Plant Physiol. (1988) 87, 651–654
0032-0889/88/87/0651/04/$01.00/0

Biochemical Studies of Paraquat-Tolerant Mutants of the Fern *Ceratopteris richardii*[1]

Received for publication August 11, 1987 and in revised form February 22, 1988

ELIZABETH W. CARROLL, OTTO J. SCHWARZ*, AND LESLIE G. HICKOK
Department of Botany, University of Tennessee, Knoxville, Tennessee 37996-0011

ABSTRACT

Enzymes and metabolites associated with mitigation of paraquat toxicity were compared in two paraquat-tolerant mutants and a sensitive wild-type strain of the fern *Ceratopteris richardii* Brongn. In 21-day-old gametophytes, the specific activities of superoxide dismutase, catalase, peroxidase, glutathione reductase, dehydroascorbate reductase, and ascorbate peroxidase showed no differences that would explain mutant tolerance. Constitutive levels of ascorbate and glutathione also did not differ significantly in the three strains. An experiment testing the inducibility of paraquat tolerance revealed no change in the dose response of mutant or wild type gametophytes after exposure to sublethal concentrations of the herbicide. Uptake of paraquat by whole gametophytes was also equivalent in mutants and wild type. These data suggest that the physiological basis for tolerance in these mutants, unlike several other tolerant biotypes reported, does not lie in the oxygen radical scavenging system, in an inducible stress response, or in a block to whole-plant uptake.

Paraquat-tolerant mutants of the fern *Ceratopteris richardii* Brongn. have been selected by means of an *in vitro*, whole-plant screening system (14, 15). Genetic analyses of two mutant strains, HαPQ2 and HαPQ45, indicate that a 10- to 20-fold increase in tolerance is conferred in both by different recessive mutations in the same locus (14). We are conducting a series of biochemical, structural, and physiological studies of these mutants to determine the mechanism of tolerance.

Investigations of paraquat tolerance and resistance in other species suggest several possible explanations for the trait in *Ceratopteris*. Because paraquat kills green tissue via increased production of superoxide and its peroxide products (11, 27), tolerance could be due to improved catabolism of active oxygen through a series of reactions located in the chloroplast (7, 8, 10, 12, 16)[2]:

$$O_2^- + O_2^- + 2H^+ \xrightarrow{\text{SOD}} O_2 + H_2O_2$$

$$H_2O_2 + \text{ascorbate} \xrightarrow{\text{ascorbate peroxidase}} \text{DHA} + H_2O$$

$$\text{DHA} + \text{GSH} \xrightarrow{\text{DHA reductase}} \text{ascorbate} + \text{GSSG}$$

$$\text{GSSG} + \text{NADPH} \xrightarrow{\text{glutathione reductase}} \text{GSH} + \text{NADP}^+$$

A mutation increasing the activity of one of these enzymes or of the level of an intermediary electron carrier (glutathione or ascorbate) essential to this detoxification scheme could explain tolerance. Although they are extrachloroplastic, catalase and peroxidase could be also involved in the removal of H_2O_2, and enhanced activities of superoxide dismutase, catalase, and peroxidase have been reported for polygenic mutants of *Lolium perenne* (13). Mutant enhancement of an inducible (rather than constitutive) defense system is also possible; for example, in *Spirodela oligorrhiza*, paraquat tolerance has been induced by adaptation to low levels of benzyl viologen, a nontoxic structural analog of paraquat (18).

While oxygen detoxification mutations may alter the organism's response to paraquat-mediated superoxide formation, other mutations could prevent paraquat's reduction to the monovalent radical either by changing the ability of PSI to reduce paraquat or by limiting the herbicide's access to its site of reduction in the chloroplast. Some seedling-lethal mutations of maize reported by Miles (21) show paraquat resistance associated with alterations in PSI or in photosynthetic electron transport to the primary acceptor of PSI.

Mutations that alter paraquat uptake or movement have been implicated in several resistant biotypes. A resistant mutant of *Escherichia coli* showed decreased paraquat uptake (17). In plants, the weed biotypes that have developed paraquat resistance in response to long-term spraying appear to limit paraquat movement within the plant. The resistant strain of *Hordeum glaucum* exhibits paraquat exclusion from the symplast (23). Autoradiography has demonstrated reduced translocation of paraquat in resistant lines of *Erigeron philadelphicus* (28) and the related species *Conyza bonariensis* (9). However, definite identification of the *Conyza* line as a translocational mutant is confused by reports that the strain also shows higher activities of chloroplast SOD, ascorbate peroxidase, and glutathione reductase (25).

