The Emergence of a Atrazine Resistant Black Nightshade (*Solanum nigrum* L.) Biotype and Molecular Basis of the Resistance

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Abstract

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Seeds from atrazine resistant plants of black nightshade (*Solanum nigrum* L.) were collected at the railway station Prague-Vršovice, seeds from susceptible plants in Vyšehořovice (Prague East district). Tests on emergence showed that in both resistant and susceptible biotypes it was highest at a seeding depth of 1 mm, and that at the same seeding depth there were statistically significant differences in emergence between the resistant and susceptible biotypes. The resistance or susceptibility to atrazine was tested by both a chlorophyll fluorescence assay and spraying with atrazine. A region of the gene encoding D1 protein of photosystem II was sequenced and compared between the resistant and susceptible biotypes. Resistance to atrazine in the *S. nigrum* biotype from Vršovice was conferred by a glycine for serine substitution at residue 264 of the D1 protein. In the plants of the biotypes there was excellent correspondence between the presence of the mutation and herbicide resistance. The assay based on restriction analysis of PCR products can be used for rapid detection of the mutation in populations of black nightshade.

Keywords: black nightshade; *Solanum nigrum* L.; resistance; atrazine; chlorophyll fluorescence; emergence; genetic variation

Black nightshade (*Solanum nigrum* L.) is widespread in warmer regions, it occurs often in wide-row crops, especially and with high abundance in maize, but can be found also in cereal crops (Holec *et al*. 2003).

In 2000, a biotype of *S. nigrum* resistant to atrazine was found at the railway station Prague-Vršovice where this herbicide had been applied for 5 years.

Germination characteristics may have major implications in the persistence of these populations in the field. The germination of *S. nigrum* is promoted by light and soil temperature above 15°C (Kremer & Lotz 1998b).

Gasquez *et al*. (1981) found that the proportion of seeds of triazine-resistant biotypes of *S. nigrum* that germinated at different temperatures up to 25°C was lower than that of susceptible biotypes. In a study by Bulcke *et al*. (1985), resistant and susceptible biotypes of *S. nigrum* of different geographical origins showed similar germination at 20°C.

Atrazine has been a commonly used herbicide in crop fields, along roads and railways. Experi-

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ments in 1979 have shown that *S. nigrum* from corn was resistant to atrazine in France (Gasquez *et al*. 1981). The rise of resistant biotypes of *S. nigrum* has been reported in Austria, Belgium, France, Switzerland, Germany, the Netherlands, Hungary and Italy (Stankiewicz *et al*. 2001).

The primary action of atrazine is to inhibite photosynthetic electron transport in chloroplasts. Atrazine binds to D1 protein (32 kDa) of the photosystem II competing with plastoqinone (QB), the primary electron acceptor of the electron-transport chain (Arntzen *et al*. 1982). Triazine resistance in many higher plant species, including *S. nigrum*, is caused by a mutation at position 264 in 32 kDa protein (single amino acid substitution resulting in the change of Ser to Gly) (Goloubinoff *et a*l. 1984). The relative frequency of the resistance gene in natural populations without atrazine applications ranges from 10^{-6} to 10^{-10}. The resistance characteristic is maternally inherited (Jaseniuk & Maxwell 1994). In resistant biotypes, photosynthetic rate and biomass production was lower than in susceptible biotypes (Holt *et al*. 1993).

This paper presents the results of trials dealing with the emergence and molecular genetic confirmation of the atrazine resistance in the black nightshade biotype from Prague-Vršovice.

MATERIAL AND METHODS

Plant material. Seeds of black nightshade (*Solanum nigrum*) were collected in September 2000 in the field in Vyšehořovice (Prague East district) where common herbicides had been used in the crop rotation, and at the railway station Prague-Vršovice where atrazine had been applied for the 5 previous years. Seeds were extracted from berries by washing 3 d at laboratory temperature, dried at laboratory temperature and stored at 5°C. The collected berries represented 20 plants per biotype. In January the seeds were sown into plastic containers 10 × 10 cm in size. These were placed in a growth chamber maintained at 22 ± 2°C, humidity 65–70% and illumination 150 μmol/m^2/s at plant level over a 12 h photoperiod in all experiments and during seed production.

