Fluoride pollutants as causative agents for nitrotoxins generated in some legume plants

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(Received 7 January 2009; final version received 17 February 2009)

Leaflets of six collected legume species including \textit{Alhagi camelorum} Fisch., \textit{Cercis siliquastrum} L., \textit{Glycyrrhiza glabra} L., \textit{Medicago sativa} L., \textit{Robinia pseudoacacia} L., and \textit{Sophora alopecuroides} L. from an aluminum reduction plant area in Iran were analyzed for presence of toxic aliphatic nitro compounds. In addition, studies were conducted to determine fluoride (Fl) levels in these plants. Controls were from 10 km distance of the factory. Results showed high Fl concentration in all polluted samples. Phytochemical changes in all polluted leaves, with the exception of \textit{C. siliquastrum} and \textit{G. glabra}, were observed with presence of nitrotoxins.

Keywords: fluoride; nitrotoxin; pollutant; legumes; Iran

Introduction

Fluoride (Fl) is the most phytotoxic of the common air pollutants emanating from many industrial processes, mostly aluminum (Al) smelters. Fl is considered harmful for both humans and plants (Fornasiero 2001). More is known about the biological effects of Fl on vegetation than any other air pollutant. The ecological effects of Fl are well-established (Cape, Fowler, and Davison 2003), and a summary of symptoms and sensitive species is provided by Weinstein, Davison, and Arndt (1998). At lower ambient concentrations, a number of physiological and biochemical changes may be initiated by Fl in plants without the appearance of visible injury symptoms (Maclean and Schneider 1981).

Most plant material in the absence of any pollution sources contains between 2 and 20 \(\mu\)g Fl per gram on a dry weight basis. Fl concentrations in pastures range from 0.7 to 16 \(\mu\)g Fl per gram (Robinson and Edgington 1946; Manley et al. 1975; Cronin et al. 2000). Noori (1991) showed Fl concentrations in collected \textit{Medicago sativa} from pot room area in the Iranian Aluminum Company (IRALCO) to be 210 and 94 parts per million (ppm) for \textit{Cercis siliquastrum}. Haidouti, Chaonopoulou, and Chronopoulos (1993) reported that the average levels of Fl in vegetation ranged from 621.2 to 257.2 ppm in severely damaged areas and from 144.3 to 64.1 ppm in lightly damaged areas. Fl has long been recognized as a potent metabolic inhibitor, which interferes with the metabolism of proteins, lipids, and carbohydrates (Wilde and Yu 1998).

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 (Kutchan 2001; Noori, Chehregany, and Hatami 2007). Nitrotoxins are relatively stable...
as they decompose slowly over several decades, and may be detected in plants for up to 50 years (Williams 1981b). Majak (2001) showed that the concentrations of toxic glycosides in plants are significantly affected by physiological stress. Further conditions of environmental stress, such as industrial chemicals, may restrict plant growth and photosynthetic rates. Such conditions lead to the synthesis of compounds derived from secondary metabolism, with one of the group being the nitrotoxins (Rama Devi, Pellissier, and Prasad 1997; Chaves and Escudero 1999).

Nitrotoxins or nitroglycosides are aliphatic nitro compounds with chemical or structural glucose esters of 3-nitro-1-propionic acid (nitropropionic acid = 3-NPA) and 3-nitro-1-propanol (nitropropanol = 3-NPOH), which were detected in some legumes (Majak 2001). Ebrahimzadeh, Nikhnam, and Maassoumi (1999) found nitro compounds in 37 species of Astragalus. Glucose esters of NPA such as karakin were the first nitro compounds to be isolated (Majak 2001). Miserotoxin, the β-D-glucoside of 3-NPOH, was first isolated from Astragalus miser var. oblongifolius Dougl. ex Hook. (Stermitz, Norris, and Williams 1969; Majak 2001). The functions of nitrotoxins include the attraction of pollinators or seed disperses and the repulsion or inhibition of herbivores and microorganisms (Majak 2001). Three new 3-nitropropanoyl-D-glucopyranoses: (1) corollin, (2) coronillin, and (3) coronarian, were isolated from the aerial parts of Coronilla varia L. (Moyer et al. 1977). Further karakin and cibarian were identified in C. varia by Majak and Bose (1976). Hutchins and others (1984) studied nitro compounds in Lotus pedunculatus and species of Indigofera (Williams 1981a). Hipkin and others (1999) detected nitro compounds in Hippocrepis comosa. Ebrahimzadeh, Nikhnam, and Maassoumi (1999) reported approximate equivalents of NO2 in (mg g\(^{-1}\)) plant in examined Astragalus, ranging from 4 to 25 mg g\(^{-1}\). Noori, Chehreghany, and Hatami (2007) 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 that nitrotoxins are increased in dry and/or drought conditions. Noori, Chehreghany, and Hatami (2007) suggested that nitroxs play a defensive role in plants against stressed environmental conditions. Tang and others (1995) showed drought stress of Cyperus rotundus L. resulted in an increased release of toxic secondary metabolites to the rhizosphere.

