Mode of Evolved Photooxidant Resistance to Herbicides and Xenobiotics

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Z. Naturforsch. **45c,** 463–469 (1990); received November 9, 1989

Paraquat, Acifluorfen, Atrazine, Photoinhibition, Superoxide Dismutase

A few species have evolved resistance to paraquat after repeated selection. As paraquat still inhibited NADP reduction, we hypothesized that resistance might be due to (a) detoxification of the paraquat-generated active oxygen species and (b) that resistant plants would have some cross resistance to other xenobiotic oxidants as well as to photoinhibition, which we subsequently demonstrated. The levels of plastid isozymes of the oxygen detoxification pathway: (CuZn) superoxide dismutase, ascorbate peroxidase and glutathione reductase were genetically higher in the resistant than in the sensitive biotype of *Conyza bonariensis* through the F_2 generation. Resistance was suppressed by chelators of copper and/or zinc. Intact chloroplasts from resistant plants had less membrane damage with and without paraquat, than those from sensitive plants. Resistant *Conyza* plants recover from paraquat inhibition of photosynthesis in 3–4 h in high light, whereas sensitive plants died. Both resistant and sensitive plants recovered from paraquat in 3–4 h in low light intensities. Paraquat-resistant *Conyza* plants were cross-tolerant to SO_2, atrazine, acifluorfen and to photoinhibition. Drought-tolerant maize inbreds were cross-tolerant to paraquat, SO_2 and acifluorfen (compared to sensitive lines) and they also possessed higher levels of (Cu/Zn) superoxide dismutase and glutathione reductase.

The tolerance to oxidant stresses in *Conyza* and maize increases with plant age, suggesting that the shift to resistance is a constitutive, earlier expression of the genes normally expressed later in development.

Introduction

Weed species have evolved resistance to paraquat under field conditions. This may seem surprising as paraquat is a contact herbicide that immediately and irreversibly adsorbs to soil colloids. Thus, it lacks residual activity. Single annual applications of paraquat should not exert the same selection pressure as single annual applications of atrazine having high residual activity. Indeed, paraquat-resistant populations only evolved where paraquat was applied 3–10 times per year, for 6 or more years, giving it the same effective selection pressure as annual atrazine applications.

Paraquat interacts with photosystem I, generating superoxide. No cases of paraquat resistance are known to be due to PS I target-site mutations. This may mean that the binding site of paraquat (if it does directly interact with a protein) is even more conserved than the 32 kDa protein that interacts with atrazine. It may *also* mean that paraquat interacts with *more* than PS I; indeed paraquat can interact with mitochondrial electron transport, as well as directly interact with mammalian enzymes.

PS I in isolated thylakoids of paraquat-resistant plants is totally susceptible to paraquat [1, 2]. This has been construed by some as implying that resistance could not be at the level of the chloroplast. Various groups have found by petiole feeding of labelled paraquat, that the radioactivity in resistant plants was localized near veins and cell walls. The distribution was more diffuse in susceptible plants [3–5]. This was interpreted as paraquat being sequestered and/or its translocation blocked before reaching chloroplasts, even though photosynthesis was not measured *in vivo*. Unfortunately, the contention that paraquat was sequestered was never demonstrated by showing that the radiolabel was in paraquat and not in a metabolite. Label from paraquat *should* eventually be sequestered, but the question is whether this can be a *primary* (first occurring) reaction in resistant plants. The first time points for sequestration were always hours after application. Thus, any different feature between resistant and sensitive plant appearing before this time is semantically more "primary".

We will describe evidence below that paraquat quickly gets to the chloroplast and that isolated in-

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Verlag der Zeitschrift für Naturforschung, D-7400 Tübingen
0341–0382/90/0500–0463 $ 01.30/0

tact chloroplasts of the resistant biotype of *Conyza bonariensis* are generally resistant to photooxidative membrane damage, compared to the wild type. We will also present evidence that these chloroplasts possess constitutively higher levels of three enzymes known to participate in detoxification of active oxygen species. These enzymes are genetically linked with paraquat resistance. Elevated levels of the same chloroplast enzymes confer varying levels of tolerance to other herbicides generating active oxygen species, as well as photoinhibition, SO_2, O_3 and drought. We also present evidence that there is a mutation in developmental control, conferring these cross tolerances, which is probably an evolutionary phenological adaptation.

