity (WHC). Characteristics of the soil are listed in Table 1. The soil texture (loamy sand) was determined using laser diffractometry [21]. Soil pH was determined in a 1:5 soil to distilled water slurry after 1 hour of agitation using a digital pH–meter (Model 691, Metrohm AG Herisau Switzerland) [22]; electrical conductivity (ECe) using an EC–meter (DDS-307, Shanghai, China) [23]. Available phosphorus (AP) was determined by the method of Bray and Kurtz [24]. Total soil nitrogen was analyzed calorimetrically with a continuous flow ion analyzer following wet digestion in sulfuric acid using Kjeldahl [25]. Available potassium (AK) was measured by flame photometry method [26].

Table 1 Soil physical and chemical characteristics used in the experiments

Texture	N(total) (%)	P (mg kg^{-1})	K (mg kg^{-1})	EC (dSm^{-1})	pH
Loamy sand	0.09	0.23	90	1.06	8.46

In order to prepare the plant extracts, tissues of *T. alexandrium* L., were collected from Tighab rangeland in Iranshahr city. Plant samples were dried in the shade, and were then ground. Then, 190 g of plant powder was placed in a plastic bottle. One liter of ethanol was poured on powder samples and was placed on shaker for 24 hours. The resulting solution was filtered out, and the extract was obtained. After soil sieving, 3 kg of dried soil were stored in plastic pots (diameter10×diameter 15×height 45 cm). Moisture contents were adjusted to 70% water–holding capacity (WHC). Seeds were disinfected with fungicide solution. Due to deep root of *P. harmala* L., in all treatments, 30 seeds of the plants were buried evenly throughout each pot at least 3 cm from the edge. The treatments comprised the extract of *T. alexandrium* (0.002 extract (2 ml per

1000 ml of distilled water) and 0.004 extract) and distilled water (control). Pots were irrigated with tap water as needed and the experiment terminated 14 days after cultivation. The parameters measured included germination percentage and rate, radical and pedicle length, seedling dry weight, photosynthesis pigment and nutrient uptake of *P. harmala* from the soil.

Calculation of plant properties

Germination seeds were counted and recorded daily [27]. This was done each 24 hours until the germination had been completed. On the last counting day (the 14^{th} day), the radical and pedicle length (using a caliper) and the dry weights of seedling were measured (digital balance with 0.0001 g precision). After 14 days, having fixed the number of the germinated seeds and having finished the growth period, the germination properties, including the germination rate and germination percentage measured according to Eqs. of 1 to 2.

$$GR = \sum Ni / Di \quad (1)$$

where GR, Ni and Di were germination rate, number of germinated seeds in each day and: counted day, respectively [28].

$$GP=(n/N) \quad 100 \quad (2)$$

GP: germination percentage, n: total number of the germinated seeds during counting, N: total number of the seeds in each petri dish [29].

In each pot, 10 seedlings were randomly chosen and the radical and pedicle lengths were calculated by a caliper. Then in order to determine the dry weight, first the samples were washed with distilled water then the plant were placed in the oven (Dena-Iran) in 70 ºC for 48 hours and the seedlings dry weights were measured. To measure chlorophyll content a, b, total chlorophyll, and carotenoids, initially 100 mg of fresh tissue was pulverized inside a porcelain mortar with 5 ml of acetone 80%. Then, the solution was transferred to centrifuge tubes, and remains in the mortar, were washed twice with 5 ml of acetone 80%, and the solution was added to the tube. Then, the tubes were centrifuged for 10 min at 6000 rpm. Material solution was transferred to the 250 mm flask, and its volume by acetone 80% reached to 25 ml. Measurement of chlorophyll was performed using spectrophotometric method (model WPA-S2000). Thus, the amount of solution uptake was read at wavelengths of 470, 663, 645 nm, and the content of chlorophyll a, b and carotenoids were measured using Eqs 3, 4, 5. Total chlorophyll was calculated by sum of chlorophyll a and b in terms of milligrams per gram of sample weight [30].

$$\text{Chlorophyll a} = (19.3 \times A663 - 0.86 \times A645)\, v/100w \quad (3)$$

$$\text{Chlorophyll b} = (19.3 \times A645 - 3.6 \times A663)/ V \quad (4)$$

$$\text{Carotenoides} = 100(A470) - 3.27\ (\text{mg chl.a}) - 104\ (\text{mg chl.b})/227 \quad (5)$$

V = volume of filtrated solution (upper solution of centrifuges)

A = absorption of light at wave lengths of 663, 645 and 470 nm

W = wet weight of sample (g)

