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Paraquat Resistance in *Conyza*[1]

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ABSTRACT

A biotype of *Conyza bonariensis* (L.) Cronq. (identical to *Conyza linefolia* in other publications) originating in Egypt is resistant to the herbicide 1,1′-dimethyl-4,4′-bipyridinium ion (paraquat). Penetration of the cuticle by [^{14}C]paraquat was greater in the resistant biotype than the susceptible (wild) biotype; therefore, resistance was not due to differences in uptake. The resistant and susceptible biotypes were indistinguishable by measuring *in vitro* photosystem I partial reactions using paraquat, 6,7-dihydrodipyrido [1,2-α:2′,1′-c] pyrazinediium ion (diquat), or 7,8-dihydro-6H-dipyrido [1,2-α:2′,1′-c] [1,4] diazepinediium ion (triquat) as electron acceptors. Therefore, alteration at the electron acceptor level of photosystem I is not the basis for resistance. Chlorophyll fluorescence measured *in vivo* was quenched in the susceptible biotype by leaf treatment with the bipyridinium herbicides. Resistance to quenching of *in vivo* chlorophyll fluorescence was observed in the resistant biotype, indicating that the herbicide was excluded from the chloroplasts. Movement of [^{14}C] paraquat was restricted in the resistant biotype when excised leaves were supplied [^{14}C]paraquat through the petiole. We propose that the mechanism of resistance to paraquat is exclusion of paraquat from its site of action in the chloroplast by a rapid sequestration mechanism. No differential binding of paraquat to cell walls isolated from susceptible and resistant biotypes could be detected. The exact site and mechanism of paraquat binding to sequester the herbicide remains to be determined.

Weed resistance to paraquat[5] (Fig. 1) has been reported in annual bluegrass (*Poa annua* L.), Philadelphia fleabane (*Erigeron philadelphicus* L.), and hairy fleabane (*Conyza bonariensis*) in England, Japan, and Egypt, respectively (6). In every case, paraquat was applied several times per year for more than 5 years. Paraquat-tolerant lines of perennial ryegrass [*Lolium perenne* L. (Causeway)] have also recently been developed (8).

The resistant biotype of *Conyza* originated in the Tahrir irrigation area in Egypt. An intensive paraquat spraying program was undertaken in vine and citrus plantations in 1970 and difficulties in controlling this weed were first observed in the mid 1970s (8). Members of the genus *Conyza* are common weeds in the United States and have become a locally serious agronomic problem in reduced tillage systems (3). These weed species, therefore, proliferate under conditions where weed control often depends upon the use of paraquat. It is possible that paraquat resistance may become a problem in this genus in the United States due to the trend toward reduced tillage and heavier reliance upon paraquat.

The mechanism of paraquat action involves the PSI-mediated reduction of the paraquat di-cation. This results in the formation of the mono-cation radical. The mono-cation radical reduces O_2 to $O_2^{\cdot-}$, the superoxide anion radical, resulting in the regeneration of the paraquat di-cation. Subsequently, H_2O_2 and the hydroxyl radical ($OH\cdot$) may be produced by a variety of reactions (4, 5). Hydroxyl radicals are known to cause peroxidation of fatty acids. This is apparently a cause of the observed loss of membrane integrity (5, 7, 9). Superoxide and H_2O_2 may not directly cause the paraquat-induced loss of membrane integrity (9). In addition to the formation of reactive forms of O_2, the presence of paraquat causes the diversion of electrons which normally reduce NADP, and maintain the reduced state of α-tocopherol, glutathione, and ascorbate which function in cellular protection mechanisms. The action of superoxide dismutase, catalase, and peroxidase would presumably remain unaffected by this electron diversion.

Several hypotheses could explain the mechanism of paraquat resistance in *C. bonariensis*. These are: (a) detoxification of the superoxide anion radical or other reactive forms of O_2 produced in the presence of paraquat; (b) alteration in the redox potential of the PSI primary electron acceptor such that the potential herbicide would be a less efficient electron acceptor; or (c) altered compartmentation of paraquat, resulting in reduced localization of the herbicide at the active site. Metabolic detoxification of paraquat is unlikely since there are no known metabolic products of paraquat in higher plants (13).