Chloroplasts as the Site of Oxidant Resistance

Two groups have performed kinetic experiments on different *Conyza* spp. to ascertain whether paraquat affects chloroplast reactions in leaves. Both found that at all light intensities paraquat immediately affects photosynthesis, measured as CO_2 fixation (Fig. 1) and fluorescence quenching [7] in resistant as well as susceptible leaves. Obviously paraquat gets to chloroplasts in both biotypes. The difference between resistant and susceptible biotypes at high light intensity is that the resistant plants fully recover in a few hours, and the susceptible plants die (Fig. 1 A).

There is a difference in the natural tolerance of isolated intact chloroplasts from paraquat-resistant and susceptible *Conyza* to photooxidative membrane damage; about half as much ethane emanates from resistant plastids under illumination (Table I). When paraquat is added, there is only a slight increase in ethane evolution in resistant chloroplasts, whereas the sensitive ones are severely damaged (Table I). Thus the chloroplasts,

Table I. Light-induced ethane production by intact class A chloroplasts from *Conyza* biotypes.

Treatment	Paraquat-susceptible biotype	Paraquat-resistant biotype
	(pl ethane/mg chlorophyll)	
No paraquat	34.9	12.2
10 μM paraquat	107.8	24.4

Conditions: 3 h at 1.3 mE m^{-2} s^{-1}. Source: Tabulated from data in [6].

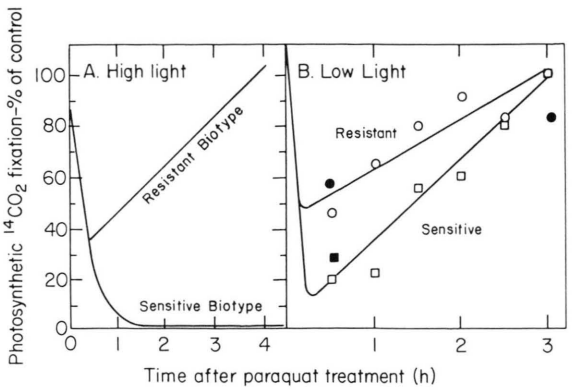

Fig. 1. Recovery of *Conyza bonariensis* leaves from paraquat damage in (A) high light and (B) low light intensities. (A) Juvenile (rosette) plants were sprayed with 100 μM paraquat and placed in 540 μE m^{-2} s^{-1} white light. Leaves were removed at intervals and placed in a chamber with $^{14}CO_2$ and fixation measured. Source: Redrawn from [6]. B. Similar leaves from fully grown rosettes were dipped in 100 μM paraquat for 2 min and then floated on distilled water under low light intensity: 20 W m^{-2} (75 μE m^{-2} s^{-1}) for the durations indicated. Leaf discs were taken and exposed to $^{14}CO_2$ in light. ○ resistant; □ sensitive leaf discs in the light. Resistant and susceptible leaves, similarly treated were incubated in the dark and photosynthesis measured (closed symbols). Source: Jansen, Malan, and Gressel, previously unpublished.

when intact are resistant [6], whereas isolated thylakoids are not [1, 2], suggesting that soluble chloroplast enzymes confer resistance.

There had been early intimations that chloroplasts of paraquat-resistant biotypes had elevated levels of superoxide dismutase [8]. We found that only the plastid isozyme of this enzyme is elevated; this was shown both by measuring activity from isolated intact chloroplasts and by fractionation on activity gels, as well as immunologically using an antibody specific for the chloroplast isozyme (Fig. 2). Superoxide dismutase alone should be insufficient to confer resistance. The product of this enzyme, H_2O_2 is far less toxic than superoxide, yet it can react chemically with Fe^{+2} yielding highly toxic hydroxyl radicals by the Fenton reaction. Chloroplasts are rich in iron ions, and do not contain catalase. Thus, there is a need to prevent hydroxyl radicals from forming, or a way to quench them once formed.