Both 3-nitropropionic acid and 3-nitro-1-propanol are the most important representatives of nitrotoxins, which are toxic principles of many leguminous plants. Namely plants of some Astragalus spp. contain considerable amounts of these toxins and they are often a caustive agent for intoxication of cattle, sheep, and horses. Nitrotoxins produced lethality and may be harmful for humans (Patočka et al. 2000). The aim of this study was to determine levels of Fl in six collected legume species: Alhagi camelorum Fisch., C. siliquastrum L., Glycyrrhiza glabra L., M. sativa L., Robinia pseu-do-acacia L., and Sophora alopecuroides L. grown in a pot room area in IRALCO and identification of the qualitative test and quantitative determination for aliphatic nitrotoxins in their leaves. The occurrence of nitrotoxins in polluted A. camelorum, M. sativa, R pseu-do-acacia and S. alopecuroides in plants in Iran are reported for the first time.

Materials and methods

Study site

The IRALCO is situated on the northeastern region of Arak, Iran (lat. 34°06′N, long. 49°46′E) with a 232 hectares area. The mean temperature varies between +39.4°C and −23.6°C (IRO 2000).
Collection of plant material and preparation
Mature fresh leaves of six legume species (A. camelorum Fisch., C. siliquastrum L., G. glabra L., M. sativa L., R. peseudo-acacia L., and S. alopecuroides L.) in similar size and age were collected from the IRALCO area during 2007. Control samples of each species were also collected up to a distance of 10 km from the factory. Specimens of each sample were prepared for reference as herbarium vouchers. Samples were air-dried for Fl content as well as detection and identification of nitrotoxins.

Determination of fluoride
Air-dried leaves samples were oven-dried in paper sacks at 80°C for 48 h, finely ground and stored in a dry place until used. A potentiometric method outlined by the Association of Official Analytical Chemists was followed in preparing plant samples for Fl determinations and in making Fl standard curves (AOAC 1980). Analysis and digestion of all prepared plant material were conducted based on Jacobson and others (1966) and Campbell (1987). Digested aqueous solution were analyzed using a Fluoride Combination Electrode (P/N: FQ0502-QQ3B) and a pH meter (Consort SER. NO: 013076) after calibration. Then the amount of Fl in each sample was calculated as ppm dry weight of plant material.

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 (1955) and modified by Williams and Parker (1974) 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 were 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 (Cooke 1955), was then added to one test tube. Two milliliters of glacial acetic acid was 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 NO2 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)
Cecil 4400 UV visible double beam scanning spectrophotometer with 10 mm matched quartz cells was used for recording the absorption spectrum between 400–800 nm, after the colored reaction mixture was filtered through No. 1 filter paper.

Results
All samples in this study contained leaf Fl concentrations, but nitro compounds were found in four polluted species’ responses to Fl emissions as evidenced from nitrotoxin values.
Data in Table 1 shows the leaf Fl (ppm) and nitrotoxin concentrations based on a scale of 1–4 of legume species from control and the IRALCO polluted area. The species *M. sativa* had the highest Fl concentration (226 ppm) in the polluted area and *A. camelorum* Fisch. had the lowest (88 ppm). Nitro compounds were not found in all of the control examined taxa. All of the polluted studied samples, with the exception of CMJ149 (*C. siliquastrum*) and CMJ150 (*G. glabra*), showed variation in NO₂ mg g⁻¹ (nitrotoxins) approximately equivalent to that in their leaves. Four of the spectra are provided in Figure 1. The figure shows absorption spectra of nitro compounds of a sample with rank scores (lines show means): (a) polluted *A. comelorum* with a score 3, (b) *M. sativa* L. with a score 1, (c) *R. peseudo-acacia* L. with a score 2, and (d) *S. alopecuroides* L. with a score 3. Figure 2 shows a comparison of leaf Fl concentration (ppm) and approximate equivalent in NO₂ mg g⁻¹ in six legume species from the IRALCO polluted area.

