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Research Article
Nitrotoxins in 13 Species of Papilionoideae (Leguminosae) Trees in Khuzestan Province, Iran

Mitra Noori, Mahdi Talebi and Mehrnoosh Kalantar

Department of Biology, Faculty of Science, Arak University, 38156-8-8349 Arak, Iran

Abstract

Background and Objective: Nitrotoxins or nitroglycosides are aliphatic nitro compounds, which were detected in some legumes (Papilionoideae). They are important due to mammalian toxicities, attraction of pollinators or seed disperses and repulsion or inhibition of herbivores and microorganisms. Legumes nitrotoxins studies have importance from the phytochemistry, chemotaxonomy, domestic nutrition, ecological adaptations and chemodiversity aspects. In this study nitrotoxins of *Acacia farnesiana* (L.) Willd., *Bauhinia purpurea* L., *Cassia aphylla* Cav. and *Prosopis stephaniana* Kunth species were reported for the first time that is important for their chemotaxonomy, toxicity and pollination. **Materials and Methods:** Thirty populations of 13 legume species were collected and identified from various parts of Iran. Their dried leaves analyzed for presence of aliphatic nitro compounds. The qualitative test and quantitative determination for aliphatic nitrotoxins were done using the developed Cooke and modified Williams-Parker methods. Recording the absorption spectrum between 400 and 800 nm was done using Cecill 4400 UV-visible double beam scanning spectrophotometer. **Results:** Nitrotoxins were detected in 5 species (*Acacia farnesiana*, *Bauhinia purpurea*, *Cassia aphylla*, *Prosopis stephaniana* and *Robinia pseudoacacia*) at concentrations ranging from 9-25 NO_2 mg g^{-1} plant. Other examined plant species lacked any nitrotoxins. **Conclusion:** Nitro compounds studies can show plant chemodiversity throughout the Papilionoideae as chemotaxonomic, toxicity and pollination character.

Key words: Nitrotoxins, Leguminosae, Papilionoideae, chemotaxonomy, toxicity, pollination

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Corresponding Author: Mitra Noori, Department of Biology, Faculty of Science, Arak University, P.O. Box 38156-8-8349, Arak, Iran
Tel: 0098 86 34173401-5 Fax: 0098 86 34173406

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Among the chemical compounds in plants, secondary metabolites, e.g., nitrotoxins are of great importance in plant-environment relationship and play a role in plant defense[1,2]. Their functions include the attraction of pollinators or seed disperses and repulsion or inhibition of herbivores and microorganisms[3]. Nitrotoxins are relatively stable as they decompose slowly over several decades and may be detected in plants for up to 50 years[4]. Nearly a century has passed since Marsh and Clawson[5] first reported livestock poisonings caused by plants now known to contain nitrotoxins. Since then, poisonings in cattle, sheep, goats, horses and insects by plants containing 3-nitro-1-propanol (nitropropanol) or 3-nitro-1-propanoic acid (nitropropionic acid) have been documented[6,7]. Some studies showed that environmental stresses such as pollutant, rays, dry and/or drought conditions affect nitrate concentrations and increased nitrotoxins. It is believed that nitro compounds are increased against stresses injuries for defense, confronting and adaptation. Nitro compounds are suggested as playing a defensive role in plants against stressed environmental conditions and also are important as chemotaxonomic character in legumes chemodiversity[8-11].