Of these known modes of tolerance to paraquat, the one that seems least likely in *Ceratopteris* is a translocational mechanism. Mutant tolerance is apparent not only in the vascular sporophyte but also in the nonvascular cordate gametophyte, most of which is a one-cell-thick, green thallus (14). Comparisons of mutants and wild type to explore several of the remaining possibilities have been completed and are presented here.

MATERIALS AND METHODS

Gametophytes of the homozygous wild type (Hn-n), HαPQ45, and HαPQ2 strains of *Ceratopteris richardii* Brongn. were cultured axenically at 30°C under continuous fluorescent illumination of about 15 W m^{-2}. By the use of techniques previously described (15), large populations of homozygous gametophytes can be grown under uniform conditions in a single growth chamber, minimizing differences attributable to variations in culture conditions.

Twenty-one d after sowing, gametophytes were removed from

[1] Financial support for this research was provided by a grant from Martin Marietta Corporation.

[2] Abbreviations: SOD, superoxide dismutase; DCPIP, 2,6-dichlorophenolindophenol; DHA, dehydroascorbate.

the culture plates, weighed, extracted, and assayed for one or more enzymes or metabolites as described below. All assays were conducted on at least two separate harvests that included gametophytes of all three strains.

Extraction Procedures. Extractions were carried out on ice with chilled buffers and equipment, and extracts and fractions were kept on ice. When Chl levels were to be read, samples were also kept in the dark. Several extraction buffers were used in the course of the experiments.

Catalase and peroxidase, which are not chloroplast-specific, were assayed in gametophytes extracted in 50 mM Tris-HCl buffer, 1 mM dithiothreitol (pH 7.5) (1 g tissue/6 ml buffer). Crude extracts were strained through a layer of Miracloth (Calbiochem) and were centrifuged at 4°C in a Beckman J21C at 10,000 rpm for 15 minutes. The supernatant was assayed.

Chloroplast-rich fractions to assay for ascorbate peroxidase and glutathione reductase were obtained by extracting plants in a hypertonic (0.3 M sucrose or sorbitol) Hepes buffer (10) (1 g tissue/3 ml buffer). The extracts were strained through Miracloth and centrifuged in a Beckman J21C for 2 min at 3700 rpm. The chloroplast-rich pellets were resuspended in extraction buffer without osmoticum. Strainate, supernatant, and resuspended pellet were assayed. To assay DHA reductase, chloroplast-rich fractions were prepared using the protocol of Jablonski and Anderson (16).

SOD activity was assayed in extracts prepared by the methods described for ascorbate peroxidase and glutathione reductase and also in density-gradient purified chloroplasts, which were prepared by grinding gametophytes in 50 mM Tris-HCl, 5 mM dithiothreitol, 0.4 M sucrose (pH 7.5). This extract was strained and centrifuged for 2 min at 1700 rpm. The pellet was layered on a 55, 45, 15% sucrose density step gradient (a modification of Ref. 6) and centrifuged at 10,000 rpm for 15 min to separate a chloroplast fraction that collected in a band above the 45% sucrose layer. This fraction was resuspended in Tris buffer without osmoticum and assayed for SOD activity.

Enzyme Assays. Enzyme assays were run at 25°C. Catalase (5) and DHA reductase (16) activities were assayed by monitoring change in oxygen concentration, using a temperature-jacketed Clark-type oxygen electrode. The four other enzymes were assayed spectrophotometrically using a Beckman DU fitted with a Gilford photometer. Peroxidase was assayed by the reduction of orthodianisidine (24). SOD was assayed by the xanthine/xanthine oxidase Cyt *c* reduction method (20), glutathione reductase by Halliwell and Foyer's method (12), and ascorbate peroxidase by the method of Asada (1).

Protein Assay. In order to compare specific enzyme activities, protein was assayed in all enzyme samples by the Bradford method (3), using a Bio-Rad protein assay kit and a standard curve of bovine serum albumin.