Calculation of Plant Nutrient Uptake

The wet oxidation method was used in order to measure the elements absorbed by the plant and plant samples digestion. For this purpose, 0.3 g of plant was transferred to digest pipes, and then 2.5 ml of a mixture of sulfuric acid, salicylic acid, selenium and hydrogen peroxide were added. The sample was shaken until all the particles were wet. The samples were immobilized 2 hours. Then, they were heated for 2 hours at 100 °C, and the tubes were removed. After cooling of the samples, hydrogen peroxide (1 ml) was added. After 10 seconds, pipes were heated (330 °C), digestion is ended when the color of the extract is colorless or pale yellow. This usually lasts 2 hours. After drying, 48.3 ml of distilled water were added to the pipes and, stirred. The next day, the stirring operation was repeated, and was put to itself to be deposited. In the extract obtained, the elements of nitrogen, phosphorous, potassium, zinc and manganese were measured [31]. Manganese and Zn measurements in extract obtained from wet digestion of plant samples using atomic absorption spectrophotometer (GBC Avanta, Australia). Nitrogen was measured by titration, by Kjeldahl method (model Gerhardt 9801/Ac), phosphorus using the colorimetric method using a spectrophotometer (model (JENWAY 640) and K were measured using flame photometer [32].

Data Analysis

Statistical analyses of the experimental data were performed using the SPSS. 20. All reported results are the means of four replicates and deviations were calculated as the standard error of the mean

(SEM). The statistical processing was mainly conducted by analysis of variance (ANOVA). Duncan test post hoc analysis was performed to define which specific mean pairs were significantly different. A probability of 0.05 or lower was considered as significant.

Results

Seed Germination and Morphological Properties

The analysis of variances of *P. harmala* seed germination (Table 2) revealed that *T. alexandrium* extract had significant effect on the seed germination rate and percentage (P<1%). The maximum and minimum germination rate of *P. harmala* were respectively, observed at 0.002 and 0.004 treatments (Table 3). Similar result was observed for germination percentage. The highest and lowest germination percentages were calculated in 0.002 and 0.004 treatments, respectively. The effects of *T. alexandrium* extract on the pedicle length, radicle length and total dry weight (P<1%) were significant (Table 2). The highest radical and pedicle length of the plant were obtained in the 0.002 treatment, and the minimum radical and pedicle length were related to 0.004 treatments (Table 3). The extract of *T. alexandrium* significantly reduced the dry weight of *P. harmala*, and by increasing the concentration of *T. alexandrium* extract, dry weight of *P. harmala* L. decreased. Maximum plant dry weight was measured in the control treatment and, the lowest value was related to 0.004 treatments (Table 3).

Photosynthetic Pigment

T. alexandrinum extract had significant effect on the photosynthetic pigments of *P. harmala* L. (Table 2). The maximum and minimum chlorophyll a contents of *P. harmala* L. were respectively, observed at 0.002 and the control treatments (Table 4). The same trend was observed for total chlorophyll contents. Maximum total chlorophyll was related to the 0.002 treatment, and the minimum value was obtained at the control treatment. The highest and lowest chlorophyll b and carotenoid contents were calculated in 0.002 and 0.004 treatments, respectively (Table 4).

Nutrient Uptake

T. alexandrinum extract had significant effect on uptake of N, P, K, Zn and Mn by tissues of *P. harmala* (P<0.05, P<0.01) (Table 5). The maximum amount of nitrogen uptake was obtained in the extract concentration of 0.002. The minimum amount of N uptake was related to the 0.002 treatment (Table 6). The maximum and minimum P uptakes were observed in the control and 0.004 treatments, respectively. However, there was no significant difference between the control and 0.002 treatments. The highest K uptake was related to the control treatment and with increasing the concentration of *T. alexandrinum* extract the amount of K was decreased. The maximum and minimum Zn and Mn uptake were observed in the control and 0.004 treatments, respectively (Table 6).

Discussion

The most common and unavoidable interaction among the plant communities is the plant-environment interaction. External factors quantitatively affect the plant's growth through their effects on plant development and partitioning of assimilates into vital metabolites [33,34]. Environmental factors often have an especially large influence on the plants yield [35]. Since plants cannot escape from the environmental extremes of light, temperature, and drought, nor move to regions with better nutritional conditions, they have thus evolved highly complex mechanisms to integrate physiology and metabolism in order to adapt to the conditions to which they are exposed [1].