Chlorophyll fluorescence assay. Fully expanded intact leaves were incubated in 10^{-5}M atrazine solution at laboratory temperature for 3 h. To adapt, the leaves were left for 2 min in the dark before fluorescence determination. The relative fluorescence of a leaf was measured on the fluorometer SF-30 (Richard Brancker, Canada). Illumination time was 10 s, irradiance 70 W/m^2. Susceptibility or resistance of plants to atrazine were estimated according to fluorescence curve readings (Ahrens *et al*. 1981).

Whole plant response. When plants had 2–3 adult leaves, atrazine was applied at a dose of 2000 g/ha of active ingredient (in Gesaprim 500 FW) with a hand sprayer, using 50 ml water per m^2. Plants that did not show any symptoms of wilting or drying within 2 weeks after treatment were evaluated as resistant individuals.

Germination. The seeds were placed in Petri dishes on moist filter paper to germinate at 20°C with a day/night regime 12/12 h. Thirty seeds were evaluated in one trial in four replicates.

Emergence. Seeds were sown into 10 × 10 cm containers filled with a mixture of topsoil and potting substrate, 2:1 ratio, pH 6. Thirty five seeds were sown per container, either onto the soil surface (0 mm) or seeds were covered with soil to a depth of 1 mm (with finely sifted substrate). For sowing depths of 10 and 20 mm, PVC frames 10 or 20 mm high and fitting the top of the container were placed on the soil and the seeds were evenly covered with sifted substrate to the top of the frame. Containers were incubated in a climate box (temperature of 20 ± 1°C, humidity 65–80%, and photoperiod 12 h dark and 12 h light). Every day, emerged seedlings were counted and removed. Assessments continued until no emergence was observed for 3 weeks. Emergence of 140 seeds was evaluated for each sowing depth in one trial. The trial had four replicates (total 560 seeds).

Statistical analysis. The results were evaluated by analysis of variance ANOVA, and minimum difference was determined by Tukey's test using the Program Statistica 6.0 (StatSoft).

DNA extraction. DNA was extracted from young leaves of individual plants using the DNeasy Plant Mini Kit (QIAGEN) following the manufacturer's instructions.

Polymerase chain reaction (PCR). Based on the sequences of the photosystem II protein D1 (*psbA*) gene of *Solanum nigrum* (GenBank accessions X01651 and U25659) primers psbA2F (3′-AATCGGTCAAGGAAGTTTTTC-5′) and psbA2R (3′-TTCCATACCAAGGTTAGCACG-5′) were designed using the Primer3 program (Rozen & Skaletsky 2000). Primers were synthesised by the Sigma-Genosys Ltd.

For PCR 1 μl of template DNA, 1 μM of each primer, 200 μM of dNTPs, 2.5 μl of 10 × PCR buffer

(Promega), 1.5 mM of $MgCl_2$ and 0.5 U of *Taq* DNA polymerase (Promega) were mixed. The amplification was carried out in an automated thermal cycler Progene (Techne) using a 3-min incubation at 97°C, 30 cycles of 0.5 min at 97°C, 1 min at 55°C, and 1.5 min at 72°C; and an 8 min incubation at 72°C. The amplification products were detected on 1.5% agarose gels using a Kodak electrophoresis unit.

Sequencing of the psbA gene. Sequencing was performed directly on PCR products. If only one fragment of the expected length was amplified, the PCR products with the corresponding primers were analysed at the Institute of Molecular Genetics, Academy of Sciences of the Czech Republic, Prague, Czech Republic. Purification of the PCR products was performed with the ExoSAP-IT® kit (USB). Sequencing was done on a Beckman automated sequencer using cycle sequencing with the DTCS Quick Start Kit (Beckman Coulter) according to the protocols recommended by the manufacturer. Sequences of the susceptible and resistant biotypes were compared using the program BLAST (Altschul *et al*. 1997).

