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Structure–Phytotoxicity Relationship: Comparative Inhibition of Selected Nitrogen-Containing Aromatics to Root Elongation of *Cucumis sativus*

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Terrestrial macrophytes are primary producers of O_2 and useful energy and organic substances and they are essential in the processes of nutrients cycling and soil and sediment stabilization. The effect on plants can directly affect structure and function of an ecosystem, resulting in oxygen deletion, decreased primary productivity, increased surface runoff and soil erosion and degradation of wildlife habitat (Freemark and Boutin 1994). Phytotoxicity tests, especially the seed germination and root elongation test have several advantages over other toxicity systems (Mayer and Poljakoff-Mayber 1982), which can be summarized as their high sensitivity due to the rapid metabolism, nutrient transport and cell division occurring in germination period, suitability for toxicity studies of unstable compounds or samples with renewal or flow-through methods besides static method, easiness to be stored for long time and minimal maintenance cost. In addition, the test can be activated quickly and does not need plant nutrients and adjutants in the water control (Wang 1991).

Due to the many origins and magnitude of uses, nitrogen-containing aromatic compounds are widespread in ecosystem and consequently have a high potential for environmental pollution. Toxicity of nitrogen-containing aromatic compounds to *Tetrahymena pyriformis* and/or *Pimephales promelas* were extensively studied (Moulton and Schlutz 1986; Schlutz et al.1987; 1989; 1991; Arnold et al. 1990; Jaworska and Schlutz 1994; Cronin et al.1998). Schlutz et al. (1989; 1991) and Arnold et al. (1990) studied toxicity of substituted anilines to *Tetrahymena pyriformis* and polar narcosis proved to be main mechanism of toxic action. Veith and Broderius (1987) investigated lethal effect of selected anilines on fish. Toxicity of nitro- substituted phenols and nitro- substituted benzenes were proved to be generally reactive (Jaworska and Schlutz 1994; Schlutz 1997; Cronin et al.1998). Toxicity of pyridines to *Tetrahymena pyriformis* were also studied (Moulton and Schlutz 1986; Schlutz et al. 1987). However compared to the comparatively extensive study of toxicity to aquatic organisms, few studies, to our knowledge, are available on toxicity and mechanism of nitrogen-containing aromatic compounds to higher terrestrial plants (Hulzebos 1993).

This paper presents the comparative inhibition effect of selected nitrogen-

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containing aromatic compounds on root elongation of *Cucumis sativus*. The Quantitative Structure-Activity Relationship (QSAR) method was employed to study structural features affecting phytotoxicity and investigate relationship between phytotoxicity and molecular descriptors of nitrogen-containing aromatic compounds.

MATERIALS AND METHODS

All tested chemicals were provided by Department of Chemistry, Nanjing University (Table 1). All were of sufficient purity (analytical purity) and further purification was not necessary.

The seeds of *Cucumis sativus* (sprout containing ≥ 95%, purity ≥ 95%), which are recommended by the Organization for Economic Cooperation and Development and the U.S. Environmental Protection Agency as test species in terrestrial environmental assessment, were employed for tests and were purchased commercially. The seeds were sterilized with 0.1% NaClO solution for 20 min, soaked for 10 minutes and washed three times with deionized water before use.

Stock solutions of test chemicals were prepared with deionized water for all chemicals after range finding experiments. The tests were conducted using 100× 15 mm disposable petri dishes and Whatman No.1 filter paper. Each dish was filled with 5 mL test solution or deionized water in control. Six concentrations in geometric series were set, ranging from no effect to 100% inhibition concentration and four replicates were set for each concentration. Fifteen pretreated, undamaged and plump seeds with almost identical size were placed evenly on the filter paper in each dish. Solutions were renewed every 12 hr to achieve semi-static exposure. Deionized water without test compounds served as control. After 48hr of incubation in the dark at 25±1°C, the root elongation of each seed was measured to 1 mm. Inverse logarithm of the concentration (Mol/L) for each chemical on which the average root length was 50% in the control, expressed as $\log 1/RC_{50}$, was calculated for each chemical as its toxic potency. A chemical persistence experiment was not undertaken because Arnold et al. (1990) and Cronin et al.(1998) demonstrated that abiotic loss of test compounds had no significant effect on QSAR analysis.