Table 2 Results of analysis of variance of germination, morphological traits and photosynthetic pigments of *Peganum harmala* L. treated with *Trifolium alexandrinum* L. extract

Source of Variation	df	Mean Square								
Treatment	2	Germination rate	Germination percentage	Radical length	Pedicle length	Total dry weight	Chlorophyll a	Chlorophyll b	Total chlorophyll	Carotenoid
		2075.69**	73.44**	27.66**	0.35**	0.52**	34.58**	34.58*	4.31**	4.30**
Error	6	20.57	13.66	0.008	2.15	0.0058	0.02	0.15	0.12	0.02

****** and *significant at 0.01 and 0.05 probability level, respectively.

Table 3 Effect of *T. alexandrinum* L. extract on means of seed germination and morphological traits of *P. harmala* L.

Extract concentration	Germination rate (n/day)	Germination percentage	Radical length (mm)	Pedicle length (mm)	Total dry weight (g pot^{-1})
0.004	8.50 ±0.50 b	28.00 ±2.00 b	4.05±0.50 c	9.40±1.00 c	1.32±0.01 b
0.002	19.30±2.00 a	54.00±4.21 a	11.27±0.73 a	19.10±2.15 a	1.52±0.03 b
Control	18.70 ±1.51 a	52.50±4.00 a	8.26±0.50 b	13.23±2.11 b	3.35±0.10 a

Values within a column followed by the different letters are significantly different (P<0.05, means±SE).

Table 4 Effect of *Trifolium alexandrinum* L. extract on means of photosynthetic pigments of *Peganum harmala* L.

Extract concentration	Chlorophyll a (mg g^{-1} fresh weight)	Chlorophyll b (mg g^{-1} fresh weight)	Total Chlorophyll (mg g^{-1} fresh weight)	Carotenoid (mg g^{-1} fresh weight)
0.004	10.40 ±1.50 b	4.34 ±1.40 b	14.75±2.30 b	2.69±0.20 b
0.002	15.79±2.24 a	6.08±1.43 a	21.79±2.20 a	4.89±1.00 a
Control	6.94±1.33 c	6.00±1.12 a	12.95±2.30 b	3.86±1.00 b

Values within a column followed by the different letters are significantly different (P<0.05, means±SE).

Table 5 Results of analysis of Variance of nutrient uptake in *Peganum harmala* L. treated with *Trifolium alexandrium* L. extract

Source of Variation	df	Mean Square				
Treatment	2	N (%)	P (%)	K (%)	Zn (%)	Mn (%)
		1.84^{**}	0.0098^{**}	0.084^{**}	0.0078^{*}	0.982^{**}
Error	6	0.00007	0.00014	0.00005	0.00009	0.3583

** Significant at the 0.01 probability level.

Table 6 Effect of *Trifolium alexandrinum* L. extract on mean nutrient uptake of *Peganum harmala* L.

Extract concentration	N (%)	P (%)	K (%)	Zn (%)	Mn (%)
0.004	2.57 ±0.02 b	0.21 ±0.01 b	0.22±0.01 b	0.14±0.01 b	0.15±0.01 b
0.002	3.77±0.02 a	0.28±0.01 ab	0.14±0.01 c	0.20±0.01 a	0.18±0.01 a
Control	3.29±0.02 a	0.30±0.01 a	0.43±0.01 a	0.20±0.01 a	0.19±0.01 a

*****Values within a column followed by the different letters are significantly different (p<0.05, means±SE).

Medicinal plants are grown in different ecosystems and sites under the influence of different potential factors, including the biochemicals as the vital determinants in the quantity of the plants [36].

In the present study, the highest seed germination of *P. harmala* happened at 0.002 treatment. The lowest seed germination was related to the 0.004 treatment. Allelopathy effects are most often described based on visual changes in the plant [37]. There is a delay or inhibition of seed germination [38], growth inhibition or stimulation of plants [39]. These actions depend on the concentration of the active substances contained allelochemicals [37]. Plant germination stage has higher the sensitivity than other plant growth stages. Low concentration of *T. alexandrinum* extract had a positive effect on the germination. Because the allelopathy phenomenon much depends on the concentration of the allelochemicals, and may change in the amount of these materials leads to different inhibition and stimulation effect [40]. Ricki Maryshany [36] reported that, allelopathic substances in low concentrations may have positive effects on the target species, but high concentrations are always a deterrent. The reason for reduction of plant germination at 0.004 treatments can be related to the activity of enzymes such as amylase that plays an important role in seed germination [41]. The results indicate that allelopathic compounds reduce plant germination with effect on hormones, such as gibberellins, which is important in plant germination, as well as the effect on the activity of special enzymes, such as amylase and proteinase, which are essential in the process of germination. In addition, reduction in the germination stage may be attributed to

change in the activity of enzymes that affect the transfer of storage compositions during germination [42]. Kazerooni Monfared *et al*. [43], studied the germination of some plant species under the effect of *T. alexandrium* extract, and reported that, with increasing extract concentration, germination percentage decreased. Ghorbanli *et al*. [42] reported that germination percentage significantly was affected by different concentrations of *Artemisia* spp. extracts.

