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Effects of Copper Chelating Agents on Diquat Activity in Diquat Resistant Landoltia

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ABSTRACT

Organic chelating agents have previously been used to reduce activity of elevated antioxidant enzyme levels in some bipyridylium resistant plant biotypes to overcome herbicide resistance. The activity of the chelating agents: ethylenediamine, ethanolamine, and triethanolamine (commonly used in chelated copper algaecides and herbicides) were determined on a resistant biotype of the duckweed species landoltia [*Landoltia punctata* (G. Meyer) D.H. Les and D.J. Crawford]. The three chelating agents significantly increased ion leakage from landoltia at concentrations exceeding 1.0 mg L^{-1}, and did not visually reduce chlorophyll content. They did not enhance the activity of diquat (1,1'-ethylene-2,2'-bipyridylium dibromide) at any of the concentrations evaluated, although membrane permeability was altered either through direct action on the membrane or as a secondary response to phytotoxicity. If elevated antioxidant enzymes were the cause of bipyridylium resistance and chelating agents deactivate these enzymes, the results of the studies reported here do not support elevated enzymes as the mechanism of resistance in landoltia.

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INTRODUCTION

Diquat is a contact herbicide first registered federally for aquatic use in 1961. Diquat's mechanism of action in plants involves electron interception from photosystem I during light reactions of photosynthesis (Yocum and San Pietro 1969) resulting in the formation of a reduced diquat radical. The reduced diquat radical is oxidized by molecular oxygen to form a superoxide anion (O^{2-}) and regenerates cationic diquat. Additional oxygen radicals are formed when superoxide reacts with H_2O_2 to form $^{\bullet}OH$ (hydroxyl radicals) via the metal catalyzed Fenton reaction (Eq. 1 and 2) or Haber-Weiss reaction (Eq. 3).

$$O_2^- + Fe^{3+} = O_2 + Fe^{2+} \quad (1)$$

$$H_2O_2 + Fe^{2+} = {}^{\bullet}OH + OH^- + Fe^{3+} \quad (2)$$

$$O_2^- + H_2O_2 = {}^{\bullet}OH + OH^- + O_2 \quad (3)$$

Radical oxygen species superoxide (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl ($^{\bullet}OH$) are formed by reoxidation with molecular oxygen and are associated with physiological damage and subsequent toxicity to plants. Unsaturated fatty acids in the cell membranes and chloroplasts are broken down by chain reactions caused by the free radicals. This leads to their destruction and formation of malondialdehyde, rupturing of the tonoplasts, and almost immediate change in osmotic potential (Calderbank 1966, Dodge 1971).

The toxic effects of O_2^- and H_2O_2 can be neutralized by plant enzymes [Halliwell-Asada system (Halliwell 1978)], but $^{\bullet}OH$ and singlet oxygen cannot be neutralized (Rabinowitch and Fridovich 1983). Carotenoids can facilitate the return of singlet oxygen to its ground state by quenching or neutralization of O_2^-. Cells also contain antioxidants to minimize reactions amplifying the effect of $^{\bullet}OH$ (Rabinowitch and Fridovich 1983). Superoxide dismutase (SOD) can eliminate O_2^- (Bielski 1978) and together with catalases (remove H_2O_2) and peroxidases functions to prevent the creation of $^{\bullet}OH$ in the plant cell (Rabinowitch and Fridovich 1983). The action of NADPH-specific enzyme glutathione reductase (GR) also reduces the presence of radical oxygen species in chloroplasts (Hartel et al. 1992). Three types of SOD (Cu-Zn, Fe, and Mn) occur in most plants, dependent on the ion cofactor associated with the enzyme (Rabinowitch and Fridovich 1983). Cu-Zn is the most common type located within chloroplasts (Asada et al. 1976).