MATERIALS AND METHODS

Cuticular Penetration. Leaves were excised under water. The petiole was supported in a water-filled 18 × 150 mm test tube with the leaf blade exposed to air. A 50 μl solution, containing 0.16 μCi [^{14}C]paraquat (0.13 mCi/mmol) and 0.5% surfactant (X-77 from Chevron Corp.), was applied uniformly to both surfaces of the leaf in 0.5 μl droplets. This dose of paraquat causes injury in the susceptible biotype but not the resistant biotype (data not shown). After 4 h, leaves were dipped in water

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[5] Abbreviations: paraquat, 1,1′-dimethyl-4,4′-bipyridinium ion; diquat, 6,7-dihydrodipyrido [1,2-α:2′,1′-c] pyrazinediium ion; triquat, 7,8-dihydro-6H-dipyrido [1,2-α:2′,1′-c] [1,4] diazepinediium ion; TMPD, *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine; PPFD, photosynthetic photon flux density.

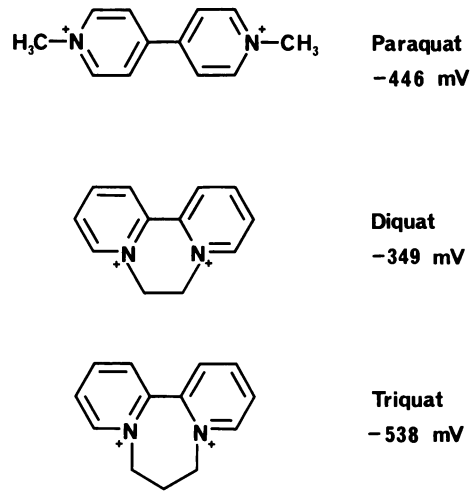

FIG. 1. Structures and oxidation-reduction midpoint (redox) potentials of the three bipyridinium herbicides tested.

for 2 min, blotted dry, dipped in chloroform three times, and then wiped with a glass filter paper wetted with chloroform. The chloroform extract was allowed to dry. The purpose of the chloroform dips and wipes was to remove the cuticle. The amount of [^{14}C]paraquat penetrating the cuticle was estimated by subtracting the amount of [^{14}C]paraquat obtained in the washes from the amount applied. Liquid scintillation spectrometry was used to measure radioactivity. Leaf areas were determined on a model LI-300 portable area meter (Lambda Instruments Corp.). There were three replications for each biotype, and a different plant was used for each replicate.

PSI Partial Reaction. Chloroplast thylakoid membranes from resistant and susceptible biotypes of *C. bonariensis* were isolated as previously described (12). Rates of PSI-mediated electron flow from reduced TMPD to paraquat, diquat, and triquat were monitored as O_2 uptake using an O_2 electrode. Rates of electron transport were measured under saturating light by continuous recording of O_2 uptake using a water-jacketed Clark-type O_2 electrode maintained at 20°C. The assay buffer contained 50 μg Chl·ml^{-1}, 50 mM Tricine-NaOH (pH 7.8), 100 mM sorbitol, 1 mM NH_4Cl, 0.1 μM gramicidin, 5 mM $MgCl_2$, 10 mM NaCl, 100 μM NaN_3, 10 μM diuron, 2.5 mM sodium ascorbate, 25 μg/ml superoxide dismutase, and 100 μM TMPD. The bipyridinium herbicides selected represent a wide range of redox potential (Fig. 1). Herbicide concentrations were varied as indicated in the results.

Dose-Response Effects on Excised Leaves. Leaves were excised under water and dipped in solutions containing a range of herbicide concentrations and 0.5% (v/v) surfactant. Excised leaves were supported in a test tube as described earlier. There were four replications of each treatment and a different plant was used for each replicate. Leaves were placed in darkness for 4 h to allow uptake of the herbicides.

In vivo Chl fluorescence was monitored with a model SF-10 fluorimeter (Richard Brancker Research Ltd.) as described previously (1). Transients were recorded with a Nicolet Explorer digital oscilloscope. The variable fluorescence values reported represent $(F_p\text{-}F_O)/F_O$, where F_p is the peak fluorescence value (recorded at 2 s in this experiment) and F_O is the fluorescence level measured at 1 ms after onset of illumination (Fig. 4). After recording the fluorescence transients, the leaves were moved to a chamber at 25°C provided with white light (450 μE·m^{-2}·s^{-1}) for 5 h. Leaves were then placed in darkness for 24 h to allow drying of injured tissue. Injury was evaluated by visual estimation of the percent green leaf area. Per cent moisture was determined by measuring leaf weights before and after drying at 70°C for 24 h.