Logarithms of the 1-octanol/water coefficient ($\log K_{ow}$) were computer estimated or retrieved as measured values from SRC-WSKOW for Microsoft Windows (version 1.26). Molecular orbital parameters were calculated from the semi-empirical molecular orbital package MOPAC6.0. Molecular Connectivity Indices (MCIs) were calculated according to the method outlined by Kier and Hall (1977).

STATISTICA for WINDOWS software (version 5.0) was employed for QSAR analysis. The quality of QSAR models were characterized by the number of observation (n), the square of correlation coefficient (r^2), the standard error of estimate (SE), the Fisher criterion (F) and the significance level (P).

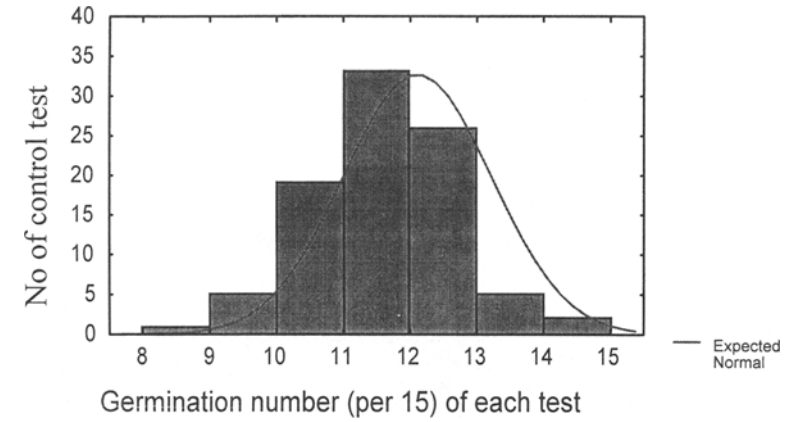

Figure 1. Normal distribution of germination rate of *C. sativus* in control test

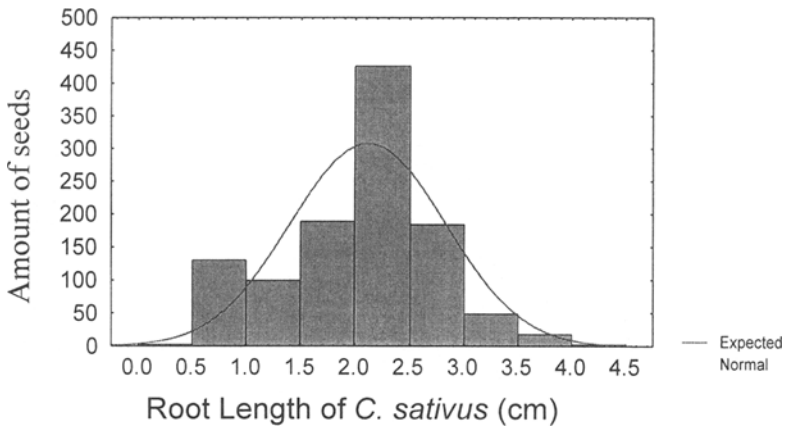

Figure 2. Normal distribution of root length of *C. sativus* in control test

RESULTS AND DISCUSSION

The suitability of *Cucumis sativus* seeds as test species was assured by the stability and reproducibility of the germination rate and root length in the control test, regularity of dose-response relation for all test compounds, and comparably high species sensitivity as well as much less exposure time needed than other higher plant test systems, detailed in our previous study (*unpublished work*). The first aspect is explained in the next paragraph.

The control test was investigated to observe the reproducibility and stability of the

Table 1. Chemicals, phytotoxicity and regularity of dose-response relations.