Restriction digests. The PCR products were cleaned using the QIAquick PCR Purification Kit (QIAGEN) as recommended by the manufacturer. Ten microlitres of the purified products was transferred to a new 0.5-ml tube and digested with 5U of the *Bfa*I restriction enzyme (New England BioLabs) over night at 37°C. The digested products were size-fractionated by electrophoresis on 4% MetaPhor® agarose gels (Cambrex), and the DNA fragments were visualised by ethidium bromide staining. GeneRuler™ DNA markers (Fermentas UAB) were used to estimate fragment sizes.

RESULTS AND DISCUSSION

The triazine resistant character of the *Solanum nigrum* biotype was confirmed by *in vivo* measurement of chlorophyll fluorescence emitted by leaves treated with atrazine. The induction curves of susceptible and resistant biotypes *S. nigrum* treated with atrazine are shown in Figure 1. After atrazine treatment, leaves from the resistant biotype do not show any changes in the fluorescence curve pattern when compared with untreated control leaves, and points Fp and Ft can be clearly distinguished. Leaves from susceptible biotypes show typically changed fluorescence curve patterns without distinguishable points Fp and Ft. This value was maintained, revealing complete blockage of the transfer of electrons between Q_A and Q_B (Domínguez *et al*. 1994) (Figure 1).

These results agreed with phenotypic resistance to atrazine after spraying of individual plants with this herbicide.

The number of emerged plants of *S. nigrum* in 21 d is presented in Figure 2.

A correct comparison of growth to determine the variation between populations should be made

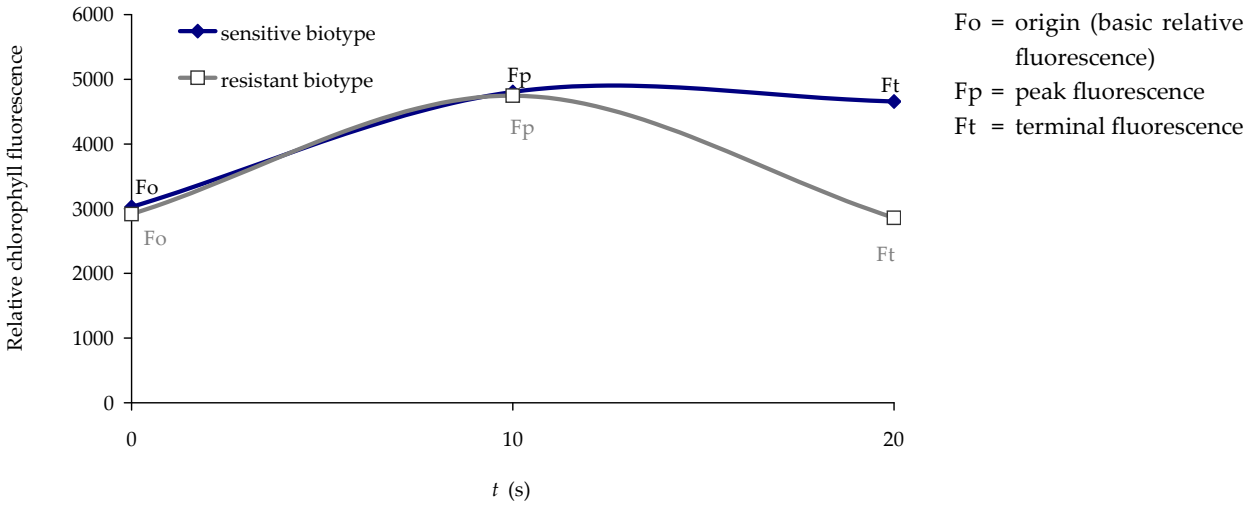

Figure 1. Chart of induction curves for slow chlorophyll fluorescence corresponding to resistant Vršovice and susceptible Vyšehořovice of black nightshade (*Solanum nigrum)* treated with 10^{-5} atrazine. Average of 10 leaves (10 plants)

Table 1. Tukey's HSD test

Biotype	Depth of sowing (mm)	Average of emerged plants	
Susceptible	20	22.75	a
	10	26.75	b
	1	32.75	c
	0	25.25	ab
Resistant	20	25.75	ab
	10	24.75	ab
	1	28.00	b
	0	26.25	b

Different letters (a, b, c) mean statistically significant differences (on 0.05 probability level)

under optimal growing conditions, using populations with both susceptible and resistant biotypes (Kremer & Kropff 1998).