Autoradiography. Leaves were excised as previously described. The petiole was placed in a 0.8 ml vial containing 50 μl of 0.064 μCi [^{14}C]paraquat (1.4 mCi·mmol^{-1}) in 10 mM phosphate buffer (pH 7). This dose of paraquat caused complete necrosis in the susceptible biotype but no injury in the resistant biotype if the leaves were subsequently placed in a PPFD of 450 μE·m^{-2}·s^{-1} (data not shown). However, for the determination of radioactive paraquat distribution, the leaves were kept in room light for 4 h, at which time onset of tissue damage was not visible. Leaves were lyophilized overnight and X-ray film was placed in contact with the leaves for 36 h. There were three replications of each treatment for each biotype and a different plant was used for each replicate. Representative autoradiograms are shown.

Binding of Paraquat to Cell Walls. Leaf material of the two *C. bonariensis* biotypes was stored at −20°C until use. Leaves were ground in pulverized dry ice in a mortar and pestle and passed through a 600 μm sieve. All subsequent operations were conducted at 4°C. Approximately 1 g of pulverized leaf material was suspended in 40 ml of 100 mM $CaCl_2$, 100 mM Tricine-NaOH (pH 7.8) at 4°C, and sonicated for 1 min at high speed in a Branson Instruments Inc. model no. S-125 Sonifier. The supernatant was removed by filtration. The solid material was washed exhaustively with water, then with ethanol, and again with water. This pellet (designated as the cell wall fraction) was resuspended in 10 ml water, and weight per unit volume was determined after drying three replicate 0.5 ml samples. Various other methods for cell wall purification, including washing in acetone or 2% (w/v) Triton X-100, substituting NaCl for $CaCl_2$ or K-phosphate (pH 7.0) for Tricine-NaOH, were also utilized for paraquat binding studies, in addition to simply measuring binding to unpurified ground leaf material. In all cases, no differential binding between susceptible and resistant biotypes was detected (data not presented).

A 1 mg aliquot of fresh cell wall material was placed in a centrifuge tube and brought to 1.5 ml with water. [^{14}C]Paraquat (0.0021 μCi; 1.4 mCi·mmol^{-1}) was followed by sufficient unlabeled paraquat to attain the desired concentrations. Ten μM $CaCl_2$ was added to half of the samples. After 10 min incubation, the samples were centrifuged for 1 min at 12,000*g*. One ml aliquots of the supernatant were removed, and radioactivity was assayed by liquid scintillation counting. The amount of bound radioactivity was determined by subtracting the amount of radioactivity determined above from the amount assayed in a similarly treated control sample which lacked the cell wall suspension. Values reported are the means of three replicates.

RESULTS AND DISCUSSION

Cuticular Penetration. Table I indicates that most of the [^{14}C] paraquat applied to *C. bonariensis* leaves was removed in the aqueous wash and that more [^{14}C]paraquat was removed in the aqueous washes of the susceptible biotype. Very little [^{14}C]paraquat was removed in the chloroform wash in either biotype.

Table I. *Cuticular Penetration of [^{14}C]Paraquat*

Means within columns followed by the same letters are not significantly different at the 5% level according to Duncan's multiple range test.

Biotype	Avg. Leaf Area	Aqueous Wash	Chloroform Wash	Amount Penetrating Cuticle
	cm²		*% of applied*	
Resistant	7.8	67 b	<1 a	33 a
Susceptible	9.6	79 a	<1 a	21 b