Studies on *Coronilla varia* L., revealed that environmental stresses affect nitrate concentrations and nitrotoxins are increased in dry and/or drought, polluted and ray conditions[2]. Nitrotoxins or nitroglycosides are aliphatic nitro compounds with chemical or structural glucose esters of 3-nitro-1-propionic acid (nitropropionic acid ¼ 3-NPA) and 3-nitro-1propanol (nitropropanol ¼ 3-NPOH), which were detected in some legumes: Papilionoideae[3]. Worldwide, thousands of plant species are known to contain nitro-compounds. Namely plants of some *Astragalus* and *Coronilla* spp., contain considerable amounts of these toxins and they are often a causative agent for intoxication of cattle, sheep and horses. Nitrotoxins produced lethality and may be harmful for human[12]. Benchadi *et al.*[13] isolated and used *Astragalus cruciatus* Link., secondary metabolites as chemotaxonomic significance for showing legume chemodiversity. A classic example of plant having nitrotoxins is *Astragalus* species. More than 450 *Astragalus* species are known to contain nitro-compounds, as either nitroproanoic acid (NPA) and it's derivatives or nitropropanol and it's derivatives[14]. Ebrahimzadeh *et al.*[15] found nitro compounds in 37 species of *Astragalus*. Glucose esters of NPA such as karakin were the first nitro compounds to be isolated[3]. Miserotoxin, the β-D glucoside of 3-NPOH (3-nitro-l-propyl-β-D-glucopyranoside) is synthesized in large quantities by timber milkvetch (*Astragalus miser* var. *serotinus*)[16] and by many other species of *Astragalus*[14]. It was first isolated from *Astragalus miser* var., *oblongifolius* Dougl. ex Hook.[3,17]. Miserotoxin and its aglycone, 3-nitropropanol (NPOH) have been detected in about 50 species and varieties of *Astragalus* (Leguminosae) primarily from the temperate regions of North and South America[14,18]. Other legumes such as *Coronilla*, *Indigoferra* and *Lotus* have been found to synthesis NPA[19]. Three new 3-nitropropanoyl-D-glucopyranoses: (1) Corollin, (2) Coronillin and (3) Coronarian were isolated from the aerial parts of *Coronilla varia* L.[20]. Further karakin and cibarian were identified in *C. varia* by Majak and Bose[21]. Hutchins *et al.*[22] studied nitro compounds in *Lotus pedunclatus* and species of *Indigofera*[23]. Hipkin *et al.*[24] detected nitro compounds in *Hippocrepis comosa*[24]. Ebrahimzadeh *et al.*[15] reported approximate equivalents[14] of NO_2 in mg g^{-1}. Noori *et al.*[25] compared nitro compound quantities in different populations of *C. varia* L., *Lotus corniculatus* L. and *Astragalus agubensis* Bunge from various parts of Markazi province in Iran. Data showed that environmental conditions affect nitrate concentrations and nitrotoxins are increased in dry and/or drought conditions. They suggested that nitroxins play a defensive role in plants against stressed environmental conditions. Both 3-nitropropionic acid and 3-nitro-1-propanol are the most important representatives of nitrotoxins, which are toxic principles of many leguminous plants[2]. Ebrahimzadeh *et al.*[26] studies on 111 specimens from 82 legume species showed existing nitro compounds in 4 *Ammodendron* species: *Ammodendron ammodendroides* Bornm., *Cystium mazandaranus* Bunge, *Incani robustus* Bunge and *Uliginosi odoratus* Lam. They reported presence of nitro toxins in the first three species for the first time[26]. The presence of these compounds in *A. odoratus* Lam., has been reported previously[23]. In this study identification of the qualitative test and quantitative determination for aliphatic nitrotoxins in 30 populations of 13 legume species (*Acacia coriacea* DC., *A. farnesiana* (L.) Willd., *A. saligna* (Labill.) Wendl., *Albiziza lebbeck* (L.) Benth., *Alhagi manifera* Desf., *Bauhinia purpurea* L., *Caesalpinia gilliesii* (Hook.) Dietr., *Cassia aphylla* Cav., *Leucaena leucephala* Lam., *Prosopis juliflora* DC., *P. stephaniana* Kunth., *Robinia pseudoacacia* L. and *Sesbania sesban* (L.) Merrill.) leaves were done that some of them are reported for the first time.