There is conflicting evidence that elevated antioxidant enzyme levels play an active role in conferring resistance to the bipyridylium herbicides (BP), diquat and paraquat. This resistance mechanism may be species dependent. Increased levels of SOD, GR, or ascorbate peroxidase (AP) have been documented in paraquat-resistant *Lolium perenne* L. (Harper and Harvey 1978), *Conyza bonariensis* (L.) Cronq. (Shaaltiel and Gressel 1986), and *Nicotiana tobacum* cv. Xanthi (tobacco) (Shaaltiel et al. 1988, Kwon et al. 2002). In addition, plant biotypes resistant to BPs that have elevated enzyme levels were also cross-resistant to other compounds (ozone, SO_2, acifluorfen, and atrazine) that produce radical oxygen species (Shaaltiel et al. 1988). The BP resistant biotypes overcame transient effects of paraquat exposure, and after dark exposure, plants respond to BP under light. This suggests that BPs are not sequestered, and that they become active in plants under light conditions. Therefore, it has been hypothesized that resistance may be the result of detoxification (elevated enzyme hypothesis) of radical oxygen species generated by the action of diquat and paraquat in the protoplasts (Lehoczki et al. 1992).

Cross-resistance to BPs occurs in most BP resistant biotypes, but the resistance factors (resistance factor = resistant biotype effective concentration/susceptible biotype effective concentration) are not equal. This poses a potential discrepancy in the elevated enzyme hypothesis. A biotype of *Conyza canadensis* was 100× more resistant to paraquat, but only 10× more resistant to diquat when compared to susceptible biotypes. This biotype was not resistant to morfamquat or other structurally related chemicals (Vaughn et al. 1989). Other studies have documented differential resistance to BPs (Preston et al. 1992, Szigeti et al. 2001). Similar resistance factors would be expected for these chemically similar herbicides if enzyme detoxification were the cause of resistance. This is assuming radical oxygen production resulting from BPs mode of action occurs at similar rates and gene expression levels for elevated enzymes are similar.

Chelating agents have been used in combination with BPs to overcome the resistance mechanism in some BP resistance plants that have elevated antioxidant enzyme levels (Gressel and Shaaltiel 1988). Chelators inactivate Cu-Zn SOD and AP (Cu containing) by chelating the ion cofactor associated with the enzyme (Youngman et al. 1979, Shaaltiel and Gressel 1986). Upon addition of a metal chelating agent to BPs, increased activity of the herbicide was noted in resistant biotypes. Diethyldithiocarbamate (chelating agent) increased the activity of paraquat on resistant plants. However, it did not enhance efficacy on susceptible biotypes (Bewick et al. 1991), in contrast to the results of Gressel and Shaaltiel (1993).

Increased levels of antioxidant enzymes did not always correlate with increased resistance (Polos et al. 1988, Norman et al. 1993). A biotype of *Ceratopteris richardii*, containing elevated levels of Cu-Zn SOD, was not resistant to paraquat (Carroll et al. 1988). Turcsanyi et al. (1998) reported that diethyldithiocarbamate was used to suppress activity of SOD by 50 to 70%, and amitrole was used to inhibit catalase by 80-90% in resistant plants. However, these biotypes were unaffected by exposure to paraquat.

Organic chelating agents (substances that can bond to a metal ion) have been used in an attempt to improve efficacy of copper, and to reduce rapid precipitation of Cu^{2+} when used as a herbicide or algaecide in aquatic environments. Anderson et al. (1987) reported that chelating agents increased the uptake of copper in hydrilla (*Hydrilla verticillata* (L.f.) Royle) grown in Hoagland's solution; with greater accumulation from copper-ethylenediamine than copper-triethanolamine and copper sulfate. Additionally, triethanolamine reduced the precipitation of copper in hard water (Masuda and Boyd 1993). Copper levels in hydrilla tissue increased after exposure to copper triethanolamine when compared to copper sulfate (Sutton and Blackburn 1971).

A diquat resistant biotype of landoltia (R) was recently identified (50-fold resistance factor) (Koschnick et al. 2006). Assuming that chelating agents can neutralize the enzymes providing

resistance, experiments were conducted to determine if chelating agents applied in combination with diquat could overcome the resistance mechanism in landoltia. The chelating agents used are currently formulated in copper herbicides and algaecides federally registered for aquatic weed control.

MATERIALS AND METHODS

A resistant biotype of landoltia (50-fold diquat resistance) and a susceptible biotype (S) were maintained in culture (isolation described in Koschnick et al. 2006). The chelating agents ethylenediamine, ethanolamine, and triethanolamine were purchased from Fisher Scientific[3] and technical grade diquat dibromide (290 mg ml^{-1})[4] was used for all treatments. Experimental procedures were completed using plastic (e.g., high-density polyethylene) to prevent diquat binding to glass surfaces (Connard and Criddle 1975), and a completely randomized design was used.