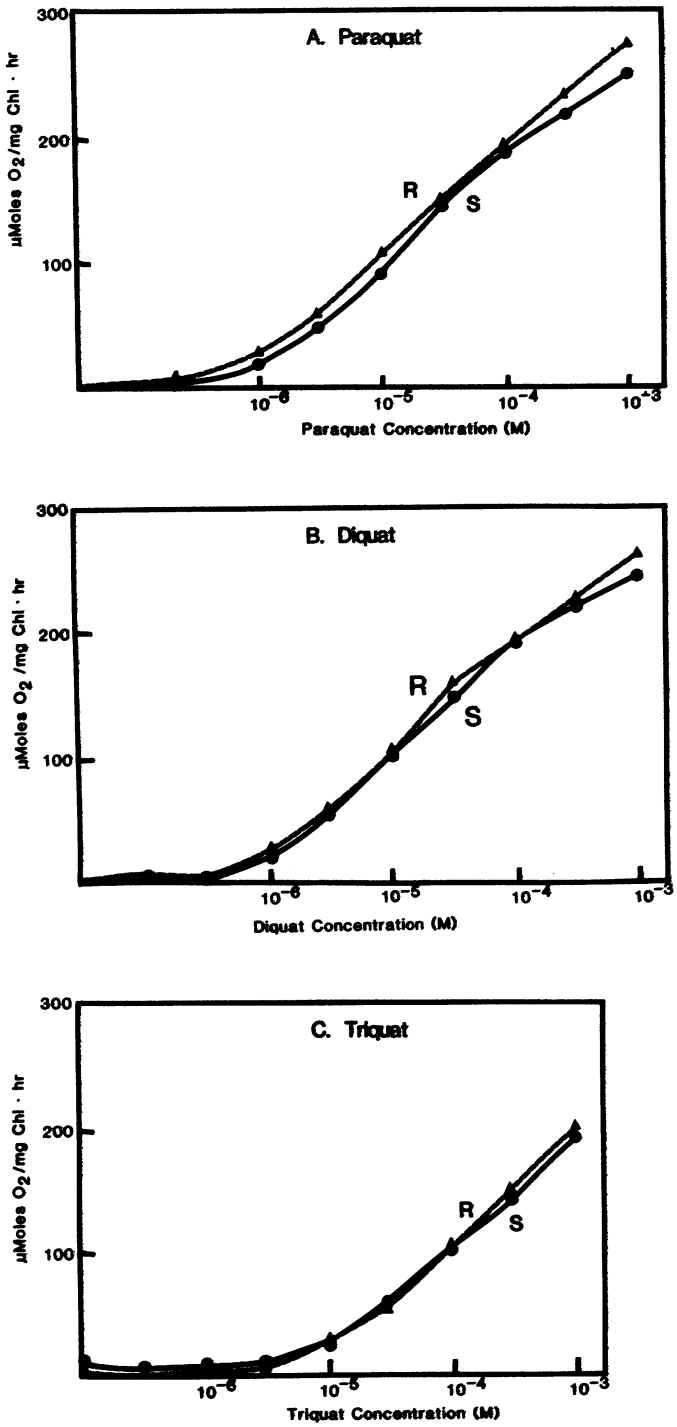

FIG. 2. Effect of bipyridinium herbicide concentration on PSI-mediated electron transport in resistant (R) and susceptible (S) biotypes, using TMPD as the electron donor. A, B, and C refer to response to paraquat, diquat, or triquat, respectively.

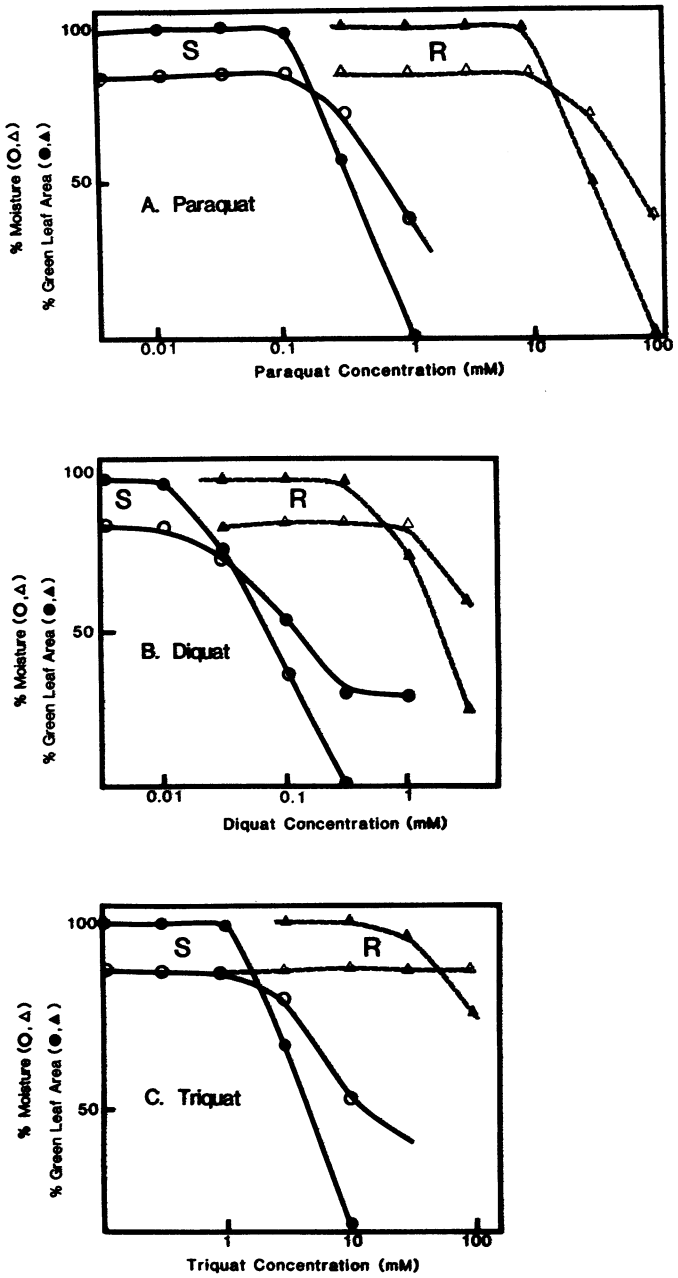

FIG. 3. Response of per cent leaf area remaining green (●,▲) and per cent moisture (O,Δ) to bipyridinium herbicide concentration in excised leaves of resistant (R) and susceptible (S) biotypes. A, B, and C refer to response to paraquat, diquat, or triquat, respectively.