Chlorophyll Assay. Specific activities are expressed here on a protein basis. Chl levels of the extracts, chloroplast-enriched fractions, and samples of whole gametophytes were also routinely measured using 80% acetone as a solvent and calculating Chl *a* + *b* from absorbance at 652 nm with the extinction coefficient of Bruinsma (4).

Metabolite Assays. Ascorbate was assayed spectrophotometrically by the reduction of DCPIP (22). Both total glutathione and oxidized glutathione were assayed spectrophotometrically by the glutathione reductase method (26). Each metabolite was assayed in 3 to 5 separate harvests, and standard curves of authentic ascorbate or glutathione (Sigma) were conducted with each assay.

Chloroplast Intactness. In order to monitor the degree of chloroplast intactness, samples of the chloroplast-enriched fractions were examined under phase contrast optics (19).

Activity Stain for Superoxide Dismutase. To identify possible isozyme differences, nondenaturing polyacrylamide gels were developed for extracts and chloroplast-enriched fractions from all three biotypes and stained for SOD activity (2).

Induction Experiment. From preliminary experiments comparing Chl loss of 21-d-old gametophytes of Hn-n and of HαPQ45, 0.01 μM paraquat (commercial preparation supplied by the Ortho Division of Chevron) was identified as a concentration just sublethal to wild type (I_{50} = 0.023 μM) while 0.1 μM paraquat had a comparable effect on Chl loss in HαPQ45 (I_{50} = 0.6 μM). The appropriate sublethal dose was then added to the nutrient medium on which spores of each biotype were sown. At 21 d the gametophytes were harvested, and 0.1 g samples were floated for 48 h on 25-ml aliquots of half-strength nutrient medium (15) or on half-strength nutrient medium supplemented with 1, 10, 20, and 50 μM paraquat. Controls were grown on medium containing no paraquat and floated on the same range of concentrations. Three lots of paraquat-grown gametophytes and two of gametophytes grown on plain medium were assayed. Five 0.1 g samples of gametophytes were floated on each paraquat concentration and assayed for Chl content (4).

Uptake Experiment. To study whole-plant uptake, two time-course experiments were conducted. Three replicate samples (per time point and strain) of 21-d-old gametophytes were floated on 2.5 μM ^{14}C-methyl paraquat (a gift of Chevron Corporation) for times ranging from 30 min to 10 h. Plants were rinsed twice for 30 s, first in a 25 μM solution of nonlabeled paraquat and then in distilled water. The samples were digested using the Unisol system (Isolab, Inc.) and were counted in a Beckman LS-200B scintillation counter.

RESULTS AND DISCUSSION

Table I shows the specific activities of six oxygen-detoxifying enzymes in wild type and both mutants. Catalase and peroxidase activities are shown for whole-plant extracts; SOD activities for whole-plant extracts, strainate from the chloroplast-rich pellet, and density-gradient purified chloroplasts; and glutathione reductase, ascorbate peroxidase, and DHA reductase activities for chloroplast-rich fractions and supernatants from their preparation.

In almost all of the data, the specific activities of the three biotypes do not differ significantly at the 5% confidence level when compared by analysis of variance. In four cases, the mean for one biotype does differ significantly from the other two. The purified chloroplast fraction of HαPQ45 shows a significantly lower SOD activity than HαPQ2 or wild type. Since HαPQ45 is the slightly more tolerant of the two mutants, it seems unlikely that the somewhat lower SOD activity seen in this set of experiments could relate to increased tolerance. A similar anomaly exists in the glutathione reductase activities of the supernatant fractions and in the ascorbate peroxidase activity of the chloroplast-rich fractions. In both cases, mutant HαPQ2 showed a somewhat higher activity than HαPQ45, and HαPQ45 was not significantly different from wild type. The mean DHA reductase activity of HαPQ2 is significantly lower than the means of the other two biotypes, which do not differ from one another.

The absence of enrichment of SOD, ascorbate peroxidase, DHA reductase, or glutathione reductase activities in any of the chloroplast fractions may be due to chloroplast breakage and consequent loss of soluble enzymes. Although more than half of the chloroplasts in the chloroplast fractions appeared intact by the phase contrast brightness test, the loss of stromal enzymes from the remainder or breakage and resealing during preparation may account for the lower values.

Activity staining of superoxide dismutase revealed a single band (photo not shown) at the same location for all fractions and strains. The band did not appear in comparable gels treated

Table I. *Specific Activities of Six Enzymes in 21-d-Old Gametophytes of Paraquat-Tolerant Ceratopteris Mutants and of Wild Type*

Preparation of extracts and fractions and enzyme assay techniques are described in "Materials and Methods."