Ion Leakage. The responses of the two biotypes of landoltia to the chelating agents and/or diquat were analyzed by measuring conductivity (µmhos cm^{-1}) of treatment solutions over time using a conductivity bridge[5] (Koschnick et al. 2006). Conductivity was used as a measure of non-specific ion leakage caused by loss of membrane integrity (O'Brien and Prendeville 1978, MacDonald et al. 1993). Ten landoltia colonies of each biotype (4 to 6 fronds per colony) were placed into individual 20-ml scintillation vials containing 15 ml deionized (DI) water.

Treatments were applied to the vials after landoltia transfer. Treatments, replicated 4 times, consisted of untreated controls, diquat at 25 µg L^{-1} on S and R biotypes, and ethylenediamine, ethanolamine, or triethanolamine each alone at 1, 10, and 100 mg L^{-1} and in combination with diquat (25 µg L^{-1}). Landoltia was exposed to chelating agents 0.5 h prior to adding diquat to the treatment solutions. After treatments were applied, vials were covered with Parafilm M[6] and inverted 3-times. Initial conductivity (Ci) was immediately measured from duplicate treatment solutions containing no plants (conductivity contributed by addition of chelating agent and/or diquat) and appropriate corrections were made when determining total ion leakage.

Vials were placed in a shaker bath (60 oscillations/min) and the temperature of treatment solutions was maintained at 28 ± 4°C. Continuous light was supplied at 175 ± 20 µmol m^{-2} s^{-1}. Conductivity of the treatment solutions was monitored at time 0, 1, 2, 4, 6, 8, 12, 18, 24, and 48 hours after treatment (HAT).

Following the final conductivity measurements, treatment vials containing landoltia were frozen and thawed 3 times to ascertain 100% ion leakage (Ct). At each time (Cx), ion leakage is reported as percent conductivity according to the formula: % conductivity = [(Cx – Ci)/(Ct – Ci)]*100 (MacDonald et al. 1993). A repeated measures analysis was conducted, means separated using a Fisher's Protected LSD, and standard errors estimated using LSMEANS (SAS 1999).

Chlorophyll. Chlorophyll content was also used to measure the effects of ethylenediamine, in combination with diquat, on R biotypes of landoltia. Ten colonies (4 to 6 fronds per colony) were placed into individual 20-ml scintillation vials containing 10 ml DI water. Treatments were: untreated controls, 100 µg L^{-1} diquat, and each of 0.1, 1.0, 10.0, and 100.0 mg L^{-1} ethylenediamine alone and in combination with diquat (100 µg L^{-1}) replicated three times. Ethylenediamine was again added to the treatment solutions 0.5 h prior to diquat addition. After herbicide was added to the solutions, vials were covered with Parafilm M and inverted three times.

Treated plants were placed in a growth room under continuous light maintained at 150 ± 10 µmol m^{-2} s^{-1} for 48 h. Air temperature was maintained at 26 ± 4°C. Plants were removed at the end of the treatment period, excess water removed by blotting on paper towels, and fresh weights were determined. Chlorophyll was extracted with dimethylsulfoxide (DMSO) (Hiscox and Israelstam 1979) and was completed after 3 h in a water bath at 65°C (Koschnick et al. 2006). Chlorophyll content was determined spectrophotometrically (Arnon 1949), and expressed as mg kg^{-1} fresh weight. An ANOVA was conducted to compare the effects of the treatments, and means separated using Fishers Protected LSD.

RESULTS AND DISCUSSION

Ion Leakage. There was significant interaction between chelating agent and concentration. Therefore, the data were separated by chelate and concentration to determine if the chelating agents enhanced diquat activity. The three chelating agents had similar effects on ion leakage from the R biotype of landoltia at 1 and 10 mg L^{-1}, and caused small increases in conductivity compared to untreated controls (Figures 1-3).

Chelating agents ethylenediamine, ethanolamine, and triethanolamine at 100 mg L^{-1}, caused significant increase in ion leakage compared to untreated controls; at this concentration the chelating agents were phytotoxic to the R biotype of landoltia. Ethylenediamine (Figure 1) and ethanolamine (Figure 2) were slightly more toxic than triethanolamine (Figure 3) when applied alone, and caused greater than 50% ion leakage by 12 HAT. Depending on the concentration of chelating agent in commercially available formulations of copper and the amount applied, the chelating agents may cause some phytotoxicity to landoltia.

