METABOLIC CHANGES IN GLADIOLUS DUE TO AIRBORNE FLUORIDE

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SUMMARY

Gladiolus plants were exposed to airborne fluoride in order to study effects at the metabolic level. Two varieties of gladiolus, one sensitive and the other resistant to fluoride, were grown on uniform soil at a clean-air site. One set of plants was then exposed to ambient air in the vicinity of an aluminum smelter plant. A second set of control plants was placed at a location free from fluoride-polluted air. At the end of the exposure period of two weeks, plants were harvested and analyzed.

The fluoride content in exposed plants was about four times higher than that in control plants. Chlorophyll content remained unchanged, but the soluble protein content of exposed plants decreased by up to 50% relative to the control plants. Activities of glutamine synthetase, glutamate dehydrogenase, malate dehydrogenase and enolase in the tissue of exposed plants were reduced to 30–50% of the activities in control plants. These effects of fluoride were more pronounced in the sensitive variety than in the resistant one. The results demonstrate that gladiolus plants can be used as a sensitive bioindicator for fluoride pollution.

INTRODUCTION

Persisting fluoride pollution at low levels may injure agricultural plants. Characterization of foliar injury is usually done by describing visible symptoms such as necrosis. Little is known about metabolic changes in plant tissue due to uptake of gaseous fluoride [1]. Disturbed metabolic processes may lead to losses in crop yield [2]. The gladiolus plant is a very sensitive bioindicator for both visible and subtle effect at a metabolic level.

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The aim of this work was to study the effects of airborne fluoride at a subnecrotic level on the pathway of nitrogen assimilation, the related enzyme activities and the effects on the synthesis of proteins in the plant. It was investigated whether the uptake of airborne fluoride:

- influences the production of chlorophyll
- influences the synthesis of protein
- disturbs the activities of N-assimilating enzymes
- inhibits or enhances activities of glycolytic enzymes.

It is generally believed [3,4] that three metabolic steps are very important for the assimilation of nitrogen in plants: reduction of nitrate to ammonia, the production of a C skeleton and the incorporation of nitrogen into this C skeleton. Important metabolic pathways are shown in Fig. 1.

a) the production of a C skeleton: 2-P-D-Glycerate is one of the resulting products of photosynthetic activity (Calvin Cycle) and enters the

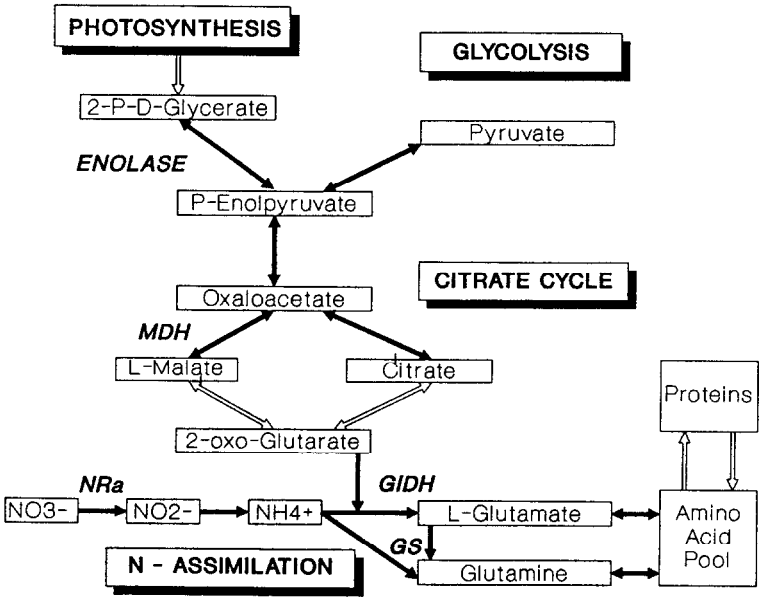

Fig. 1. The pathways for the production of a C skeleton and the assimilation of nitrogen.