Fraction and Strain	Specific Enzyme Activity (mg protein^{-1})[a]	
	Peroxidase	Catalase
	A/min	*mmol O_2/min*
Whole-plant extract		
Wild type	1.4 ± 0.43	1.1 ± 0.06
HαPQ2	1.4 ± 0.12	1.2 ± 0.19
HαPQ45	1.5 ± 0.20	1.1 ± 0.41

	Superoxide Dismutase
	units SOD
Strainate	
Wild type	16 ± 3.5
HαPQ2	18 ± 2.8
HαPQ45	15 ± 5.7
Supernatant from crude chloroplast pellet	
Wild type	12 ± 2.8
HαPQ2	12 ± 2.1
HαPQ45	14 ± 1.4
Density-gradient purified chloroplasts	
Wild type	4.7 ± 0.42
HαPQ2	4.3 ± 1.80
HαPQ45	2.8 ± 1.50[b]

	Glutathione Reductase	Ascorbate Peroxidase	DHA Reductase
	μmol NADPH oxidized min^{-1}	*μmol ascorbate oxidized min^{-1}*	*μmol O_2 taken up min^{-1}*
Supernatant			
Wild type	0.22 ± 0.047	10.0 ± 2.30	1.3 ± 0.34
HαPQ2	0.30 ± 0.026[b]	9.7 ± 1.91	1.0 ± 0.12[b]
HαPQ45	0.19 ± 0.045	9.9 ± 2.29	1.3 ± 0.42
Chloroplast-rich pellet			
Wild type	0.11 ± 0.019	1.6 ± 0.55	0.46 ± 0.107
HαPQ2	0.14 ± 0.030	2.2 ± 0.64[b]	0.47 ± 0.164
HαPQ45	0.12 ± 0.038	1.8 ± 0.24	0.54 ± 0.131

[a] For glutathione reductase, DHA reductase, and SOD in purified chloroplasts, values are the means (± SD) from assays made on fractions from two separate harvests. All other means are for three separate harvests. Each extract was assayed three times, and the means were compared at the 5% confidence level by analysis of variance. [b] Mean differs significantly from the other two strains at the 5% confidence level.

with 1 mM KCN, suggesting that only the Cu-Zn form of superoxide dismutase is present in *Ceratopteris* gametophytes.

Table II compares the endogenous levels of ascorbate and glutathione in the three strains. Again, no statistically significant differences were seen in levels of either metabolite. Moreover, no detectable differences were observed in the percentage of oxidized glutathione, which was always quite low (3% or less) in the gametophytes.

Figure 1 shows the results of an experiment testing the effects of exposure to sublethal doses of paraquat on subsequent tolerance to paraquat in both wild type and HαPQ45. These results suggest that prior exposure to low levels of paraquat did not affect the level of paraquat tolerated by either wild type or the HαPQ45 mutant.

Figure 2 shows the mean (±SD) counts per minute of six replicate samples for each strain and each time point in the experiments on whole gametophyte uptake of ^{14}C-methyl paraquat. The paraquat uptake profiles for the wild type and mutants are similar. All three strains accumulate paraquat to the same extent after 10 h of incubation, suggesting that differences in

Table II. *Ascorbate and Glutathione Levels of 21-d-Old Gametophytes*

Strain	Ascorbate[a]	Total Glutathione[b]
	μg/g fresh wt	*μg/g fresh wt*
Wild type	61 ± 15.8[c]	4.1 ± 0.25
HαPQ2	57 ± 9.3	3.7 ± 0.66
HαPQ45	61 ± 6.6	3.9 ± 1.04

[a] Assayed spectrophotometrically by the reduction of DCPIP (22). [b] Total glutathione and oxidized glutathione assayed spectrophotometrically by the glutathione reductase method as modified by Smith (26). [c] Each value is the mean (± SD) of assays of three different extracts. Each extract was assayed three to five times. The means for the three strains reported in this table do not differ significantly at the 5% level when compared by analysis of variance.