Therefore, considerably more [^{14}C]paraquat penetrated the cuticle of the resistant biotype. The mean leaf areas of the two biotypes differed; however, the smaller area in the resistant biotype does not explain the greater uptake. It was previously stated (8) that paraquat adsorption on leaf tissue was greater in the resistant biotype of this species. This is consistent with our observation. Therefore, resistance to paraquat cannot be explained by a cuticular barrier in the resistant biotype.

PSI Partial Reaction. Since the bipyridinium herbicides act as electron acceptors, there was a stimulation of electron transport (measured *in vitro* as O_2 consumption using isolated chloroplasts) as herbicide concentration increased (Fig. 2). The responses of the two biotypes were indistinguishable for all three herbicides. Therefore, an altered electron acceptor site of action does not appear to be responsible for resistance. The concentration requirement for triquat activity (Fig. 2C) was greater than that of paraquat (Fig. 2A) or diquat (Fig. 2B) in both biotypes. This is presumably due to its more negative redox potential (Fig. 1).

Dose-Response Effects on Excised Leaves. Resistance to the bipyridinium herbicides was observed by desiccation and by visual estimation of the per cent green leaf area (Fig. 3). Since diquat and triquat are structurally quite similar but differ greatly in their redox potentials (Fig. 1), a larger degree of resistance to triquat would be anticipated if resistance is due to an altered site

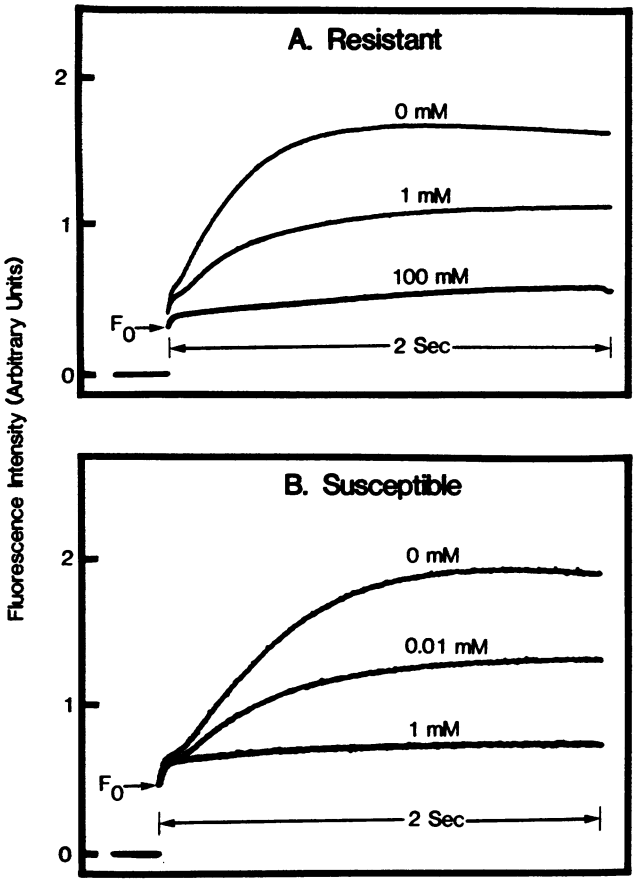

FIG. 4. Response of *in vivo* fluorescence transients to paraquat in excised leaves of resistant (R) and susceptible (S) biotypes.

