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Similar Stress Responses are Elicited by Copper and Ultraviolet Radiation in the Aquatic Plant *Lemna gibba*: Implication of Reactive Oxygen Species as Common Signals

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Metals and ultraviolet (UV) radiation are two environmental stressors that can cause damage to plants. These two types of stressors often impact simultaneously on plants and both are known to promote reactive oxygen species (ROS) production. However, little information is available on the potential parallel stress responses elicited by metals and UV radiation. Using the aquatic plant *Lemna gibba*, we found that copper and simulated solar radiation (SSR, a light source containing photosynthetically active radiation (PAR) and UV radiation) induced similar responses in the plants. Both copper and SSR caused ROS formation. The ROS levels were higher when copper was combined with SSR than when applied with PAR. Higher concentrations of copper plus PAR caused toxicity as monitored by diminished growth and chlorophyll content. This toxicity was more pronounced when copper was combined with SSR. Because the generation of ROS was also higher when copper was combined with SSR, we attributed this enhanced toxicity to elevated levels of ROS. In comparison to PAR-grown plants, SSR treated plants exhibited elevated levels of superoxide dismutase (SOD) and glutathione reductase (GR). These enzyme levels were further elevated under both PAR and SSR when copper was added at concentrations that generated ROS. Interestingly, copper treatment in the absence of SSR (i.e. copper plus PAR) induced synthesis of the same flavonoids as those observed in SSR without copper. Finally, addition of either dimethyl thiourea or GSH (two common ROS scavengers) lowered in vivo ROS production, alleviated toxicity and diminished induction of GR as well as accumulation of UV absorbing compounds. Thus, the potential of ROS being a common signal for acclimation to stress by both copper and UV can be considered.

Keywords: Chalcone synthase — Flavonoids — Glutathione reductase — Oxidative stress — Reactive oxygen species — Superoxide dismutase.

Abbreviations: CHS, chalcone synthase; DMF, dimethylformamide; DMTU, dimethyl thiourea; DTNB, 5,5′-dithiobis-2-nitrobenzoic acid; H_2DCFDA, 2′,7′-dichlorodihydrofluorescein diacetate; GR, glutathione reductase; GSSG, oxidized glutathione; PAR, photosynthetically active radiation; RO, reverse osmosis; SOD, superoxide dismutase; SSR, simulated solar radiation; ROS, reactive oxygen species.

Introduction

Metals and UV radiation often co-exist in the environment. Some metals are used by plants for nutrition (Fox and Guerinot 1998). However, even metabolic metals beyond a threshold concentration are harmful to biological systems (Williams et al. 2000). One such metal is copper. Because both copper and ultraviolet (UV) radiation can cause oxidative stress in plants (Foyer et al. 1994, Green and Fluhr 1995, Caro and Puntarulo 1996, Giardi et al. 1997, Fry 1998, Surplus et al. 1998, A-H-Mackerness et al. 1999, Babu et al. 2001, Galatro et al. 2002, Hartley-Whitaker et al. 2001, Estevez et al. 2001, Hideg et al. 2002), there is a potential for common response to these two effectors.

Copper being a redox active metal can participate in a variety of reactions that generate reactive oxygen species (ROS) (Aust et al. 1985, Aruoma et al. 1991, Fry 1998). Copper has been implicated in damage to proteins (Weckx and Clijsters 1996), lipids (Sandmann and Böger 1980), polysaccharides (Fry 1998) and photosynthesis (Yruela et al. 1996, Babu et al. 2001). Recently, we have shown that copper can reduce molecular oxygen to ROS in higher plant chloroplasts when the redox state of the chloroplast has been altered (Babu et al. 2001). Similarly, iron has been shown to generate ROS in soybean roots and green algae (Caro and Puntarulo 1996, Estevez et al. 2001). Within the chloroplast, the accelerated turnover of the D1 protein by other stressors (UV and herbicides) has also been proposed to be due to the accumulation of semiquinone radicals (Greenberg et al. 1989, Jansen et al. 1998, Babu et al. 1999). Interestingly, another divalent metal, cadmium, and other abiotic stressors have been shown to alter the turnover of the D1 protein of photosystem II in plants (Giardi et al. 1997, Geiken et al. 1998). Thus, copper and other metals cause damage to plants at a variety of targets and the key underlying mechanism appears to be formation of ROS.