Halliwell and Asada [11, 12] have described a pathway for detoxification of oxygen radicals, by

enzymes known to be in chloroplasts. Ascorbate peroxidase competes with the Fenton reaction for H_2O_2, yielding dehydroascorbate. This is recycled back to ascorbate by reduced glutathione, either by a direct chemical reaction or by dehydroascorbate reductase. The chemical reaction is faster than the enzymatic reaction at the pH of chloroplasts. The oxidized glutathione is reduced by NADPH utilizing glutathione reductase. If paraquat were to completely block NADPH reduction, this reaction could not occur, unless NADPH could be mobilized by other means. We assayed the levels of these other enzymes of the pathway and found that they too were highly elevated in resistant plants (Fig. 2).

The enzymes of the Halliwell-Asada pathway are well known in plastids because of their housekeeping properties. They are needed to scavenge active oxygen species formed during "mistakes" or "leaks" in photosynthetic electron transfer. Presumably, plants normally do not make much more of these enzymes than needed, thus additional amounts can confer a large surplus scavenging capacity. The amount of radicals formed in the presence of paraquat is a function of light intensity, *i.e.* magnitude of electron flow. Even sensitive *Conyza* can recover from paraquat effects when the light intensity is low (Fig. 1 B), *i.e.* the normal level of enzymes can cope with the excess of radicals produced at low light intensities. Paraquat is dissipated during this period at about the same rate in both biotypes at low light intensities, when the sensitive biotype is not damaged by paraquat.

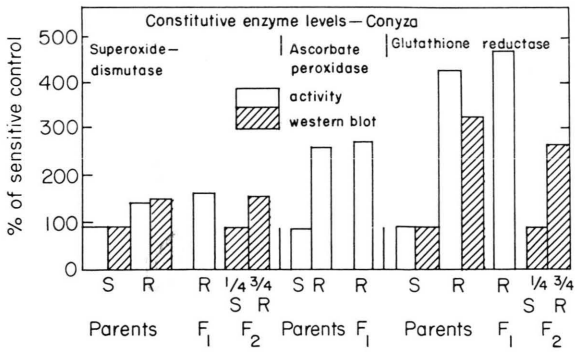

Fig. 2. Constitutively elevated levels of the Halliwell-Asada oxygen detoxification pathway in paraquat-sensitive and resistant *Conyza bonariensis* plants. Enzyme activity data in the parents are from [9, 10] and activity and immunological determinations of enzymes from the F_1 and F_2 generations are from [10]. The F_1 plants from reciprocal crosses were all resistant, and there was a 3:1 resistant: susceptible segregation in the F_2. The F_2 enzyme determinations are on single plants, following resistance determinations made on single leaves [10].

Paraquat is *not* dissipated in the dark in the sensitive plants (Fig. 1 B). This suggests that paraquat dissipation may require enzymes active in the light, or substrates or cofactors produced only in the light. It can also mean that only the paraquat radical (predominantly produced in the light) is the form of paraquat that can be more easily dissipated. The paraquat radical is probably more labile than paraquat, supporting this last possibility.

Correlations between enzyme levels in different biotypes and a trait such as resistance can be spurious, unless it can be shown that the traits are tightly linked through genetic segregation. Paraquat resistance is inherited as a single gene dominant trait in *Conyza* spp. [10, 13].

The levels of all three enzymes were high in the F_1 generation. Leaf discs of plants of the F_2 generation were tested to ascertain which plants were resistant and which susceptible. All of the resistant plants when tested singly had high levels of the two enzymes for which antibodies were available, and all susceptible plants had low levels (Fig. 2). There was no segregation of high enzyme levels away from resistance, indicating a pleiotropic control.

Further indirect support for the theory that the Halliwell-Asada oxidant detoxifying pathway is responsible for paraquat tolerance in *Conyza* and other species is supported by inhibitor studies. The chloroplast superoxide dismutase is a copper and zinc containing enzyme. Strong chelators of copper and zinc both inhibit this enzyme *and* lower the I_{50} of paraquat in many species [14]. Not too many plant enzymes contain copper and fewer contain zinc.

These data are not unique. High levels of the oxygen detoxifying enzymes (singly or together) have been correlated with both constitutive and induced tolerance to a variety of oxidant stresses (Table II). Genetic studies though had not previously shown the tight linkage of the pathway to oxidant resistance. Other antioxidants such as carotenoids, tocopherol, *etc.*, have *also* been correlated with tolerance to the same stresses, reminding us of the diversity of biology.