glycolytic cycle. Enolase (E.C. 4.2.1.11) dehydrogenates it to P-enol-pyruvate which, in turn, is decarboxylated to oxaloacetate.
Malate dehydrogenase, MDH (E.C. 1.1.1.37), reduces oxaloacetate to L-malate with the aid of NADH (reduced nicotinamidadenine-dinucleotide). Following the pathway of the citrate cycle, 2-oxo-glutarate is produced which serves as a C skeleton for the assimilation of nitrogen.
b) the assimilation of nitrogen: nitrate is reduced in the cytosol by nitrate reductase, NRa (E.C. 1.6.6.1), to nitrite and the latter is reduced to ammonia by nitrite reductase (E.C. 1.7.7.1) in the chloroplasts. This ammonia can be assimilated by glutamine synthetase, GS (E.C. 6.3.1.2.), and then transferred from the amide into the α-amino position by glutamate synthetase (E.C. 1.4.7.1). The oxidation of glutamate by glutamate dehydrogenase, GlDH (E.C. 1.4.1.2) was proposed to be a major source of ammonia during leaf senescence as a result of an increased activity of this enzyme [5] .

METHODS

Plant material: Two varieties of gladiolus (Gladiolus gandavensis L.), one sensitive (var. Snow Princess) and one resistant (var. Flowersong) to fluoride, were grown on a uniform, sandy loam soil (pH 6.4) in flat vegetable pots (40 cm diameter, 15 cm height, mounted 40 cm above ground level) at a clean-air site. One set of plants was exposed in the vicinity of an aluminum smelter plant (530 m above sea level). Another set of plants was placed at a location away from the main wind direction and thus protected from fluoride-polluted air. The distance from the source was 1.2 km and equal for both locations. At the end of the exposure period of three weeks, plants were harvested. The green, healthy portion of the leaf tissue (from 4 cm above soil to the top) was excised. One part served for the analysis of fluoride in dry matter, the rest was rapidly frozen in liquid nitrogen. The frozen material was then used for the analysis of chlorophyll and soluble protein and for the determination of the activities of GS, GlDH, MDH and enolase. Activities are expressed on a fresh weight basis.

Enzyme extraction: The plant material was homogenized with a Polytron mixer (Kinematica, Luzern, Switzerland) in four volumes of chilled extraction medium (100 mM phosphate buffer pH 7.5 containing 1 mM Na_2EDTA, 5 mM cysteine neutralized with NaOH, and 1% (w/v) poly-

vinylpyrrolidone. Extracts were passed through Miracloth (Calbiochem, U.S.A.) and 2 ml were mixed with 0.4 ml casein (5% w/v) in order to stabilize the enzymes. The rest of the extract was kept frozen (-18°C) for protein and chlorophyll determinations. The crude extract was analyzed immediately for NRa activity. For the other enzymes, the extract was centrifuged for 10 min at 5000 x g. The supernatant was analyzed for GlDH, GS, MDH and enolase activity. The temperature was maintained at 0-4°C throughout extraction.

Nitrate reductase assay: NRa activity was measured in the crude, uncentrifuged extract for 15 min at 30°C according to Streit [5]. The assay mixture consisted of 30 mM phosphate buffer pH 7.5, 10 mM KNO_3 and 0.2 mM NADH as starting reagent. NADH was omitted from blanks. The reaction was stopped at 1/10 of the assay volume with a solution containing 500 mM zinc acetate and phenacine methosulfate (46 mg/l). After mixing, the mixture was allowed to stand for 20 min and was then centrifuged for 10 min at 3500 x g. The nitrite formed in the supernatant was detected with Griess-Ilosvay reagent (1.5 g sulfanilic acid and 0.3 g naphthylamine in 450 ml 30% acetic acid (w/v), mixing equal volumes. After a reaction time of 10 min the absorbance was measured at 546 nm, using 0-50 nm $NaNO_2$ for calibration.