Highest radical and pedicle lengths of *P. harmala* were observed at 0.002 treatment. The lowest radical and pedicle lengths were related to the 0.004 treatment. While, allelopathic effects of *T. alexandrium* increased on the plant dry weight, with increasing concentration of the extract. Allelopathic compounds reduce plant root and shoot growth by having an impact on the root growth, reducing the formation of capillary roots and water absorption in plants. Some mechanisms of allelopathic activities are similar to plant hormones. Allelopathic compounds through having an impact on root growth can reduce water absorption in plants and thereby reduce the length of seedling [44]. Decreased seedlings length of plant which are exposed to allelopathic compounds may be due to the negative effect of the extract on cell division or cell elongation, which in addition to longitudinal growth of the plant, inhibiting substances in extract can have a negative impact on weight of plant [45]. Kazerooni Monfared *et al*. [43] showed that root is the first plant's part that absorbs allelopathic materials directly from the environment, and so compared to other traits may be more affected by allelopathic materials. One reason for reduction of the plant growth during allelopathic stress is changes in mitochondrial respiration rate, which decreases the production of ATP. Decreased ATP production can cause changes in other cellular processes, such as ion adsorption and growth of the consumption of energy. Decreased plant growth in the presence of allelopathic compounds is associated with high mitosis stop of root and shoot's meristem cells and therefore, the length of root and shoot will be reduced [46].

Allelopathic effects of *T. alexandrium* had positive effect on the photosynthetic pigments in the 0.002 treatment (lower concentration). Reducing photosynthesis pigments affects seedling photosynthesis, root and shoot length and plant vigor. Studies have shown that decreased plant growth in the presence of allelochemicals associated with decreased chlorophyll, and decreased chlorophyll may be a secondary effect caused by the performance of special allelochemical [47]. Several authors considered that, decomposition of plant in the soil can prevent plant germination and growth [48]. The water-soluble materials prevent the roots of some plants [49]. Many studies have shown that the remains of some plants have allelopathic properties in the soil and after harvesting released compounds such as phenolic acids had negative effect on the germination of some plants or plant performance [50]. Allelochemicals effects are selective and depend on the concentration and type of residue and, may lead to stimulating the growth inhibitory effects in plants. Allelopathic materials can disrupt their neighboring plants, and affect the amount of photosynthesis pigments in the neighboring plant. The reason for reducing chlorophyll at high concentrations may be decomposition of chlorophyll and carotenoid pigments or reduce the synthesis of them [51]. Reactions and processes like cell division, hormone production, cell membrane stability and permeability, photosynthesis and respiration can be raised as the purpose and effect point for allelopathic material [52].

Results of the present study showed that in lower concentrations of the extract had the positive effect was observed. Nutrient uptake is an important factor for plant growth and development. Studies have shown that the accumulation of many allelopathic factors has an impact on the rate of nutrient uptake. Both increases and decreases in nutrient uptake have been reported for plants that are subject to the allelopathic conditions change. Unstable situation of minerals in receiver plants is created by leaching of plant debris, root exudates and allelopathic debris [53]. It is interesting to note that these effects may be directly related to plants competition and may indirectly be done through microorganisms related to stabilization of nutrients such as nitrogen. Special allelochemical (flavonoids and phenolic acids) prevent the minerals uptake by plant roots, and the mechanism of action of these compounds are done through disrupting the membrane normal actions in plant cells. Allelochemical can reduce cellular ATP content through inhibition of electron transport and oxidative phosphorylation, which are two mitochondrial membrane actions, two of which are

applied, as well as change membrane permeability property compared to inorganic ions uptake [51, 53].

Conclusion

In our study, it has been shown that seed germination, growth and nutrient uptake of *P. harmala* were affected by *T. alexandrium* extract. Inhibition effects of *T. alexandrium* extract indicated that extract of the plant had deterrent effect on the seed germination and growth of *P. harmala*, However, lower concentrations of extract, showed positive effects on the plant growth. In the present study, although the plant extract was not analyzed, but results of different studies showed that compounds such as phenolic and santonian likely be introduced as inhibitory factors of studied characteristics of *T. alexandrium*, However, a definitive statement in this field requires research in which a variety of compounds in the extract of *T. alexandrium* and their concentration to be evaluated in the influenced plants.

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