MATERIALS AND METHODS

Collection of plant material and preparation: Thirty populations of 13 species (*Acacia coriaceae* DC., *A. farnesiana* (L.) Willd., *A. saligna* (Labill.) Wendl., *Albiziza lebbeck* (L.) Benth., *Alhagi manifera* Desf., *Bauhinia purpurea* L., *Caesalpinia gilliesii* (Hook.) Dietr., *Cassia aphylla* Cav., *Leucaena leucephala* Lam., *Prosopis juliflora* DC., *P. stephaniana* Kunth., *Robinia pseudoacacia* L. and *Sesbania sesban* (L.) Merrill.) were collected from various parts of Khuzestan province, Iran. Details of the examined samples are given in Table 1. Specimens of each sample were prepared for reference as herbarium vouchers. Samples were air dried for detection and identification of nitrotoxins.

Qualitative and quantitative tests for nitro compounds: Twenty milligrams of dried leaflets of 10 specimens of each species were removed from collected samples and analyzed for presence of aliphatic nitro compounds. The qualitative test for aliphatic nitrotoxins was developed by Cooke[27] and modified by Williams and Parker[28] for quantitative determination. Ten milligrams of leaflet were placed into each of two test tubes and macerated to a fine powder with a stirring rod. One milliliter of 1 N HCl was added to each test tube and the solutions allowed to stand with frequent stirring for 2 h. One milliliter of 20% KOH was added to each tube and the test tubes were kept at room temperature for another 2 h. One milliliter of glacial acetic acid, followed immediately by 1 mL of Griess-Ilosvay reagent[27] was then added to one test tube. Two milliliters of glacial acetic acid were added to the second tube that served as control. Color was allowed to develop for 3 min. Solutions that contained nitrotoxins turned pink to red within a few seconds. The intensity of the red color was determined visually. Nitro content was ranked on a scale of T–5. Ranking and their approximate equivalent in NO_2 mg g^{-1} of plant were: T (Trace) = 2–3, 1 = 4–8, 2 = 9–13, 3 = 14–19, 4 = 20–25 and 5 = over 25.

Reading absorption spectrum (spectrophotometry): Cecill 4400 UV-visible double beam scanning spectrophotometer with 10 mm matched quartz cells was used for recording the absorption spectrum between 400 and 800 nm, after the colored reaction mixture was filtered through No. 1 filter paper.

Table 1: Collection information of 30 collected populations from 13 legume trees species from various parts of Khuzestan province, Iran