The germination at 20°C of resistant and susceptible biotypes of *S. nigrum* differed. The average germination rate of the resistant biotype was 23.3%, that of the susceptible one 60%.

The effects of biotype and depth of sowing on the emergence of plants at 20°C were investigated. The results of statistical analyses are presented in Tables 1–3. The number of emerged plants in relation to biotype and sowing depth is shown in Table 1. Depth of sowing was confirmed as a statistically significant factor in the emergence of *S. nigrum* plants (at significance level $P \leq 0.01$). A slightly lower emergence rate of the resistant biotype compared to that of the susceptible biotype has been presented by Kremer and Lotz (1998a).

Results presented in the chart (Figure 2) showed a statistically significant difference between susceptible and resistant biotypes in germination of *S. nigrum* when seeded at a depth of 1 mm. At the other sowing depths the confidence intervals overlapped, meaning that there are no significant differences between replicates and variants (see Tukey's test). In the susceptible biotype there are significant differences between number of plants from the depth of 1 mm and the other depths of sowing.

In Table 1, showing the homology groups, three statistically different groups can be discerned by their emergence. First there was the susceptible biotype at a depth of sowing of 20 mm, then a

Table 2. Tukey's HSD test – susceptible biotype

Depth of sowing (mm)	Number of emerged plants	
20	24.25	a
10	25.75	a
0	25.75	a
1	30.38	b

Different letters (a, b) mean statistically significant differences (on 0.05 probability level)

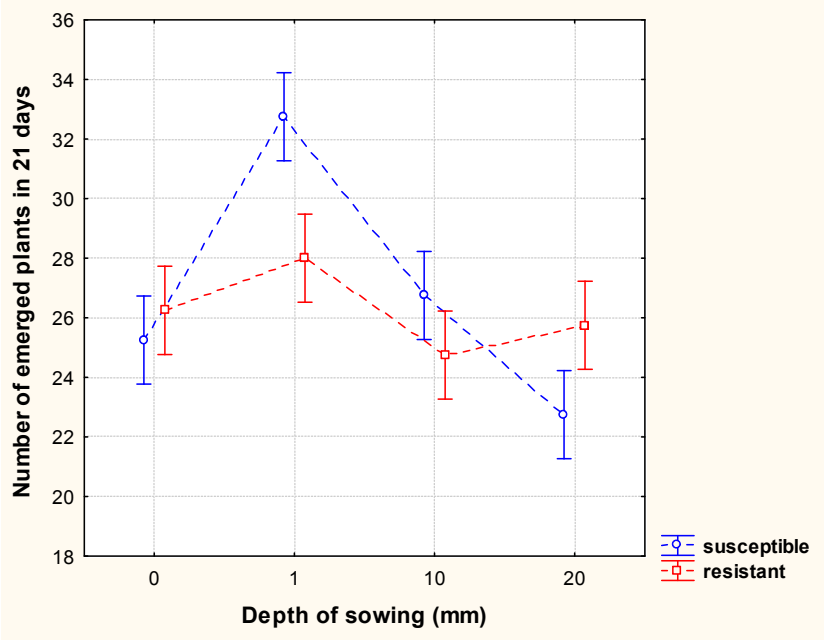

Figure 2. Number of emerged plants of susceptible biotype Vyšehořovice and resistant biotype Vršovice of black nightshade (*Solanum nigrum*)

Table 3. Tukey's HSD test – resistant biotype

Depth of sowing (mm)	Number of emerged plants	
20	24.75000	a
10	25.75000	a
0	26.25000	a
1	28.00000	a

Letter (a) mean statistically significant differences (on 0.05 probability level)

homology group of three treatments (resistant seeded at 0 mm, resistant at 1 mm, and susceptible at 10 mm), and finally susceptible at a depth of 1 mm.