When R biotypes of landoltia were treated with combinations of diquat and chelating agents, surprisingly little effect was observed considering the R biotype of landoltia is 50 times more tolerant to diquat than the S biotype. Diquat activity on R biotypes of landoltia was not consistently enhanced by the addition of ethylenediamine, ethanolamine or triethanolamine at any concentration, even though chelating agents at 100 mg L^{-1} caused significant ion leakage when applied alone. Diquat alone at 25 µg L^{-1} caused minimal ion leakage from R biotypes of landoltia, increasing only at 48 HAT compared to controls, but this concentration caused a significant increase in ion leakage from S biotypes of landoltia.

The effects of ethylenediamine, ethanolamine, and triethanolamine were not additive or synergistic when applied in combination with diquat to the R biotype, and apparently had no effect on the diquat resistance mechanism. Visually,

[3]Fisher Scientific, 2000 Park Lane Dr., Pittsburgh, PA 15275.

[4]Syngenta. Jealott's Hill International Research Centre, Bracknell, Berks. RG42 6EY.

[5]Fisher Scientific Conductivity Meter, 2000 Park Lane Dr., Pittsburgh, PA 15275; SN:41559949.

[6]Pechiney Plastic Packaging, 175 Western Avenue, Neenah, WI 54956.

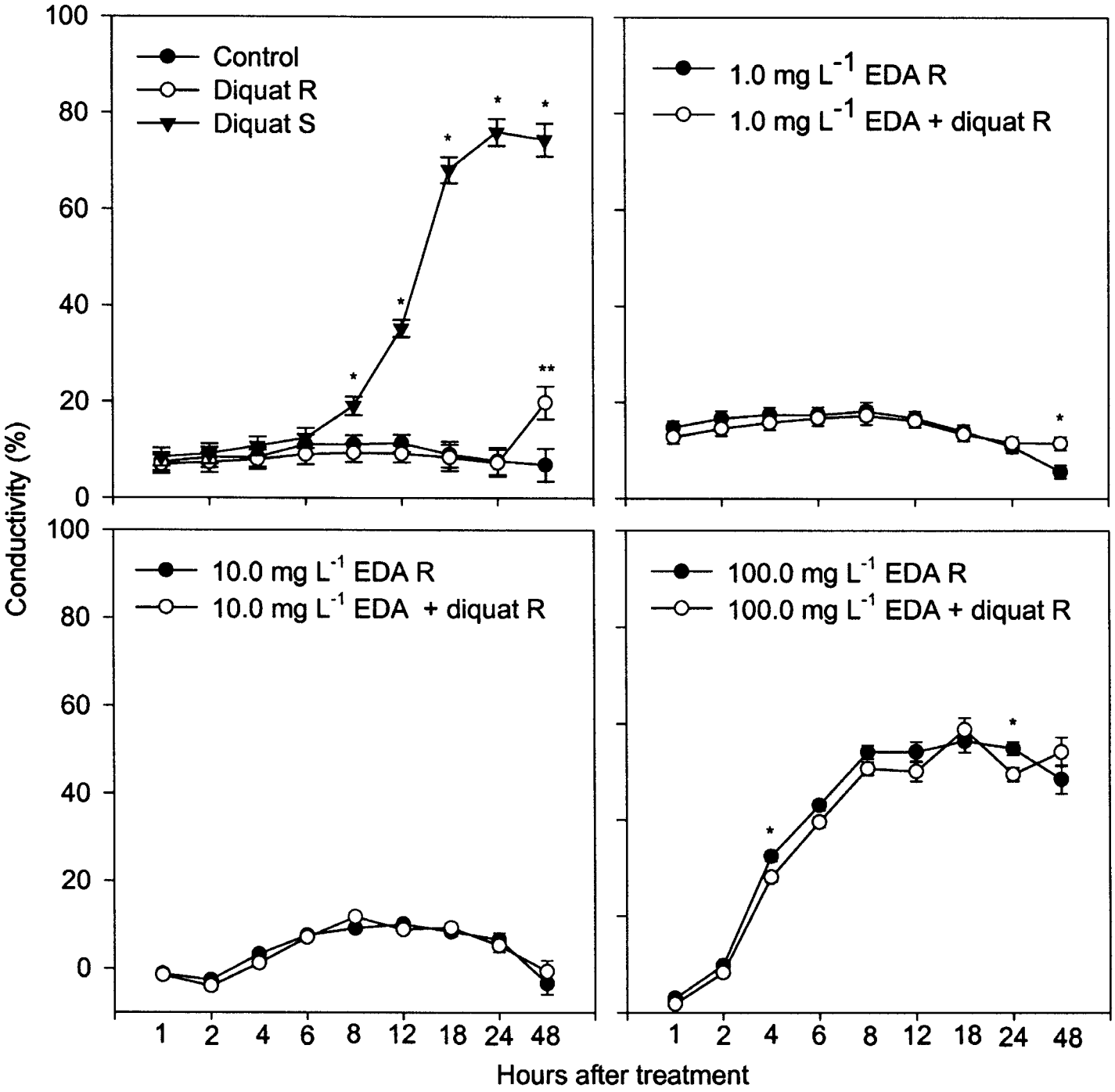

Figure 1. The effects of ethylenediamine (EDA) on ion leakage (% conductivity) from resistant (R) biotypes of landoltia alone and in combination with diquat (25 µg L^{-1}), and the effect of diquat on susceptible (S) biotypes of landoltia at the same concentration. Values presented as means ± standard error. Means with an * indicate significant differences between treatments at each concentration and time ($p < 0.05$).

chlorosis of landoltia treated with diquat alone was similar to that of landoltia treated with diquat in combination with any of the chelating agents throughout the studies. Ion leakage, caused by the addition of chelating agents, was independent of visual chlorophyll degradation.

Chlorophyll. Ethylenediamine applied alone at concentrations ranging from 0.1 to 100.0 mg L^{-1} did not cause reductions in chlorophyll levels in the R biotype of landoltia compared to control plants after a 48-h exposure (Figure 4). Ethylenediamine, applied at the same concentration in combination with diquat (100 µg L^{-1}), did not cause further reductions in chlorophyll content.

The chelating agents ethylenediamine, ethanolamine, and triethanolamine did not enhance the activity of diquat on the R biotype of landoltia at the concentrations evaluated. They caused some ion leakage from landoltia cells at con-