Glutamine synthetase assay: GS activity was determined measuring ATP-dependent formation of γ-glutamylmonohydroxamate [6]. The assay mixture consisted of 125 mM imidazole (pH 7.2) containing 100 mM $MgSO_4$, 225 mM monosodium glutamate, 18 mM ATP disodium salt. The reaction was started with 6 mM NH_2OH (blanks without NH_2OH). After 20 min incubation at 30°C the reaction was stopped with a solution containing 0.37 M $FeCl_3$, 0.2 M TCA and 6.7 M HCl. The mixture was centrifuged for 10 min at 3500 x g and the absorbance of the supernatant was measured at 540 nm. Standard curves were constructed with pure γ-glutamyl hydroxamate (0-1 mM).

Glutamate dehydrogenase assay: GlDH activity was measured according to Thomas [7] by following the decrease in absorbance at 340 nm due to NADH oxidation. The assays were conducted at 30°C in a 100 mM Tris buffer, pH 8.0 containing 150 mM NH_4Cl, 2 mM $CaCl_2$, 0.2 mM NADH and 15 mM neutralized 2-oxoglutarate. Blanks were prepared without 2-oxoglutarate.

Malate dehydrogenase assay: MDH activity was determined kinetically with the decrease of NADH absorbance at 340 nm. The reaction

mixture consisted of 100 mM phosphate buffer pH 7.5 containing 0.5 mM oxaloacetate and 0.2 mM NADH. The temperature was held at 25°C throughout the reaction period.

Enolase assay: As enolase activity cannot be measured directly, a coupled enzyme reaction was used. Enolase converts 2-phospho-D-glycerate to P-enolpyruvate, pyruvate kinase catalyzes it to pyruvate, which, with the aid of lactate dehydrogenase in the presence of NADH, is reduced to lactate. The decrease in NADH absorbance was then measured. The reaction mixture consisted of a solution of 83 mM triethanolamine/ HCl buffer, pH 7.6 containing 3.3 mM $MgSO_4$, 13.2 mM KCl, 0.9 mM glycerate-2-phosphate trisodium salt, 1.2 mM ADP monopotassium salt, 0.2 mM NADH and an excess of lactate dehydrogenase (18.3 U/ml) and pyruvate kinase (2.7 U/ml). The reaction was recorded kinetically at 30°C and at 340 nm as the decrease in NADH.

Protein and chlorophyll determination: Soluble protein was determined using the Bio Rad reagent according to Bradford [8]. Chlorophyll content was determined according to Strain [9] by bringing the extract to 80% v/v acetone, centrifugation at 3000 x g and reading the absorbance at 649 and 665 nm. The total chlorophyll (chlorophyll a + b) was calculated according to the formula:

Total chlorophyll (ug/ml)=[6.45 A(665) + 17.72 A (649)]/ml

Chlorophyll was expressed as ug chlorophyll/g fresh weight.

Statistical evaluation: The results were tested for significant differences using variance analysis and one-sided t-test. The exposed and control plants were compared separately for each variety.

RESULTS

Fluoride content of gladiolus leaves was significantly higher in exposed plants. There was not a marked difference in the uptake of fluoride in the two varieties. Fluoride uptake remained more or less unchanged during several exposure periods. Chlorophyll content seemed to not be greatly influenced by the uptake of fluoride. Protein content decreased significantly relative to the uptake of fluoride in the sensitive variety. This effect was less pronounced in the resistant variety. Enzyme activities varied over the year with different exposure periods, but ratios remained more or less the same. GS activity decrea-

TABLE I

Effect of fluoride pollution on the contents of fluoride, chlorophyll, soluble protein, and on the activities of GS, GlDH, MDH and enolase in gladiolus. Results after an exposure period of three weeks in springtime.