Voucher data	Taxon	Locality	Dates	Altitude (m)	Longitude	Latitude	NO_2
*CMK_1	*Acacia coriacea*	Ahvaz-Golestan	28.01.2015	22.50	31°20' N	48° 40' E	+
CMK_2	*Acacia coriacea*	Gotvand-Kushkak	17.02.2015	72.60	32°15' N	48° 49' E	-
CMK_3	*Acacia farnesiana*	Molasani-Kooye Ghods	08.04.2014	29.60	31°35' N	48°53' E	-
CMK_4	*Acacia farnesiana*	Shush-Shahid Mostafa Khomeyni Ave.	07.04.2014	74.80	32°12' N	48°15' E	-
CMK_5	*Acacia saligna*	Mahshahr-Chamran Suburb	07.02.2015	15.00	34°30' N	49°10' E	-
CMK_6	*Acacia saligna*	Gotvand-Kushkak	23.11.2014	72.60	32°15' N	48°49' E	-
CMK_7	*Albizia lebbeck*	Izeh-Felestin Blv.	28.04.2014	842.80	31°48' N	49°54' E	-
CMK_8	*Albizia lebbeck*	Baghmalek-Ghaletol	28.04.2014	708.00	31°31' N	49°51' E	-
CMK_9	*Albizia lebbeck*	Mahshahr-Chamran Suburb	24.04.2014	15.00	34°30' N	49°10' E	-
CMK_{10}	*Albizia lebbeck*	Ahvaz-Golestan	17.04.2014	22.50	31°20' N	48°40' E	-
CMK_{11}	*Albizia lebbeck*	Gotvand-Kushkak	18.04.2014	72.60	32°15' N	48°49' E	-
CMK_{12}	*Alhagi mannifera*	Abadan-Faiie	05.04.2014	4.10	30°22' N	48°20' E	-
CMK_{13}	*Alhagi mannifera*	Ahvaz-Golestan	05.04.2014	22.50	31°20' N	48°40' E	-
CMK_{14}	*Bauhinia purpurea*	Shush-Shahid Mostafa Khomeyni Ave.	07.04.2014	74.80	32°12' N	48°15' E	+
CMK_{15}	*Bauhinia purpurea*	Izeh-Felestin Blv.	09.04.2014	842.80	31°48' N	49°54' E	-
CMK_{16}	*Bauhinia purpurea*	Baghmalek-Ghaletol	09.04.2014	708.00	31°31' N	49°51' E	-
CMK_{17}	*Caesalpinia gilliesii*	Ahvaz-Golestan	17.04.2014	22.50	31°20' N	48°40' E	-
CMK_{18}	*Cassia aphylla*	Ahvaz-Golestan	28.01.2015	22.50	31°20' N	48°40' E	+
CMK_{19}	*Leucaena leucocephala*	Molasani-Kooye Ghods	27.04.2014	29.60	31°35' N	48°53' E	-
CMK_{20}	*Leucaena leucocephala*	Shush-Shahid Mostafa Khomeyni Ave.	18.04.2014	74.80	32°12' N	48°15' E	-
CMK_{21}	*Leucaena leucocephala*	Khoramshahr-Taleghani Alley	05.04.2014	5.20	30°29' N	48°15' E	-
CMK_{22}	*Leucaena leucocephala*	Ahvaz-Golestan	17.04.2014	22.50	31°20' N	48°40' E	-
CMK_{23}	*Prosopis juliflora*	Shush-Shahid Mostafa Khomeyni Ave.	18.04.2014	74.80	32°12' N	48°15' E	-
CMK_{24}	*Prosopis juliflora*	Mahshahr-Chamran suburb	24.04.2014	15.00	34°30' N	49°10' E	-
CMK_{25}	*Prosopis juliflora*	Khoramshahr-Taleghani Alley	05.04.2014	5.20	30°29' N	48°15' E	-
CMK_{26}	*Prosopis juliflora*	Abadan-Faiie	05.04.2014	4.10	30°22' N	48°20' E	-
CMK_{27}	*Prosopis juliflora*	Ahvaz-Golestan	17.04.2014	22.50	31°20' N	48°40' E	-
CMK_{28}	*Prosopis stephaniana*	Ahvaz-Golestan	17.04.2014	22.50	31°20' N	48°40' E	+
CMK_{29}	*Robinia peseudoacacia*	Izeh-Felestin Blv.	09.04.2014	842.80	31°48' N	49°54' E	+
CMK_{30}	*Sesbania sesban*	Ahvaz-Golestan	28.01.2015	22.50	31°20' N	48°40' E	-