Table II. Differences in higher plant oxidant-stress tolerances and enhanced enzyme activity of the Halliwell-Asada pathway.

Species	Selector or inducer	Cross tolerances	Enzymes elevated[a]	Ref.
Interspecific (biotype or cultivar) constitutive differences				
Conyza bonariensis	paraquat	atrazine, acifluorfen, SO_2	SOD, AP, GR	[10, 15]
	paraquat,	photoinhibition		[16]
	paraquat	diquat, rose bengal		[17]
Lolium perenne	paraquat	SO_2	SOD, GR	[8]
	SO_2	paraquat	SOD, GR	[15]
Nicotiana sp.[b]	O_3	paraquat	SOD, GR	[15]
Zea mays	drought	paraquat, SO_2, acifluorfen	SOD, GR	[18]
Ceratopteris sp.	acifluorfen	paraquat		[19]
Intraspecific (induced) differences				
Pinus sp.	SO_2		SOD	[20]
Cicer sp.	SO_2		SOD	[21]
Phaseolus sp.	acifluorfen		GR	[22]
Hordeum sp.	drought		GR, AP	[23]
Spinacea sp.[c]	O_3		AP	[24]
Zea mays	hyperbaric O_2		GR	[25]
Gossypium sp.	drought, paraquat		GR	[26]

[a] SOD, superoxide dismutase; GR, glutathione reductase; AP, ascorbate peroxidase.
[b] *Cv.* Florida was the only one of four O_3 tolerant varieties tested having cross resistance.
[c] Inducible in only 3 of 6 varieties.

It was first postulated in 1982 that many plants with paraquat tolerance should be constitutively tolerant to a wide variety of oxidant stresses [27]. This has been substantiated in many laboratories with many stresses (Table II). We have recently shown that this includes photoinhibition, as measured by photoacoustic spectroscopy (Fig. 3). The magnitude of resistance to inhibition of energy storage (Fig. 3A) is greater than the magnitude of inhibition of oxygen evolution (Fig. 3B) under photoinhibitory conditions, as has been shown with other stresses [28, 29].

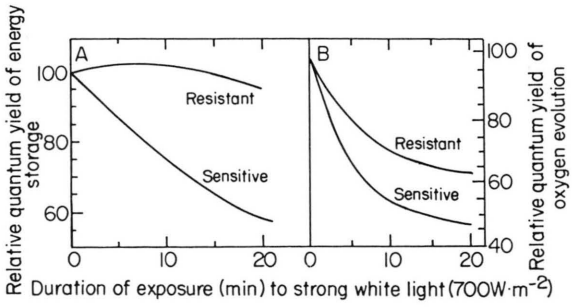

Fig. 3. The effect of photoinhibitory light on (A) quantum yield of energy storage and (B) quantum yield of oxygen evolution in paraquat-resistant and sensitive plants. The normalized quantum yields were measured after exposure to photoinhibitory white light of 700 W m^{-2} (3.2 mE m^{-2} s^{-1}) using photoacoustic spectroscopy. Source: collated and redrawn from [16].

These cross tolerances can be of practical use. It is very cumbersome and time consuming to measure drought tolerance, especially if many genetic lines are to be tested. It is far simpler to test leaf discs for paraquat tolerance. Known drought-tolerant lines of maize were found to be paraquat-tolerant. Other drought-tolerant lines were identified by screening maize inbreds for paraquat tolerance [18]. The paraquat/drought-tolerant maize lines possessed high levels of superoxide dismutase and glutathione reductase. They had cross tolerance to other oxidant stresses such as to a diphenylether herbicide generating photodynamic protoporphyrin IX and to SO_2, in a manner similar to *Conyza* [18].

Most cross tolerances are not of the same magnitude as paraquat resistance. Paraquat rapidly affects plants, but if the plant survives paraquat is quickly dissipated (Fig. 1). The oxidant detoxifying pathway needs only to protect a plant for a

short period. Other stresses continue over longer periods (*e.g.* atrazine, SO_2, diphenylether herbicides) due to slower dissipation. There may be an inverse relation between the duration of a stress and the magnitude of that stress that can be tolerated. It is also possible that the different microlocalization of active oxygen species, or the different composition of these species cause these differences in tolerance.