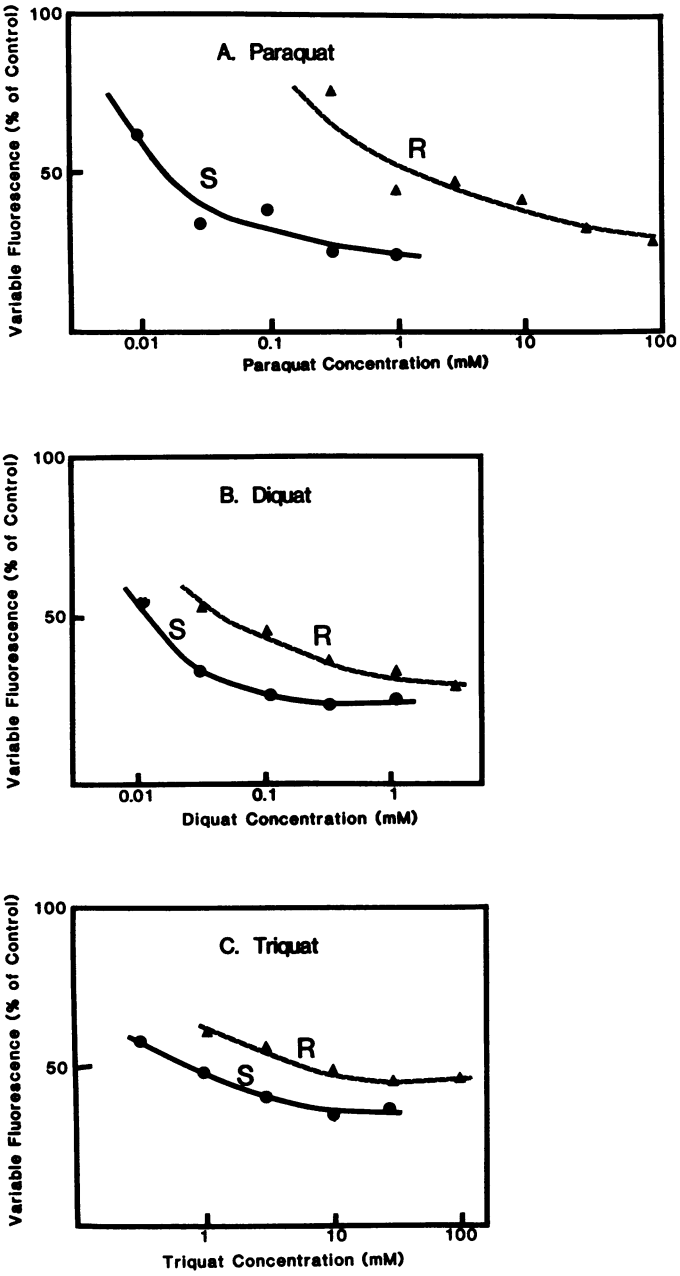

FIG. 5. Response of *in vivo* fluorescence to bipyridinium herbicide concentration in excised leaves of resistant (R) and susceptible (S) biotypes. A, B, and C refer to response to paraquat, diquat, or triquat respectively.

of action, *i.e.* an altered redox potential of the PSI primary electron acceptor which donates electrons to the herbicide. However, the degree of resistance to triquat was quite similar to that of diquat (Figs. 3 and 4; Table I). Hence, a modified site of action at the reducing side of PSI is not the basis for resistance.

Quenching of *in vivo* Chl fluorescence transients by paraquat is shown in Figure 4. Paraquat caused quenching in both biotypes, but much higher concentrations were required in the resistant biotype. All three bipyridinium compounds caused quenching of *in vivo* fluorescence (Fig. 5). Higher concentrations of all herbicides were required to cause quenching in the resistant biotype. Bipyridinium herbicides quench Chl fluorescence by efficiently accepting electrons from PSI, thereby keeping the plastoquinone pool oxidized. Quenching of Chl fluorescence by paraquat was similar in the two biotypes when evaluated in isolated chloroplasts. *In vitro* fluorescence was quenched 50% at approximately 2 μM in both biotypes (data not shown). Resistance to quenching of *in vivo* fluorescence by the bipyridinium herbicides must, therefore, be attributed to exclusion of the herbicides from the active site. This exclusion might occur by rapid compartmentation.

It was previously proposed that paraquat resistance might involve detoxification of the superoxide anion radical (14) or other reactive forms of O_2. This concept is not consistent with the Chl fluorescence measurements shown in Figures 4 and 5; the lack of fluorescence quenching is direct evidence that the herbicides do not reach their site of reduction in the chloroplast, and therefore that there are no toxic O_2 species generated in the resistant biotype.

Resistance ratios (see legend of Table II) and herbicide concentrations which caused 50% injury, as evaluated by the three techniques discussed, are shown in Table II. Resistance to paraquat and cross-resistance to diquat and triquat were observed by all methods employed. However, the resistance ratios for paraquat were larger than those for diquat or triquat. The resistance mechanism is, therefore, somewhat specific for the herbicide which provided the selective force in the field. The resistance ratios determined for paraquat by the three different methods are in reasonably close agreement (Table II). The correspondence of the ratios determined by Chl fluorescence measurements with ratios determined by per cent moisture or per cent green leaf area is consistent with the generally accepted principle that paraquat causes plant injury by its effects on the chloroplast.