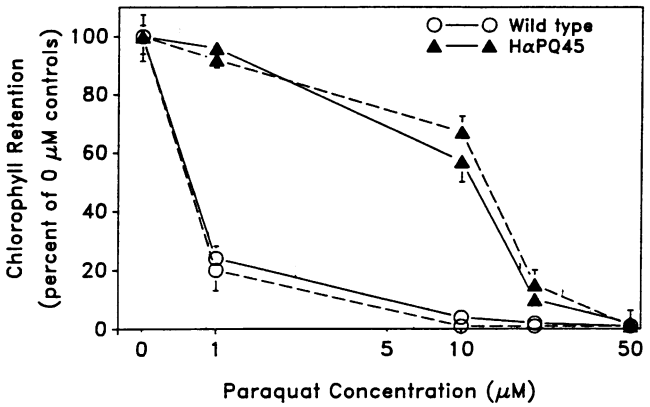

FIG. 1. Effect of previous exposure to sublethal concentrations of paraquat on the bleaching of wild-type or HαPQ45 gametophytes floated on paraquat concentrations of 1 to 50 μM. Mean (±SD) of three replicate samples are shown for each strain and concentration. The solid lines represent samples grown on nutrient medium without paraquat, and the dashed lines represent samples grown on nutrient medium supplemented with paraquat at a sublethal concentration (0.01 μM for wild type and 0.1 μM for HαPQ45).

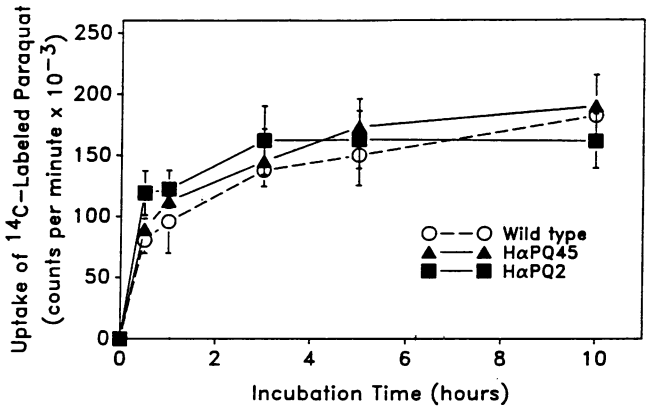

FIG. 2. Paraquat uptake by whole gametophytes of wild type, HαPQ45, and HαPQ2. Means (±SD) of three replicate samples are shown for each strain and each time point. Gametophytes were floated in the dark on 2.5 μM ^{14}C-methyl paraquat for the times indicated. After rinsing to remove surface residues of labeled paraquat, samples were solubilized and counted.

whole-plant uptake are not responsible for the observed tolerance in the mutants.

The experiments reported here suggest that the higher tolerance to paraquat conferred by single mutations in *Ceratopteris* strains HαPQ2 and HαPQ45 does not stem from higher constitutive levels of enzymes and metabolites involved in superoxide detoxification. Furthermore, experiments testing differences in whole-plant uptake, and the effects of a sublethal concentration of paraquat on a biotype's susceptibility to a lethal concentration, refute hypotheses involving enhancement of tolerance via these mechanisms. Since enhanced mutant tolerance is shown by the single layer of photosynthetic cells characteristic of gametophytic tissue, compartmentation of paraquat in certain tissue types or restricted translocation are unlikely in *Ceratopteris.*

The possibility remains that the mutants sequester paraquat away from the site of its reduction by PSI, either within the cell or in the cell wall. To explore this hypothesis, we are currently comparing the access of paraquat to PSI in mutant and wild type and the tolerance of protoplasts made from *Ceratopteris* gametophytes of both susceptible and tolerant strains.

Acknowledgments—We would like to thank Ms. Jennifer Panter and Ms. Petra Lindsey for their expert technical assistance and Dr. Thomas G. Warne for his advice on experimental design and data analysis.