**Discussion**

The Al reduction plant at Arak, Iran, has produced Fl emissions since it started in 1972. These emissions are responsible for damage to vegetation growing on areas close to the plant (Noori 1991). All polluted samples contained high Fl concentrations in their leaves and showed that there are wide variations in plant response to Fl emission. This may be attributed to different deposition rates, substrates and/or tolerance. Based on polluted samples data, it is believed that *M. sativa* and *S. alopecuroides* are accumulator plants (Table 1). More is known about the biological effects of Fl on vegetation than any other air pollutant. At lower ambient concentrations, a number of physiological and biochemical changes may be initiated by Fl in plants without the appearance of visible injury symptoms. Some of these changes may have important consequences such as reductions in growth or yield (Maclean and Schneider 1981). Fl has long been recognized as a potent metabolic inhibitor, which interferes with the metabolism of proteins, lipids, and 50 carbohydrates (Wilde and Yu 1998). Inhibition of nitrogen fixation by Fl was noted in legumes by Sheridan (1979). Although the mechanism involved in the inhibition is not completely understood, Fl blocks enzymes that require such cofactors as Ca²⁺, Mg²⁺, and Mn²⁺ ions (Wilde and Yu 1998). An effect of Fl on a physiological process may be a reflection that one or more enzyme system is affected by Fl, and metabolic steps are affected by it (Yang and Miller 1963).

Among the chemical compounds in plants, nitrotoxins are of great importance in the plant–environment relationship and play a role in plant defense (Noori, Chehreghany, and Hatami 2007). Nitrotoxins are relatively stable as they decompose slowly over several decades and may be detected in plants for up to 50 years (Williams 1981b). Majak (2001) showed that the concentrations of toxic glycosides in plants are affected significantly by physiological stress. Furthermore, conditions of environmental stress, such as industrial chemicals, may restrict plant growth and photosynthetic rates. Such conditions lead to the synthesis of compounds deriving from secondary metabolism, with one of the groups being the nitrotoxins (Rama Devi, Pellissier, and Prasad 1997; Chaves and Escudero 1999). These compounds may either be the cause or the product of abnormal metabolism. Quantitative and qualitative analyses of such accumulated metabolites could be valuable in implicating the possible site of Fl action (Yang and Miller 1963). Table 1, and Figures 1 and 2 show a wide variation in leaf nitrotoxins in studied polluted samples. All collected samples in this study were of reliable age and condition for nitro analysis. All studied control samples lacked nitrotoxins in their leaves, whereas nitrotoxins
Table 1. Nitro compounds present and concentrations of fluoride (ppm) in foliage of six studied legume species from control and the IRALCO polluted area.

<table>
<thead>
<tr>
<th>Voucher data</th>
<th>Species</th>
<th>Mean ± SD Fl content (ppm)</th>
<th>Scored NO$_2$ concentration$^a$</th>
<th>$\lambda_{\text{max}}$ mean</th>
<th>Absorption mean</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Control(Polluted)</td>
<td>C (P)</td>
<td>C (P)</td>
<td>C (P)</td>
</tr>
<tr>
<td>CMJ148$^a$</td>
<td><em>Alhagi camelorum</em> Fisch.</td>
<td>57 ± 1.67 (88 ± 2.77)</td>
<td>0 (3)</td>
<td>420 (510)</td>
<td>0.11 (0.28)</td>
</tr>
<tr>
<td>CMJ149</td>
<td><em>Cercis siliquastrum</em> L.</td>
<td>32 ± 1.49 (89 ± 1.99)</td>
<td>0 (0)</td>
<td>– (–)</td>
<td>– (–)</td>
</tr>
<tr>
<td>CMJ150</td>
<td><em>Glycyrrhiza glabra</em> L.</td>
<td>43 ± 2.16 (180 ± 2.24)</td>
<td>0 (0)</td>
<td>– (–)</td>
<td>– (–)</td>
</tr>
<tr>
<td>CMJ151</td>
<td><em>Medicago sativa</em> L.</td>
<td>24 ± 1.32 (226 ± 1.07)</td>
<td>0 (1)</td>
<td>480 (510)</td>
<td>0.043 (0.093)</td>
</tr>
<tr>
<td>CMJ152</td>
<td><em>Robinia pseudo-acacia</em> L.</td>
<td>62 ± 1.57 (175 ± 1.89)</td>
<td>0 (2)</td>
<td>430 (490)</td>
<td>0.063 (0.162)</td>
</tr>
<tr>
<td>CMJ153</td>
<td><em>Sophora alopecuroides</em> L.</td>
<td>1.5 ± 1.59 (170 ± 2.38)</td>
<td>0 (3)</td>
<td>430 (510)</td>
<td>0.152 (0.28)</td>
</tr>
</tbody>
</table>