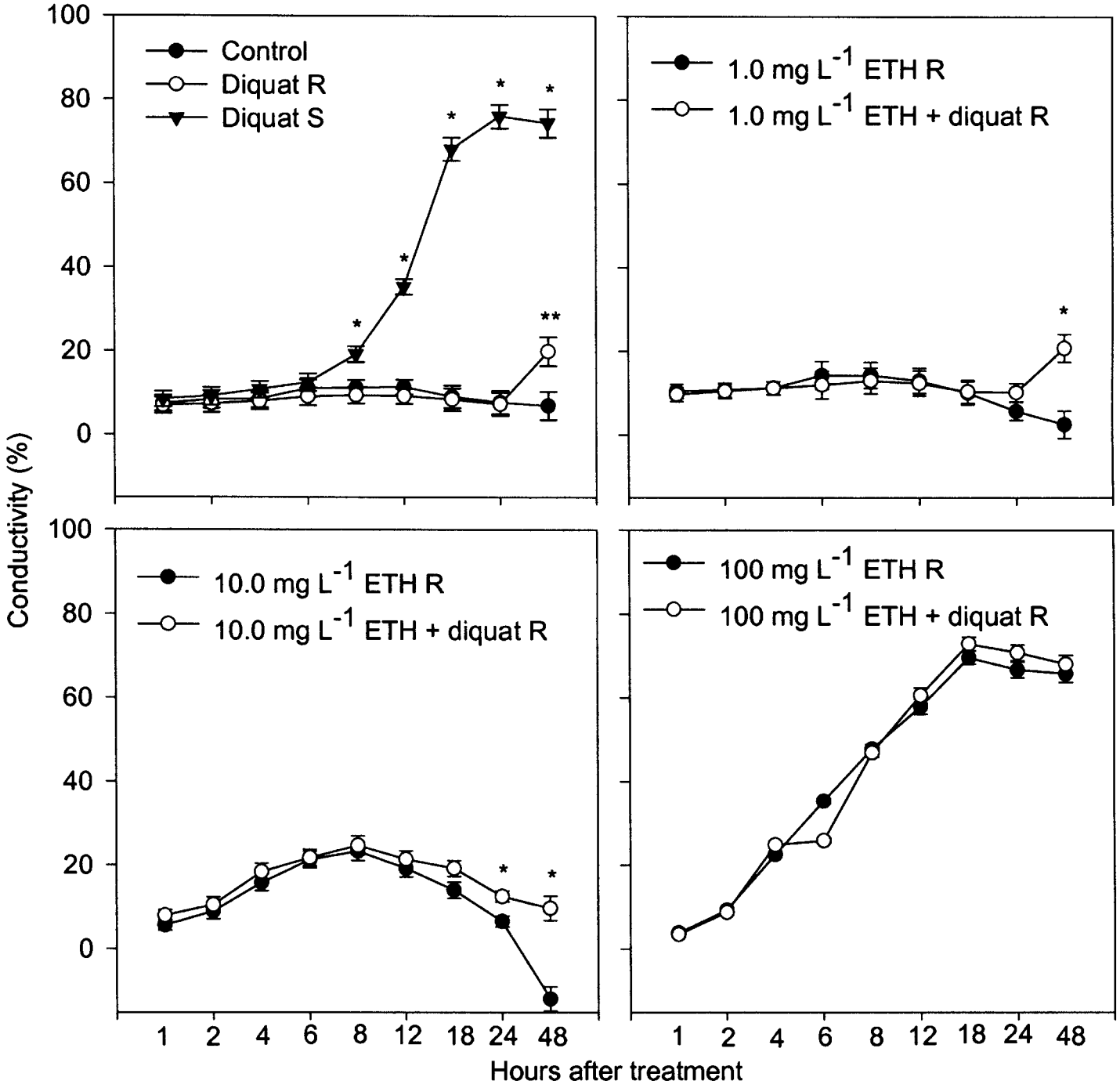

Figure 2. The effects of ethanolamine (ETH) on ion leakage (% conductivity) from resistant (R) biotypes of landoltia alone and in combination with diquat (25 µg L^{-1}), and the effect of diquat on susceptible (S) biotypes of landoltia at the same concentration. Values presented as means ± standard error. Means with an * indicate significant differences between treatments at each concentration and time ($p < 0.05$).

centrations of 1 and 10 mg L^{-1}, with significant effects on leakage at 100 mg L^{-1}. However, at these concentrations, ethylenediamine did not significantly affect chlorophyll content of landoltia after 48-h (Figure 4).

The mechanism of action of the chelating agents on landoltia is unknown, but they appear to have an effect on membrane permeability without causing chlorophyll degradation. Whether the chelating agents interact directly with the cell membrane to cause ion leakage, or leakage was the result of another mechanism, was not determined in this study.

Elevated SOD, GR and AP levels in some BP-resistant plant biotypes may increase tolerance to BPs (Harper and Harvey 1978, Shaaltiel and Gressel 1986, Shaaltiel et al. 1988, Kwon et al. 2002). Chelating agents have been used to inhibit activity of these enzymes in some R biotypes and reduce the resistance to BPs. These enzymes were not quantified in