It has been questioned why paraquat-resistant plants should have a modicum of resistance to xenobiotics such as rose bengal and to herbicides such as diphenylethers that cause accumulation of protoporphyrin IX. These are thought to cause the production of singlet oxygen [30, 31]. Various compounds thought to be singlet oxygen producers from *in vitro* studies actually produce superoxide *in vivo* [32–34]. It has been hypothesized, with considerable evidence, that this is due to the high level of strong reductants such as NAD(P)H and reduced glutathione in cells [32, 34]. Still, experiments with plant tissues perfused with D_2O, which specifically enhances the effects of singlet oxygen and does not affect other active oxygen species, show that singlet oxygen can also be active in plants [30]. Ascorbate and glutathione can also quench singlet oxygen, and high levels of the enzymes recycling these compounds should confer some tolerance to all active oxygen species.

Developmental Stage and Stress Tolerance

Plants normally need more stress tolerance at some stages of their life cycle and less at others. Stress tolerance, like many other protective traits has a "cost". A paraquat-tolerant *Hordeum* has been shown to be less productive and less competitively fit than the susceptible biotype when paraquat is not present [35]. It can thus be expected that stress tolerance is under developmental control providing tolerance only when usually needed. These controls evolved with the species and may vary in different environments. *Conyza bonariensis* germinates and forms a rosette in the cool, rainy winters of the mid-east and forms a flowering stalk in the arid hot summer. Maize germinates in wet spring and develops in drier summer. Both species thus need more stress tolerance as the plants grow older. They naturally possess greater stress tolerance as they grow older as measured by tolerance to photoinhibition in *Conyza* (Fig. 4) and tolerance to paraquat-induced photodamage of leaf membranes in maize (Fig. 5). This includes tolerance to paraquat (Table III). Leaves of flowering *Conyza* plants are far more tolerant to stresses than juvenile rosette leaves; but a differential is still maintained between the resistant and sensitive biotypes (Fig. 4 and 5a). It is nigh impossible to kill putatively paraquat-sensitive species with agriculturally used rates of paraquat application when they flower. Leaves from older maize plants are far more tolerant to paraquat damage than leaves from young plants. Others have also shown that leaves from older plants [21] or summer produced

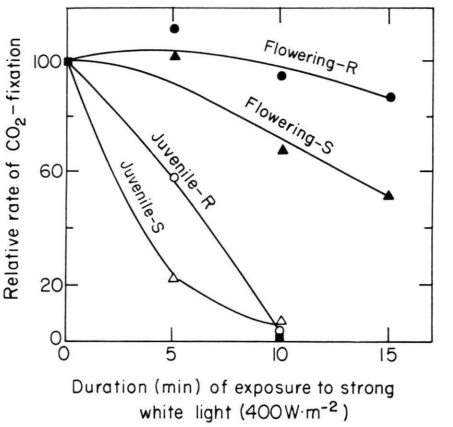

Fig. 4. The effect of photoinhibitory light on $^{14}CO_2$ fixation of paraquat-resistant (R) and sensitive (S) plants treated with 1.7 mE m^{-2} s^{-1} light without herbicide. The rates were normalized to the maximum rate for the juvenile and flowering of each *Conyza bonariensis* biotype. Source: collated and redrawn from [16].

Table III. Differential paraquat sensitivity with age of *Conyza bonariensis* plants.

Plant stage	Susceptible	Resistant
	I_{50} paraquat [μM]	
Juvenile	0.8	46.0
Flowering	6.2	187.0

Equi-aged leaves were floated for 2 h on a solution containing paraquat and 0.2% Tween 20 at 200 W m^{-2} (860 μE m^{-2} s^{-1}) white light. Rinsed leaves were then floated for 2 h on distilled water at 20 W m^{-2} (75 μE m^{-2} s^{-1}) white light. Leaf discs were then exposed to $^{14}CO_2$ under the same light conditions. Source: Jansen, Malan, and Gressel, unpublished.