Notes: Data are given as mean ± SD.

$^a$CMJ=Mehrana Jafari collection numbers; C=control; P=polluted; $^b$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; 4 = 20–25.
were found in all examined polluted taxa, except CMJ149 (C. siliquastrum) and CMJ150 (G. glabra). Therefore, these samples were deleted in Figure 1. The absence of nitrotoxins in all of the control samples and the existence of these compounds in four polluted samples show that Fl compounds suggest that some enzymes are needed for nitrotoxins synthesis. In this study, it is clear that producing nitrotoxins in leaf of four polluted plants is a
response to Fl pollutants and may play a protective defensive role against Fl pollution. Noori, Malayeri, and Jafari (2008) found phytochemical changes in polluted leaves having high Fl concentration with appearance or disappearance of some flavonoids.

There are many interactive effects of several concurrent stresses from air pollution and environmental conditions. Noori, Chehereghany, and Hatami (2007) revealed that environmental conditions affect nitrate concentration and that nitrotoxins are increased in dry and/or drought conditions. Data suggested that nitrotoxins play a defensive role in plants against stressed environmental conditions. Cowgill (1991) showed NO2 concentrations to be significantly different in the two climatic years. There was variation in nitrite production in the leaves of various species of Astragalus over a six-year period. It was discovered that, in drought years, the nitrate concentration in leaves was consistently higher than in wet years or those of adequate moisture. Tang and others (1995) showed that drought stress of C. rotundus L. resulted in an increased release of toxic secondary metabolites to the rhizosphere. Günthardt-Goerg and Vollenweider (2007) reported stress associated with a biotic (bacteria, fungi, and insects) or abiotic (frost, drought, mineral deficiency, heavy metal pollution in the soil, acidic deposition, and ozone) helpful for the validation of symptoms in broadleaved and conifer trees. Differentiation of changes in the leaf or needle physiology, through aging, senescence, accelerated cell senescence, programmed cell death and oxidative stress, provides additional clues, raising diagnosis efficiency, especially in combination with information about the target of the stress agent at the tree, leaf/needle, tissue, cell and ultra-structural level (Günthardt-Goerg and Vollenweider 2007). Secondary metabolites and their derivatives may play vital roles in plant development and defense and provide a local or a systemic defense response to pathogen, herbivore attack and other environmental stresses.

Acknowledgments

The authors would like to thank many people at the Iranian Aluminum Company (IRALCO) for allowing us to use the facilities and for help in our study. Amongst these are M. Marjani, IRALCO Manager; A. Ahmadi, IRALCO Research Manager; M. Talebi, IRALCO Research Expert; Dr. M. Ramazani, IRALCO Central Laboratory Manager; F. Mohamedi, H. Davoodzadeh, and A. Paghoheshfar, IRALCO Central Laboratory Experts. Our thanks go also to some personnel of the Biology Department in Arak University. We wish to thank Dr. J. Zolgharnin and Dr. H. Khammohamed of the Chemistry Department in University of Arak. Also, we would like to thank Miss A. Hatami, Laboratory Expert of the Plant Physiology Lab at Arak University. Our thanks to Mr. A. Bakhshi, secretary of Biology Department in Bu-Ali Sina University.

References