DETERMINATION	RESISTANT VARIETY		SENSITIVE VARIETY	
	CONTROL	EXPOSED	CONTROL	EXPOSED
Fluoride in dry matter	7.65	22.33	5.98	22.18
(ug F/g dry matter)	±0.31	±3.37	±0.66	±3.47
Significance level	0.01		0.01	
Total chlorophyll	65.17	68.38	108.89	106.68
(ug/g fresh weight)	±24.0	±18.28	±38.98	±56.34
Significance level	n.s.		n.s.	
Soluble protein	2.66	2.94	4.08	1.70
(mg/g fresh weight)	±0.81	±0.57	±0.56	±1.43
Significance level	n.s.		0.01	
GS (glutamine synthetase)	2.49	1.10	2.37	1.29
(uMol h^{-1}/g fresh weight)	±0.43	±0.16	±0.35	±0.33
Significance level	0.001		0.01	
GlDH (glutamate dehydrog.)	52.53	42.40	67.26	42.63
(mU/g fresh weight)	±11.75	±3.36	±6.82	±14.32
Significance level	n.s.		0.01	
MDH (malate dehydrog.)	3.785	3.237	5.057	3.530
(U/g fresh weight)	±0.951	±0.491	±0.863	±0.529
Significance level	n.s.		0.01	
Enolase	1.669	1.408	2.076	1.292
(U/g fresh weight)	±0.256	±0.155	±0.156	±0.276
Significance level	n.s.		0.001	

sed significantly in both varieties of exposed plants. GlDH activity only decreased in the sensitive plants. MDH activity decreased to two-thirds in the sensitive variety and four-fifths in the resistant variety. Enolase was also inhibited in the sensitive plants. Typical results of one exposure period are shown in Table 1.
Fig. 2 shows values of fluoride, chlorophyll and protein content expressed in relative units (%) averaged over six exposure periods in two years.

As enzyme activities vary greatly with different physiological stages they are expressed in relative units for convenience. From other experiments with bioindicators it is known that NRa activity in leaves is inhibited by the uptake of airborne fluoride (unpublished results). The activity of GS decreased significantly with increasing fluoride content of the leaves. This effect was more pronounced in the sensitive variety. The same was true for GlDH, but the resistant variety showed a slight increase in GlDH activity with increasing fluoride content. Fig. 3 shows the results for the activities of both GS and GlDH.

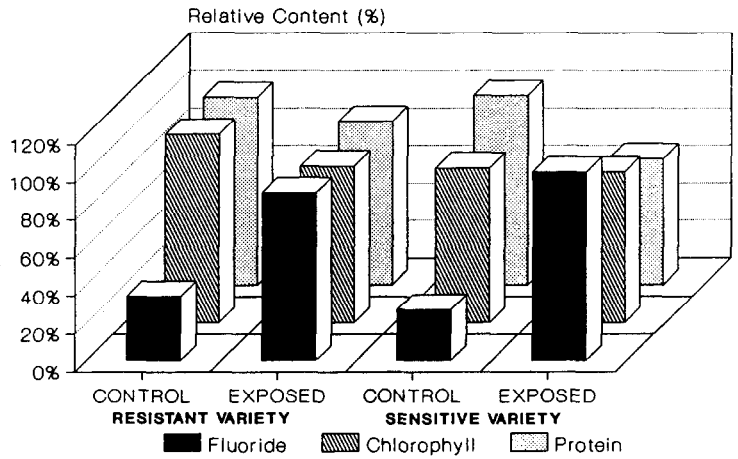

Fig. 2. Relative contents of fluoride in dry matter and relative contents of total chlorophyll and soluble protein in green tissue (for chlorophyll and protein: controls of the resistant variety = 100%; for fluoride: highest value = 100%). Values are averages of six exposure periods over two years.