*CMK: Mehrnoosh Kalantar collection number

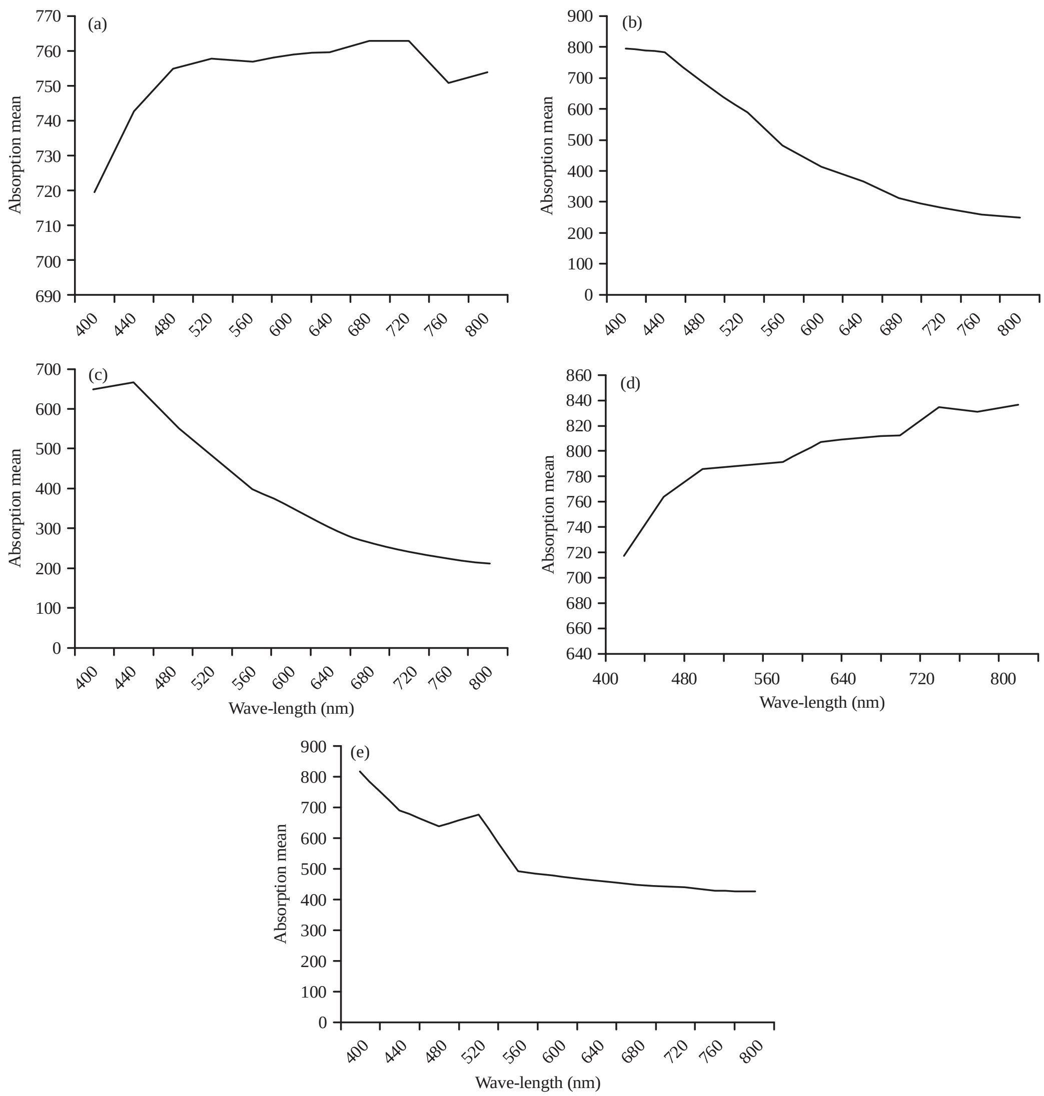

Fig. 1(a-e): Absorption spectra of nitro compounds of a sample with rank scores (lines show means) (a) *Acacia farnesiana* (CMK_1), (b) *Bauhinia purpurea* (CMK_{14}), (c) *Cassia aphylla* (CMK_{18}), (d) *Prosopis stephaniana* (CMK_{28}) and (e) *Robinia pseudoacacia* (CMK_{29})

Table 2: Nitro compounds present

Voucher data	Species	λ max mean	Absorption mean	Scored NO_2 concentration[a]
*CMK_1	*Acacia. farnesiana* (L.) Willd.	700	763	2.5
CMK_{14}	*Bauhinia purpurea* L.	400	796	4.0
CMK_{18}	*Cassia aphylla* Cav.	440	666	3.5
CMK_{28}	*Prosopis stephaniana* Kunth.	800	837	3.0
CMK_{29}	*Robinia pseudoacacia* L.	400	813	2.0

*CMK: Mehrnoosh Kalantar collection number, [a]Approximate concentration (NO_2 mg g^{-1} dry weight) represented by rank score are: 0 = 1-3, 1 = 4-8, 2 = 9-13, 3 = 14-19 and 4 = 20-25

RESULTS

Table 1 shows collection information of 30 populations of 13 legume trees species from various parts of Khuzestan province, Iran and also NO_2 presence or absence in each populations. Nitrotoxin concentrations based on a scale of 1-4 of five legume trees species have been shown in Table 2.