Quenching of fluorescence, by all herbicides tested, begins at herbicide concentrations far below those that cause loss of moisture or Chl (Figs. 3 and 5). Also, the I_{50} for fluorescence quench-

Table II. *Estimated Concentrations Which Cause 50% Injury by Reduction of In Vivo Chl Fluorescence, Per Cent Moisture or Per Cent of Leaf Area Remaining Green*

The resistance ratio was estimated by dividing the I_{50} for the resistant biotype by the I_{50} for the susceptible biotype.

Herbicide	Method of Estimation of Injury	I_{50} Resistant Biotype	I_{50} Susceptible Biotype	Resistance Ratio
		mM		
Paraquat	Chl fluorescence	2.3	0.015	150
	% Moisture	62	0.62	100
	% Green leaf area	30	0.32	94
Diquat	Chl fluorescence	0.060	0.013	4.6
	% Moisture	4.5[a]	0.12	38
	% Green leaf area	1.7	0.064	27
Triquat	Chl fluorescence	7.2	0.078	9.2
	% Moisture	—[b]	7.3	
	% Green leaf area	130[a]	3.6	35

[a] Extrapolated value. [b] No per cent moisture response to triquat at the concentrations tested.

ing was much lower than the I_{50} for per cent moisture or per cent green leaf area (Table II) for each herbicide and biotype combination. It is possible that sufficient reducing power is still generated to maintain protective mechanisms when fluorescence is only partially quenched; *i.e.* when electrons are only partially diverted from NADP. Only when Chl fluorescence was quenched to near the F_O level was there a correlation to apparent tissue injury.

Diquat had the lowest I_{50} values of the three herbicides tested (Table II). The ranking of I_{50} values within any biotype, for any method used, yields the order: I_{50}, diquat < I_{50}, paraquat < I_{50}, triquat (Table II). Therefore, herbicidal activity corresponds quite closely with the redox potential of these herbicides (Fig. 1); a more positive redox potential corresponds to a more active herbicide. High herbicidal activity in bipyridinium compounds is known to require a redox potential in the range of −350 to −450 mv (13). This explains the relatively low activity of triquat.

Paraquat is inactivated in soils by adsorption to anionic soil colloids (2). We recognized that this might be similar, in principle, to the basis for paraquat bioinactivation in resistant *C. bonariensis*, such that the herbicides could be adsorbed to cell walls. The cation exchange properties of the cell wall are primarily due to de-esterified galacturonans, a component of pectin. Divalent cations such as Ca^{2+} bind noncovalently to pectins (10), so it seemed reasonable to propose that the paraquat di-cation might also bind to pectins. The hypothesis that compartmentation in the resistant biotype is due to binding to the apoplast was tested indirectly by examining the degree of movement of [^{14}C] paraquat supplied through the xylem, and then directly by measuring binding of [^{14}C]paraquat to cell walls.

Autoradiography. [^{14}C]Paraquat at pH 7 fed through the petiole became uniformly distributed in the leaves of the susceptible biotype, but was localized in the proximity of vascular tissue, and regions of the lower petiole, in the resistant biotype (Fig. 6). This demonstrates that paraquat movement through the apoplast was restricted in the resistant biotype and that paraquat was rapidly compartmentalized as it moved out of the vascular tissue and into the photosynthetic mesophyll tissue. We emphasize that the mechanism of paraquat resistance cannot be related strictly to changes in the vascular tissue; if this were true, the labeled