Compounds	RC_{50} [a] (mg/L)	R^2 [b]	SE [c] (mg/L)	Compounds	RC_{50} [a] (mg/L)	R^2 [b]	SE [c] (mg/L)
2-Nitrophenol	99.9	0.926	6.36	4-Chloro-2-Nitrophenol	62.2	0.954	5.24
3-Nitrophenol	139.6	0.967	3.57	4-Fluoro-2-Nitrophenol	82.1	0.878	7.77
4-Nitrophenol	57.0	0.961	3.03	2-Chloro-3-aminopyridine	356.9	0.950	3.25
2,4-Dinitrophenol	10.7	0.911	4.29	2-Amino-5-metylpyridine	203.3	0.934	4.16
2-Nitroresorcinol	33.2	0.978	3.56	1-amino-naphthane	132.5	0.948	2.80
3-Aminophenol	1053.4	0.993	1.48	2,3,4-Trifluoroaniline	198.6	0.972	4.37
2-Aminophenol	75.6	0.926	6.36	3-Aminobenzoic acid	324.0	0.995	0.97
Aniline	605.6	0.995	1.98	4-Aminobenzoic acid	334.7	0.935	2.84
4-Fluoroaniline	251.0	0.930	3.65	2,4,5-Trifluoroaniline	262.8	0.913	2.92
3-Nitroaniline	122.4	0.959	2.25	4,4'-biphenylamine	122.2	0.923	4.60
4-Nitroaniline	114.8	0.998	0.91	2,4-Difluoroaniline	137.5	0.962	2.67
2-Aminopyridine	59.73	0.961	1.84	4-Aminopyridine	353.6	0.997	0.38

[a] Concentration on which 50% inhibition of root elongation for each chemical.
[b] Linear correlation coefficient, [c] Standard error of estimation.

germination rate and root elongation of *Cucumis sativus*. A range from 77.33% to 88.33% of each test and 81.26% of all control test, for germination rate was observed. A rang from 18.7 mm to 22.3 mm of each test and 21.7 mm of all control tests was obtained. Figure 1 and Figure 2 present the normal distribution of germination rate and that of root elongation of *Cucumis sativus*, respectively. In Figure 1, the germination rate (per 15 seeds) ranges from 8 to 15, and the range of 10–13 covers the majority of germination rate data. In Figure 2, the root length for each seed ranges from 6.0 mm to 40.0 mm and the range of 10.0–30.0 mm covers the majority of root length data. One can conclude that the high stability and reproducibility of germination rate and root elongation made *Cucumis sativus* seed a favorite species for phytotoxicity test.

Regularity of dose-response relation was observed for each test compound. Square of correlation coefficients (r^2) and standard error of estimates (*SE*) were used to characterize the linear relation between concentration and comparative effect of each chemical on root elongation. Dose-response relation curves were not reproduced here and all r^2 and *SE* were presented for this purpose (Table 1). For dose-response relations of root elongation, r^2 values range from 0.878 to 0.998 and *SE* values range from 0.38 to 7.77 mg/L, which demonstrated the regularity of dose-response relations for all test chemicals.

High stability and reproducibility of the germination rate in the control test, regular linear dose-response relationship for each studied compound, together with comparably high sensitivity and very short exposure time (48 hr) needed strongly assured the suitability of root elongation of *Cucumis sativus* to phytotoxicity test and the validation of the test results.