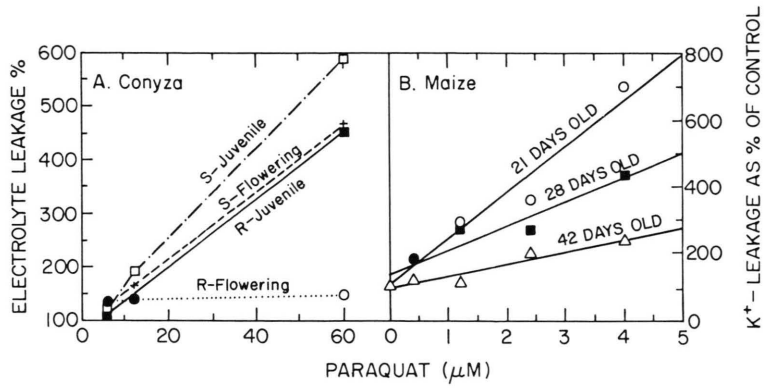

Fig. 5. Effect of plant age of (A) *Conyza* and (B) maize on susceptibility to paraquat. Three leaf discs were floated for 30 min on paraquat at 48 μE m^{-2} s^{-1} white light, rinsed in distilled H_2O, incubated for 24 h in 3 ml distilled H_2O at room temperature in the light. Electrolyte leakage was measured as conductivity. Untreated control values are 110 μmho for resistant juvenile 135 μmho for resistant flowering, 140 μmho for sensitive young juvenile and 98 μmho for sensitive flowering plants. Source: Malan, Jansen, and Gressel, previously unpublished. B. K^+ efflux was measured as percent of untreated controls of maize cultivar P473 leaf discs from the youngest mature leaves sampled from different age plants and treated with paraquat, following the protocol described in [18]. The untreated control value was 0.5 mg·l^{-1} K^+. Source: collated and redrawn from [18].

leaves [36] have higher levels of these enzymes than leaves from younger or spring grown plants, respectively. Additionally, younger tree leaves (receiving more sunlight) have higher superoxide dismutase levels than older, shaded leaves [37].

We would thus propose a hypothesis that the mutation conferring paraquat resistance in the cases we have studied, is to a changed developmental pattern of enzyme production. The mutation causes an earlier constitutive appearance of higher levels of the oxidant-stress tolerance pathway in these species. Regulatory genes controlling development often pleiotropically regulate whole pathways, as is seen here. Of course other properties may also be pleiotropic with the Halliwell-Asada pathway that later help in the dissipation of the xenobiotics, but these remain to be shown.

Concluding Remarks

It is clear from the data presented that paraquat reaches chloroplasts of resistant *Conyza,* and that the plastids possess the primary (by time) mechanism of resistance; constitutively elevated levels of the Halliwell-Asada pathway. The biochemical data were substantiated and the hypothesis strengthened by inhibitor studies and especially by genetics. The significance of the data go well beyond paraquat resistance, it lies in the cross resistances. A substantial bank of data from many labs with various species have demonstrated cross tolerances between xenobiotic and environmental oxidant stresses (Table II).

Paraquat can be used to rapidly screen for harder to determine genetic tolerances such as tolerance to drought. Enzyme assays could possibly be used to screen for lines singly high in one or two of the enzymes, allowing for controlled crosses to increase levels of tolerance by obtaining high levels of all three enzymes of the Halliwell-Asada oxygen detoxification pathway. Paraquat resistance is not always due to a single gene in other species [19, 38].

The chloroplast, by evolving a method of self-protection has evolved a mechanism allowing temporary tolerances to other oxidative stresses, until these stresses subside. We do not propose that this is the *only* mechanism of stress tolerance; its ubiquity which has only recently been realized suggests that it is an important mechanism of stress tolerance in many species.

Acknowledgements

C. M. appreciates a Department of Agriculture and Water Supply, Republic of South Africa bursary that allowed his stay in Rehovot. Drs. O.

Canaani, S. Malkin, Mr. D. Kazzes, Ms. A. Glazer, Ms. M. Greyling, and Ms. R. Shmida have participated in various aspects of our studies and their contributions are greatly appreciated. Part of the work on maize was performed at the Grain Crops Research Institute – Summer Grain Centre, Potchefstroom. J. G. has the Gilbert de Botton Chair of Plant Sciences.

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