Figure 1a-e show absorption spectra of nitro compounds of a sample with rank scores (lines show means): (a) *Acacia* *farnesiana* with a score 2.5, (b) *Bauhinia purpurea* with a score 4, (c) *Cassia aphylla* with a score 3.5, (d) *Prosopis stephaniana* with a score 3 and (e) *Robinia pseudoacacia* with a score 2. As Table 1 and 2 and also Fig. 1 show, nitrotoxins were detected in 5 species (*Acacia farnesiana*, *Bauhinia purpurea*, *Cassia aphylla*, *Prosopis stephaniana* and *Robinia pseudoacacia*) at concentrations ranging from 9-25 NO_2 mg g^{-1} plant. Other examined plant populations lacked any nitrotoxins.

DISCUSSION

In this study nitrotoxins were detected in 5 species (*Acacia farnesiana*, *Bauhinia purpurea*, *Cassia aphylla*, *Prosopis stephaniana* and *Robinia pseudoacacia*) and other examined plant species lacked any nitrotoxins. Based on the studies of Willams and Barneby[29], nitrotoxins present at levels 4 and 5 in plant taxa are more toxic than those under a 4 ranking. Then, *Bauhinia purpurea* L., with a score of 4 NO_2 concentration was more toxic than other species (Table 1, Fig. 1). Among the chemical compounds in plants, nitrotoxins are of great importance in the plant-environment relationship and play a role in plant defense[2]. Nitrotoxins are relatively stable as they decompose slowly over several decades and may be detected in plants for up to 50 years[4]. Therefore, all dried and fresh materials of nitrotoxin-bearing studied species may be poisonous to cattle and sheep.

Noori *et al*.[25] studies on six collected legume species including *Alhagi camelorum* Fisch., *Cercis siliquastrum* L., *Glycyrrhiza glabra* L., *Medicago sativa* L., *Robinia peseudoacacia* L. and *Sophora alopecuroides* L., from an aluminum reduction plant area in Iran showed all of polluted samples, with the exception of *C. siliquastrum* and *G. glabra* had nitrotoxins at concentrations ranging from 4-13 NO_2 mg g^{-1} in their leaves comparing to control. Also results of Noori and Hatami[11] studies on 20 Papilionoideae species showed detection of nitrotoxins in 4 species (*Coronilla varia* L., *Lotus corniculatus* L., *Astragalus ajubensis* Bunge and *Hyppocrepis constricta* L., at concentrations ranging from 4-25 mg NO_2 mg g^{-1} plant while o ther examined plant species lacked any nitrotoxins[11]. Ebrahimzadeh *et al*.[26] studies on 111 specimens from 82 legume species showed existing nitro compounds in 4 *Ammodendron* species: *Ammodendron ammodendroides* Bornm., *Cystium mazandaranus* Bunge, *Incani robustus* Bunge and *Uliginosi odoratus* Lam.

CONCLUSION

In this study the nitrotoxins of *Acacia farnesiana* (L.) Willd., *Bauhinia purpurea* L., *Cassia aphylla* Cav. and *Prosopis stephaniana* Kunth., species were reported for the first time. Nitrotoxins as one set of secondary metabolites are of great importance in plant-environment relationship and play a role in plant defense. They were detected in some legumes (Papilionoideae) and these toxins and they are often a causative agent for intoxication of cattle, sheep and horses. Nitrotoxins produced lethality and may be harmful for human. They are important due to mammalian toxicities, attraction of pollinators or seed disperses and repulsion or inhibition of herbivores and microorganisms. Some studies showed that environmental stresses such as pollutant, rays, dry and/or drought conditions affect nitrate concentrations and increased nitrotoxins. It is believed that nitro compounds are increased against stresses injuries for defense, confronting and adaptation.

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