Individual evaluation of *S. nigrum* biotypes is given in Table 2 (susceptible biotype) and Table 3 (resistant biotype).

The number of emerged plants was significantly influenced only by depth of sowing. A statistically higher number of plants emerged from the depth of 1 mm than from 0, 10 or even 20 mm. Thus, a depth of 1 mm seems to be the most advantageous one.

The effect of sowing depth on the resistant biotype was not statistically significant.

Gasquez *et al*. (1981) found that the fraction of triazine resistant biotypes of *S. nigrum* that germinated at different temperatures up to 25°C was lower than that of susceptible biotypes. In a study of Bulcke *et al*. (1985), resistant and susceptible biotypes of *S. nigrum* from different geographical areas showed similar germination at 20°C.

Kremer and Lotz (1998b) found that the susceptible biotype had a higher emergence rate when seeded on the soil surface, while the time to 50% emergence from the depths 10, 20 and 40 mm was similar in susceptible and resistant biotypes; the highest rate of emergence was at the depth of 1 mm with lowering tendency; emergence rate hardly differed between 10 mm and 20 mm depth of sowing.

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S: 1   aatcggtcaaggaagtttttctgatggtatgcctctaggaatctctggtactttcaattt 60
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
R: 1   aatcggtcaaggaagtttttctgatggtatgcctctaggaatctctggtactttcaattt 60

S: 61  catgattgtattccaggctgagcacaacatccttatgcacccatttcacatgttaggcgt 120
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
R: 61  catgattgtattccaggctgagcacaacatccttatgcacccatttcacatgttaggcgt 120

S: 121 ggctggtgtattcggcggctccctattcagtgctatgcatggttccttggtaacttctag 180
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
R: 121 ggctggtgtattcggcggctccctattcagtgctatgcatggttccttggtaacttctag 180

S: 181 tttgatcagggaaaccacagaaaatgaatctgctaatgaaggttacagattcggtcaaga 240
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
R: 181 tttgatcagggaaaccacagaaaatgaatctgctaatgaaggttacagattcggtcaaga 240

S: 241 ggaagaaacttataatatcgtagccgctcatggttattttggccgattgatcttccaata 300
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
R: 241 ggaagaaacttataatatcgtagccgctcatggttattttggccgattgatcttccaata 300

S: 301 tgct[a]gtttcaacaactctcgttcgttacacttcttcctagctgcttggcctgtagtagg 360
       |||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
R: 301 tgct[g]gtttcaacaactctcgttcgttacacttcttcctagctgcttggcctgtagtagg 360

S: 361 tatctggtttaccgctttaggtattagcactatggctttcaacctaaatggtttcaattt 420
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
R: 361 tatctggtttaccgctttaggtattagcactatggctttcaacctaaatggtttcaattt 420

S: 421 caaccaatctgtagttgacagtcagggtcgtgtaattaacacttgggctgatatcatcaa 480
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
R: 421 caaccaatctgtagttgacagtcagggtcgtgtaattaacacttgggctgatatcatcaa 480

S: 481 ccgtgctaaccttggtatggaa 502
       |||||||||||||||||||||
R: 481 ccgtgctaaccttggtatggaa 502
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Figure 3. Comparison of nucleotide sequences of the *psbA* gene from the atrazine-susceptible Vyšehořovice (S) and atrazine-resistant Vršovice (R) black nightshade (*Solanum nigrum*) biotypes. Homologous nucleotides are joined with a vertical bar. Boxed nucleotides in the sequences indicate the substitution

A depth of 50 mm is critical for the emergence of *S. nigrum*.

Amplification of *Solanum nigrum* genomic DNA with primers psbAF2 and psbAR2 produced a single ethidium bromide-staining fragment of the expected size (502 bp) for both biotypes.