Table 2 Chemicals, phytotoxicity and molecular structure descriptors

COMPOUNDS	Log1/RC_{50} [1]	Pred.[2]	E_{LUMO} [3]	Q_{max}^{+} [4]	^2X_p [5]
2-Nitrophenol	3.144	3.277	-1.184	0.580	3.912
3-Nitrophenol	2.998	3.113	-1.167	0.566	3.328
4-Nitrophenol	3.388	3.332	-1.064	0.574	4.264
2,4-Dinitrophenol	4.236	3.933	-1.888	0.584	5.702
4-Chloro-2-Nitrophenol	3.446	3.519	-1.227	0.580	4.803
2-Nitroresorcinol	3.668	3.525	-1.322	0.591	4.701
4-Fluoro-2-Nitrophenol	3.481	3.579	-1.447	0.580	4.803
3-Aminophenol	2.305	2.461	0.521	0.217	3.377
2-Aminophenol	3.160	2.937	-1.366	0.214	3.239
Aniline	2.187	2.246	0.636	0.183	2.743
4-Fluoroaniline	2.646	2.503	0.289	0.186	3.365
2,4-Difluoroaniline	2.973	2.738	-0.066	0.197	3.873
2,3,4-Trifluoroaniline	2.870	2.926	-0.382	0.206	4.25
4-Nitroaniline	3.187	3.258	-0.787	0.577	4.264
3-Nitroaniline	3.052	3.024	-1.194	0.402	3.328
3-Aminobenzoic acid	3.014	3.020	-0.702	0.211	4.265
4-Aminobenzoic acid	2.627	2.771	-0.377	0.350	3.328
2,4,5-Trifluoroaniline	2.870	2.926	-0.382	0.206	4.25
4,4'-biphenylamine	3.141	3.270	0.046	0.184	6.076
1-Amino-naphthane	3.062	3.312	-1.963	0.168	4.167
2-Aminopyridine	2.426	2.344	0.320	0.203	2.743
4-Aminopyridine	2.425	2.390	0.061	0.162	2.743
2-Chloro-3-aminopyridine	2.557	2.624	-0.075	0.205	3.406
2-Amino-5-metylpyridine	2.726	2.558	0.302	0.203	3.549

[1] Log1/RC_{50} is logarithm of the inverse of RC_{50} value in Mol/L.
[2] Pred. represents predicted phytotoxicity from Eq. (6).
[3] E_{lumo} is energy of lowest unoccupied molecule orbital (eV).
[4] Q_{max}^{+} is maximum net positive charge.
[5] ^2X_p is 2nd order simple path molecule connectivity indices.

Table 2 lists the comparative inhibition activity of 24 nitrogen-containing aromatic chemicals on root elongation of *Cucumus Sativus* after 48hr semi-static exposure (log1/RC_{50}), and molecular structure descriptors for QSAR analysis.

According to McFarland (1970), chemical toxicity is a combination of the penetration of the toxicant into bio-phase and the interaction between toxicants and target sites. Hydrophobicity, electric properties and steric features proved to be the most common factors affecting the toxicity of xenobiotics. In our investigation, nitro- substituted phenols, amino- substituted phenols, substituted anilines and substituted pyridines were involved. Toxicity of nitro- containing aromatics often involves their *in vivo* redox reaction, and selected nitro-substituted phenols were proved to be reactive in *Tetrahymena pyriformis* system and *Pimephales promelas* system (Jaworska and Schultz 1994; Schlutz 1997).

Most mono- substituted anilines explained their toxicity to aquatic organism by polar narcosis (Arnold et al. 1990; Schlutz et al. 1991). Schlutz' other studies showed that toxicity of pyridines was mainly affected by shape/ size and hydrophobic factors (Moulton and Schlutz 1986; Schlutz et al. 1987). In addition, due to the presence of nitrogen and other heterogeneous atom, hydrogen bonding interaction may occur. While these toxicity influencing factors are considered, four parameters were selected to model phytotoxicity of nitrogen-containing aromatics: $\log K_{ow}$ was used to characterize hydrophobicity of chemicals and model the ability of chemicals penetrating through bio-membrane and reaching action sites, energy of lowest unoccupied molecular orbital (E_{lumo}) to model electrophilicity, the 2nd order molecular connectivity index (^2X_p) to encompass steric information such as molecule shape/size features and maximum net positive atomic charge, Q_{max}^+, to reflect hydrogen bonding interaction of test chemicals with action sites.

Hydrophobicity proved to be a key factor affecting toxicity in toxicological studies. Hulzebos (1993) studied phytotoxicity of 76 priority pollutants to lettuce in soil and nutrient solution and found that phytotoxicity was well correlated with lipophilicity for almost all pollutants (including chloroanilines, chloro- substituted nitrobenzenes). Cronin et al. (1998) studied toxicity of some nitrogen-containing aromatic compounds to *Tetrahymena pyriformis* and to *Pimephales promelas* and obvious correlations of toxicity with $\log K_{ow}$ were observed with a few outliers for the two systems. The correlation of phytotoxicity with hydrophobicity in this study was investigated and a significant correlation was observed:

$$RC_{50} = 0.626 \log K_{ow} + 2.422 \qquad \text{Eq. (1)}$$
$$n = 24 \quad r^2 = 0.392 \quad F = 14.2 \quad SE = 0.373 \quad P < 0.001$$