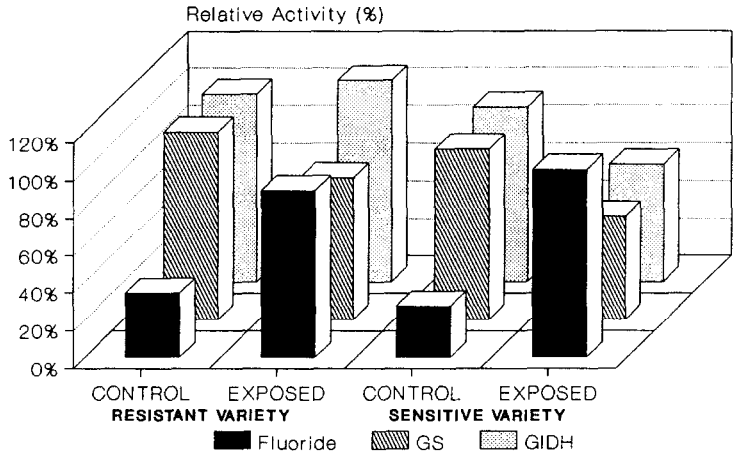

Fig. 3. Relative activities of GS and GlDH in gladiolus averaged over six exposure periods in two years (controls of resistant variety = 100%).

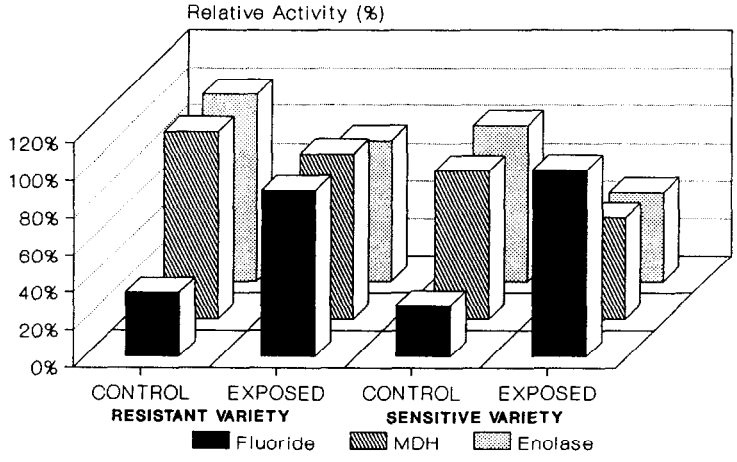

Fig. 4. Relative activities of MDH and enolase in green tissue of gladiolus (controls of resistant variety = 100%). Averages of 6 exposure periods in two years.

The enzymes of the glycolytic and citrate cycle showed a decrease in their activities with increasing fluoride content. Again, this effect was more pronounced in the sensitive variety. These findings are consistent with earlier observations made in experiments with agricultural plants [10]. In Fig. 4 results of the activities of MDH and enolase are shown.

DISCUSSION AND CONCLUSIONS

The results show clearly that both varieties of gladiolus take up the same amount of fluoride, depending on the location of exposure, thus depending on the fluoride content in the air.

Chlorophyll production is not influenced by the uptake of fluoride in both varieties at both sites. Fluoride inhibits the activity or the production of enolase. Consequently, less P-enol-pyruvate is then produced. The production of 2-oxoglutarate is reduced as the uptake of fluoride inhibits MDH activity. Both enolase and MDH are important steps in the production of a C skeleton, which must be available for nitrogen assimilation.

In the nitrogen pathway, NRa activity decreases with the uptake of fluoride. Glutamine formation is markedly inhibited as fluoride inhibits GS activity. GlDH plays an ambiguous role in plant metabolism: it may assimilate NH_4^+ with 2-oxo-glutarate to form L-glutamate in an anabolic metabolism. On the other hand, high GlDH activity is found during senescence. This process which supplies important amounts of ammonia, which at excessive concentrations in the cytosol, is toxic to the plant. This ambiguous function may explain why the activity of GlDH increased in exposed plants of the resistant variety, but decreased in plants of the sensitive variety.

All these changes in response to the uptake of fluoride have a negative effect on the production of amino acids. There is no doubt that less protein is synthesized by plants exposed to fluoride. Finally, gladiolus plants would seem to be an excellent bioindicator for monitoring fluoride pollution at a subnecrotic level because of the uptake of fluoride, the response in the activities of enolase, MDH, GS and GlDH, and the reduced formation of soluble protein.

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