LITERATURE CITED

1. ASADA K 1984 Chloroplasts: formation of active oxygen and its scavenging. Methods Enzymol 105: 422–429
2. BEAUCHAMP CO, I FRIDOVICH 1971 Isozymes of superoxide dismutase from wheat germ. Biochim Biophys Acta 317: 50–64
3. BRADFORD MM 1976 A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248–254
4. BRUINSMA J 1963 The quantitative analysis of chlorophylls a and b in plant extracts. Photochem Photobiol 2: 241–249
5. DEL RIO L, M GOMEZ ORTEGA, A LEAL LOPEZ, J LOPEZ GORGE 1977 A more sensitive modification of the catalase assay with the Clark oxygen electrode. Anal Biochem 80: 409–415
6. DOUCE R, J JOYARD 1982 Purification of the chloroplast envelope. *In* M Edelman, RB Hallick, N-H Chua, eds, Methods in Chloroplast Molecular Biology. Elsevier, New York, pp 239–256
7. FOSTER JG, GE EDWARDS 1980 Localization of superoxide dismutase in leaves of C_3 and C_4 plants. Plant Cell Physiol 21: 895–906
8. FOYER CH, B HALLIWELL 1976 The presence of glutathione and glutathione reductase in chloroplasts: a proposed role in ascorbic acid metabolism. Planta 133: 21–25
9. FUERST EP, HY NAKATANI, AD DODGE, D PENNER, CJ ARNTZEN 1985 Paraquat resistance in *Conyza.* Plant Physiol 77: 984–989
10. GRODEN D, E BECK 1979 Hydrogen peroxide destruction by ascorbate-dependent systems from chloroplasts. Biochim Biophys Acta 546: 426–435
11. HALLIWELL B 1984 Chloroplast Metabolism: The Structure and Function of Chloroplasts in Green Leaf Cells. Clarendon Press, Oxford
12. HALLIWELL B, CH FOYER 1978 Properties and physiological function of a glutathione reductase purified from spinach leaves by affinity chromatography. Planta 139: 9–17
13. HARPER DB, BMR HARVEY 1978 Mechanism of paraquat tolerance in perennial ryegrass. II. Role of superoxide dismutase, catalase, and peroxidase. Plant Cell Environ 1: 211–215
14. HICKOK LG, OJ SCHWARZ 1986 Paraquat tolerant mutants in *Ceratopteris*: genetic characterization and reselection for enhanced tolerance. Plant Sci 46: 153–158
15. HICKOK LG, TR WARNE, M SLOCUM 1987 *Ceratopteris richardii*: applications for experimental plant biology. Am J Bot 74: 1304–1316
16. JABLONSKI PP, JW ANDERSON 1981 Light-dependent reduction of dehydroascorbate by ruptured pea chloroplasts. Plant Physiol 67: 1239–1244
17. KAO SM, HM HASSAN 1985b Biochemical characterization of a paraquat-tolerant mutant of *E. coli.* J Biol Chem 260: 10478–10481
18. LEWINSOHN E, J GRESSEL 1984 Benzyl viologen-mediated counteraction of diquat and paraquat phytotoxicities. Plant Physiol 76: 125–130
19. LILLEY RM, MP FITZGERALD, KG RIENITS, DA WALKER 1975 Criteria of intactness and the photosynthetic activity of spinach chloroplast preparations. New Phytol 75: 1–10
20. MCCORD J, I FRIDOVICH 1969 Superoxide dismutase: an enzymatic function for erythrocuprein. J Biol Chem 224: 6049–6055
21. MILES CD 1980 Mutants of higher plants: maize. Methods Enzymol 69: 3–22
22. OMAYE ST, JD TURNBULL, HE SAUBERLICH 1979 Selected methods for the determination of ascorbic acid in animal cells, tissues, and fluids. Methods Enzymol 62: 3–11
23. POWLES SB, G CORNIC 1987 Mechanism of paraquat resistance in *Hordeum glaucum.* I. Studies with isolated organelles and enzymes. Aust J Plant Physiol 14: 81–89
24. SEQUI P, A MARCHESINI, A CHERSI 1970 Peroxidase isoenzymes in germinated wheat. FEBS Symp 18: 297–303
25. SHAALTIEL Y, J GRESSEL 1986 Multi-enzyme oxygen radical detoxifying system correlated with paraquat tolerance in *Conyza bonariensis.* Pestic Biochem Physiol 26: 22–28
26. SMITH IK 1985 Stimulation of glutathione synthesis in photorespiring plants by catalase inhibitors. Plant Physiol 79: 1044–1047
27. SUMMERS LA 1980 The Bipyridinium Herbicides. Academic Press, New York
28. TANAKA Y, H CHISAKA, H SAKA 1986 Movement of paraquat in resistant and susceptible biotypes of *Erigeron philadelphicus* and *E. canadensis.* Plant Physiol 66: 605–608