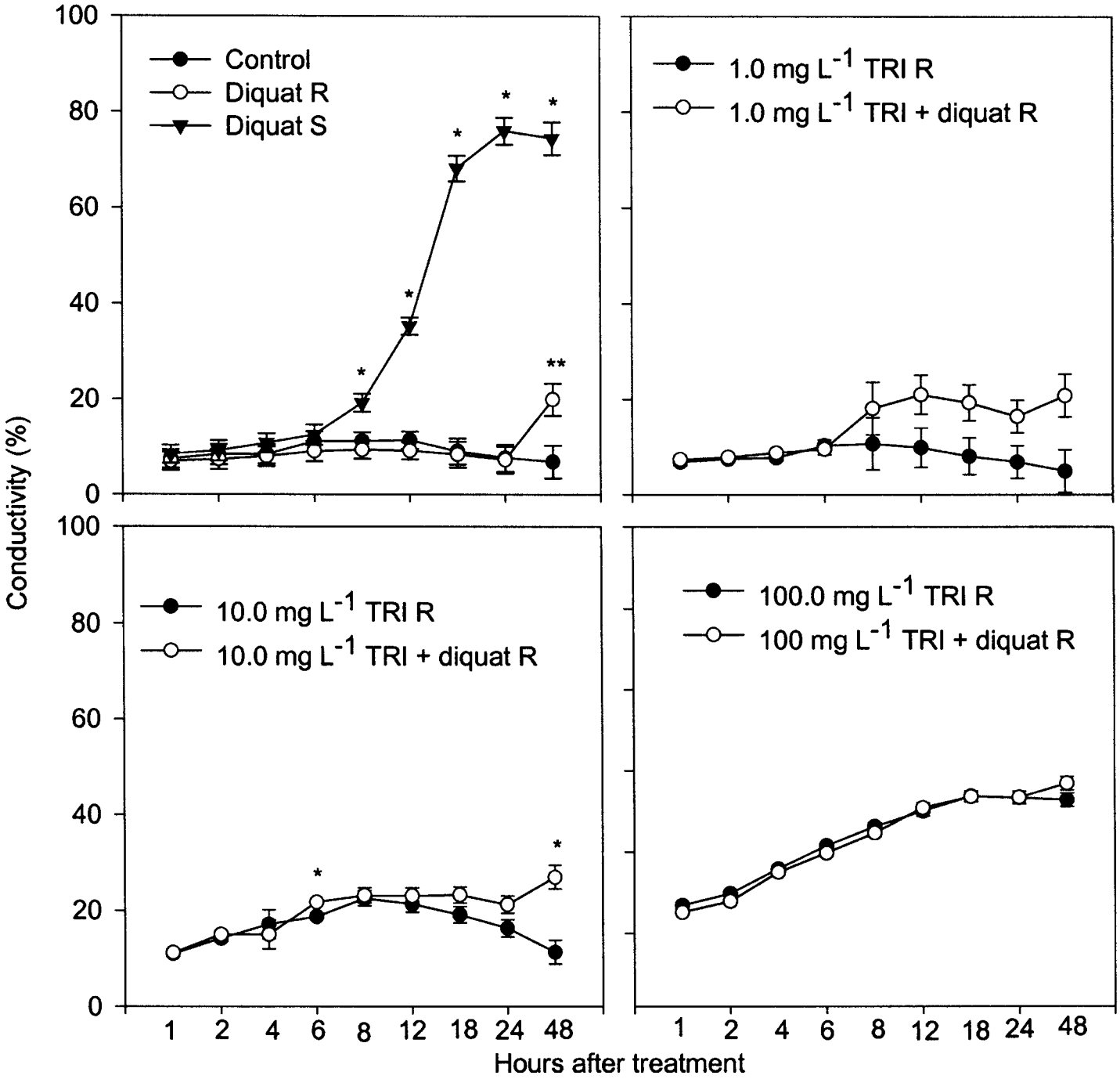

Figure 3. The effects of triethanolamine (TRI) on ion leakage (% conductivity) from resistant (R) biotypes of landoltia alone and in combination with diquat (25 µg L^{-1}), and the effect of diquat on susceptible (S) biotypes of landoltia at the same concentration. Values presented as means ± standard error. Means with an * indicate significant differences between treatments at each concentration and time ($p < 0.05$).

S and R biotypes of landoltia. However, if these enzymes are assumed to be elevated in R biotypes of landoltia, the chelating agents tested did not alter antioxidant activity or enhance diquat activity in R biotypes at the concentrations of diquat and chelating agents evaluated.

Light levels used for these studies were relatively low, but sufficient for diquat and chelating agent phytotoxicity. Membrane damage from BP activity may be slightly delayed under higher light intensity due to increased base photoprotective enzyme levels in duckweed (*Lemna minor* L.) (Artetxe et al. 2006). For these studies it was assumed that if basal photoprotective enzymes were elevated in R biotypes of landoltia to confer resistance to diquat, they would be elevated irrespective of light intensity.