Phytotoxicity was significantly but not well correlated with hydrophobicity, which implied that multiple modes of action were represented by the tested compounds. In *Tetrahymena pyriformis* and/or *Pimephales promelas* systems, some nitro-substituted phenols demonstrated as being reactive (Jaworska and Schlutz 1994; Schultz 1997). Some amino- substituted phenols may be metabolized to a quinone-like structure with subsequent elevated toxicity by electrophilic attack of proteins (Dupuis and Benezra 1982; Schlutz et al. 1992). Most mono- substituted anilines proved to elicit their toxicity by polar narcosis (Arnold et al. 1990; Schlutz et al. 1991). Simple regression analysis was performed with E_{lumo}, Q_{max}^+ and ^2X_p, individually, no satisfactory mono-variable correlation was obtained:

$$RC_{50} = -0.822\, E_{lumo} + 2.685 \qquad \text{Eq. (2)}$$
$$n = 24 \quad r^2 = 0.675 \quad F = 45.8 \quad SE = 0.272 \quad P < 0.00000$$

$$RC_{50} = 0.713\, Q_{max}^+ + 2.36 \qquad \text{Eq. (3)}$$
$$n = 24 \quad r^2 = 0.508 \quad F = 22.8 \quad SE = 0.335 \quad P < 0.00009$$

$$RC_{50} = 0.781\, {}^2X_p + 1.326 \qquad \text{Eq. (4)}$$
$$n = 24 \quad r^2 = 0.609 \quad F = 34.3 \quad SE = 0.299 \quad P < 0.00001$$

All of the 4 structure descriptors proved to be significant influencing factors. Multiple regression analysis with the combined application of the four descriptors led to another highly predictive model:

$RC_{50} = -0.48\ E_{lumo} + 0.593\ {}^2X_p + 0.242\ Q_{max}^+ - 0.176 \log K_{ow} + 1.498$ Eq. (5)
$n = 24 \quad r^2 = 0.919 \quad F = 54.2 \quad SE = 0.146 \quad P < 0.00000$

From a predictive viewpoint, this model was satisfactory. However, a stepwise variable regression revealed that log K_{ow} was not a significant variable and even could be removed from this model without weakening model quality:

$RC_{50} = -0.45\ E_{lumo} + 0.48\ {}^2X_p + 0.228\ Q_{max}^+ + 1.602$ Eq. (6)
$n = 24 \quad r^2 = 0.907 \quad F = 65.1 \quad SE = 0.153 \quad P < 0.00000$

This model can be interpreted mechanistically. E_{lumo} is related to relative electrophilicity (Veith and Mekenyan 1993). Q_{max}^+ measures hydrogen bonding interaction between toxicants and bio-macromolecule of action sites and ${}^2X_p$ encodes topological information about molecule size or molecule shape features. The inclusion of the 3 descriptors in Eq. (6) demonstrated the anticipated importance of electronic and steric parameters. There was no obvious correlation among the 3 parameters while correlation matrix was studied. From both a predictive and a mechanism interpretation standpoint, this equation is satisfactory. When residuals are studied, no outliers were found to this relationship, and predicted phytotoxicity was very close to the observed values (Table 2).

It is interesting and notable that log K_{ow} indicating hydrophobicity proved to be not a significant variable and didn't enter the stepwise regression procedure. The absence of hydrophobicity in the QSAR model implied the minor importance of hydrophobicity in determining the phytotoxicity of the studied chemicals in comparison with other factors. This suggested that phytotoxicity of the studied nitrogen-containing aromatic compounds was probably reactive and was not limited by the ability of their penetrating through bio-membrane.

In conclusion, this paper presented the phytotoxicity of some nitrogen-containing aromatic compounds to *Cucumis sativus* with the root elongation method. The QSARs analysis revealed that phytotoxicity of nitrogen-containing aromatics is mainly affected by electrophilicity, hydrogen bonding interaction and molecule shape/ size features and can be accurately modeled by E_{lumo}, Q_{max}^+ and ${}^2X_p$.

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