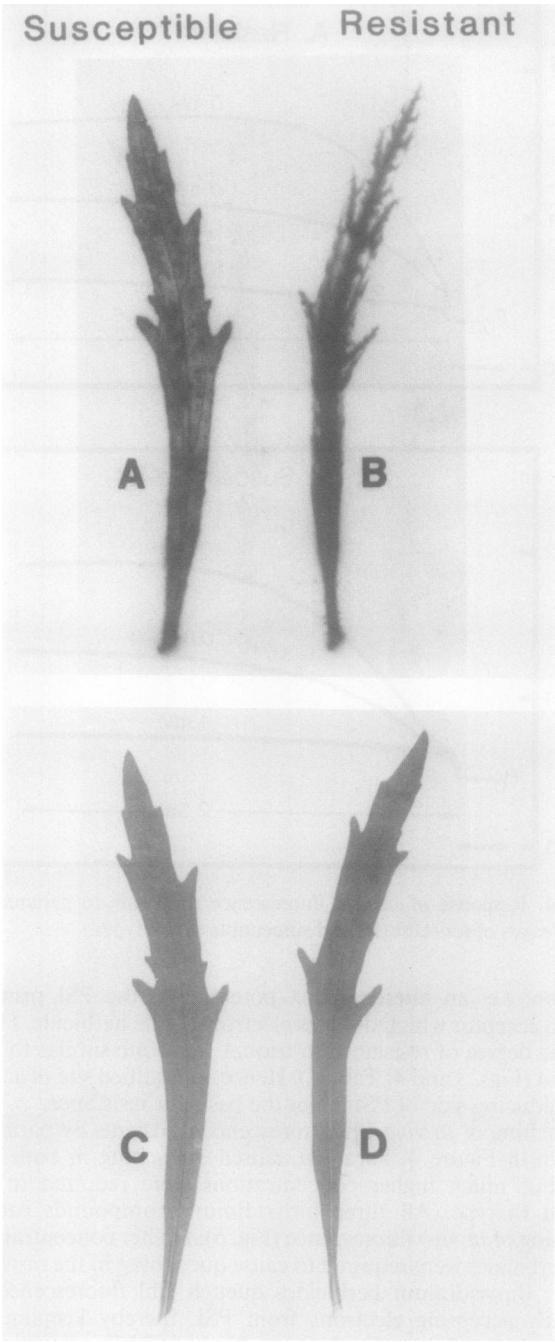

FIG. 6. Autoradiograms (A and B) and photographs (C and D) of excised leaves fed a solution of [^{14}C]paraquat through the petiole. A and C are the susceptible biotype; B and D are the resistant biotype.

areas would have shown only the vascular strands with no radioactivity in the adjacent cells. This indication that paraquat was compartmentalized at the cellular level and was excluded from the active site in the chloroplasts, even in the areas in which it was present in the resistant biotype, came from a separate experiment. When leaves treated identically to those of the autoradiogram of Figure 6 were placed in bright light (450 $\mu E \cdot m^{-2} \cdot s^{-1}$ PPFD), no visible injury could be detected even in the cells near leaf veins (data not shown). Therefore, paraquat moved out of the xylem but was rapidly immobilized as it moved through the mesophyll tissue of the resistant biotype. Such a mechanism would also function when paraquat is sprayed onto leaf surfaces in the manner in which it is used for weed control.

Table III. *Binding of [^{14}C]Paraquat to Purified Cell Walls of Conyza*

Paraquat Concn.	Calcium Concn.	Biotype[a]	Paraquat Bound[b]
μM			*ng*
1	0	R	49 ± 5
		S	57 ± 7
	10	R	35 ± 4
		S	35 ± 7
100	0	R	4800 ± 700
		S	4700 ± 810
	10	R	2200 ± 450
		S	2900 ± 400

[a] R, resistant; S, susceptible. [b] Mean ± SE.

In this situation, paraquat would penetrate the cuticle but then become rapidly sequestered in leaf mesophyll tissue before reaching the active site in the chloroplast.

The restriction of paraquat movement within the leaf of the resistant biotype suggested that compartmentation was due to a sequestering mechanism, possibly adsorption to cation exchange sites. Compartmentation cannot be readily attributed to exclusion from subcellular compartments by membrane impermeability, since such a mechanism could not explain the basis for restricted movement of paraquat in the leaf; if anything, such movement might be enhanced by membrane impermeability.

Binding of Paraquat to Cell Walls. Binding of paraquat to cell walls was similar in the two biotypes for each of the treatments indicated (Table III). Resistance cannot be attributed to binding to insoluble cell wall material, since binding was not greater in the resistant biotype than in the susceptible biotype. Calcium reduced the amount of paraquat bound, indicating that binding of paraquat was at least partially ionic, and that cation exchange sites were present. The fact that we cannot detect differential binding of herbicide to the extracted cell walls may indicate that we lost a soluble constituent (either associated with the walls or within the cell) that is the basis for the resistance *versus* susceptible response.

No differences were observed between the two biotypes to degree of staining with ruthenium red, a pectin stain (11) (unpublished observations). This also argues that resistance is not due to alteration in pectins.

We conclude that paraquat resistance cannot be attributed either to reduced cuticular penetration or alteration of the active site. Resistance is due primarily to exclusion from the site of action in the chloroplast, resulting from rapid sequestration via an unknown mechanism. Compartmentation is not simply due to ionic interactions of paraquat with water-insoluble cell wall components.

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