Copper enhances the activity of diquat on diquat-resistant landoltia (Koschnick 2006). Therefore, additional studies are warranted with chelated copper algaecides/herbicides to determine their effect on the resistance mechanism in

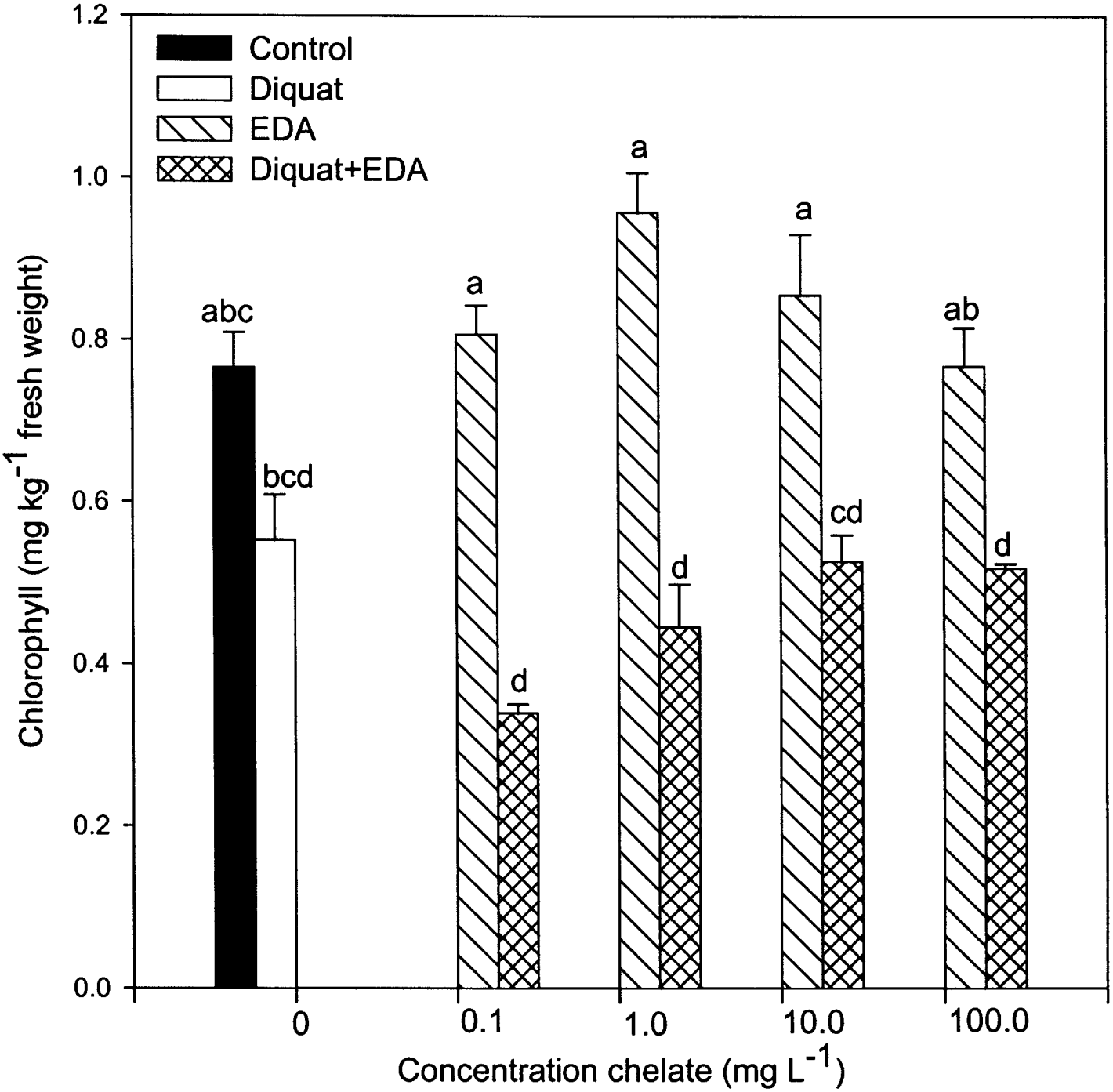

Figure 4. The effects of ethylenediamine (EDA) on chlorophyll content of resistant (R) biotypes of landoltia alone and in combination with diquat (100 µg L^{-1}). Values presented as means ± standard error. Means with different letters indicate significant differences between treatments ($p < 0.05$).

landoltia. Chelating agents may increase activity of copper/diquat combinations based on inherent phytotoxicty of the chelates, provided rates are high enough.

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