Sequences of the 502 bp psbA gene region from susceptible and resistant *Solanum nigrum* differed by a single nucleotide substitution at the variable Ser codon (AGT to GGT) at position 264, predicting a serine in the susceptible but a glycine in the resistant biotype (Figure 3).

Restriction enzyme analysis of the polymerase chain reaction product was used to confirm results of the sequence analysis. The nucleotide sequence of susceptible *S. nigrum* has three restriction sites for the enzyme *Bfa*I. The mutation results in loss of one restriction site. A change in the restriction digest pattern (RFLP-restriction fragment length polymorphism) provided a diagnostic tool to characterise the biotypes. Two possible restriction patterns could be derived from *Bfa*I digestion of PCR amplification products from *S. nigrum*: the susceptible sequence has four fragments, the resistant sequence three fragments (data not shown). Restriction digest patterns were evaluated for presence of the 324 band which was the result of mutation. The 121 bp and 126 bp fragments were not sufficiently different in size to permit resolution on the electrophoretic system employed. The band 56 bp could not be distinguished from nonspecific PCR reaction products.

There was an excellent correspondence between the presence of the mutation and phenotypic resistance to atrazine of individual plants.

Sequencing of the specific region of the *psbA* gene enabled the identification of the point mutation in the serine codon. This mutation has been shown to confer triazine resistance in *S. nigrum* (Goloubinoff *et al*. 1984; Zhu *et al*. 1989; Gadamski *et al*. 1996) and in many other plant species (Hirschberg & McIntosh 1983; Blyden & Gray 1986; Bettini *et al*. 1987; Foes *et al*. 1998, 1999; Chodová & Salava 2004). This mutation not only reduces the binding capacity of triazine herbicides to D1 protein, but also hinders electron transfer in photosystem II which results in high, initial steady-state chlorophyll fluorescence (Foes *et al*. 1998). Because no other differences were found between the atrazine-resistant and atrazine-susceptible biotypes, it can be concluded that this point mutation is responsible for the resistance of the Vršovice *Solanum nigrum* biotype.

The RFLP-PCR technique enables reliable and rapid determination of the mutation in codon 264 of the *psbA* gene in black nightshade.

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Souhrn

Salava J., Chodová D., Nováková K. (2004): **Vzcházivost biotypu lilku černého (***Solanum nigrum* L.**) rezistentního vůči atrazinu a molekulární podstata této rezistence.** Plant Protect. Sci., **40**: 94–100.

Biotyp lilku černého (*Solanum nigrum* L.), který nebyl citlivý k atrazinu, byl nalezen u železniční stanice Praha-Vršovice. Citlivý biotyp byl získán z lokality Vyšehořovice (Praha-východ). Průkazný rozdíl ve vzcházivosti mezi biotypy byl při výsevu semen do hloubky 1 mm. Nejvyšší vzcházivost byla u obou biotypů z hloubky 1 mm, nejnižší z hloubky 20 mm. Citlivost rostlin byla ověřena metodou pomalé fluorescence chlorofylu, která byla ve shodě s fenotypovým hodnocením rostlin ošetřených atrazinem. Pro stanovení molekulární podstaty rezistence byla sekvencována část genu kódujícího protein D1 fotosystému II. Rezistence k atrazinu byla u biotypu lilku černého z Vršovic způsobena záměnou serinu glycinem v pozici 264 aminokyselinového řetězce proteinu D1 fotosystému II. Výsledky sekvenční analýzy byly potvrzeny štěpením produktu PCR restrikčním enzymem *Bfa*I. Byla nalezena úplná shoda mezi rezistencí k atrazinu a přítomností mutace. Vyvinutá metoda RFLP-PCR může být použita pro rychlou detekci mutace v populacích lilku černého.

Klíčová slova: lilek černý; *Solanum nigrum* L.; rezistence vůči atrazinu; fluorescence chlorofylu; vzcházivost; genetická variabilita

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