UV radiation by itself or in combination with photosynthetically active radiation (PAR) has been shown to impact on

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plants (Jordan 1996, Jansen et al. 1998). The most damaging part of the UV spectrum reaching earth's atmosphere is UV-B (290–320 nm). For example, UV-B radiation has been shown to impair several biochemical and physiological processes including gene expression (Greenberg et al. 1989, Melis et al. 1992, Strid et al. 1994, Teramura and Sullivan 1994, Wilson et al. 1995, Jansen et al. 1996a, Surplus et al. 1998, A-H-Mackerness et al. 1999, Babu et al. 1999, Gerhardt et al. 1999, Booij-James et al. 2000). Stress from UV-B radiation in many cases is thought to proceed via a ROS mechanism, although the mechanisms of ROS generation by UV are not as well understood as with copper (Green and Fluhr 1995, Surplus et al. 1998, A-H-Mackerness 2000, A-H-Mackerness et al. 2001, Hideg et al. 2002). Thus, like copper, UV radiation seems to cause oxidative stress through ROS pathways.

Metals and UV also have been shown to induce acclimation processes that include production of antioxidant enzymes (Xiang and Oliver 1998). However, it is not clear what signals the induction of such acclimation processes. One possibility is that generation of ROS signal acclimation to metals and UV. It is clear that both UV and metals can generate ROS. In this context, UV-B-generated ROS have been shown to induce gene expression (Green and Fluhr 1995, Surplus et al. 1998, A-H-Mackerness 2000, A-H-Mackerness et al. 2001). However, most of these studies were related with pathogenesis-related proteins, not UV-B-specific responses. An important acclimation response of UV-B in plants is the biosynthesis of flavonoids (Caldwell et al. 1983, Mazza et al. 2000, Wilson et al. 2001). Flavonoids are also induced in plants by pathogens and ozone (Dixon and Paiva 1995, Booker and Miller 1998). However, there is little information on how these stressors all signal the accumulation of flavonoids (Jordan 1996, Frohnmeyer et al. 1999). For instance, there are few instances where UV-B-induced ROS have been examined in conjunction with chalcone synthase gene expression, a key enzyme in the flavonoid biosynthetic pathway (e.g. A-H-Mackerness et al. 2001). In this context, it would be interesting to study the induction of similar responses by different stressors that can both generate ROS.

The present study was undertaken to investigate the possibilities of common responses in plants subjected to different stressors. Because copper and UV radiation often co-exist in the environment and both have been implicated in ROS production, it would be interesting to compare the effects of these two stressors on plants when applied alone or simultaneously. There is currently little information on the combined effects of heavy metals and UV radiation on plants or algae (Dube and Bornman 1992, Estevez et al. 2001, Larsson et al. 2001). More importantly, information on the induction of similar responses by different stressors is very vague. It is also not known if there is a common signal transduction mechanism involved in the responses such as copper and UV. In this study, we examined the impacts of copper plus simulated solar radiation (SSR) (a light source containing UV-B [290–320 nm], UV-A [320–400 nm] and PAR [400–700 nm]) on the aquatic higher plant

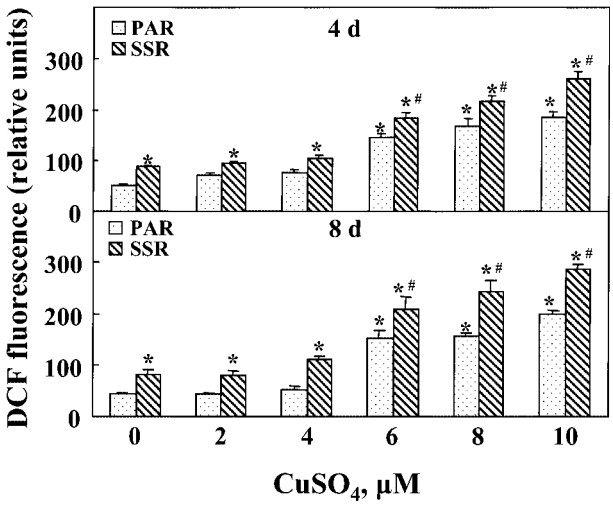

Fig. 1 Effect of copper on DCF fluorescence of *L. gibba* grown under PAR or SSR. Plants were treated with 0–10 μM copper in the presence of PAR or SSR for 4 or 8 d. Thereafter, plants were washed and subjected to 5 μM DCF for 30 min. Plants were washed three times with nutrient medium and the DCF fluorescence was measured. DCF fluorescence emission was collected at 530 nm with an excitation wavelength at 485 nm. * indicates significant difference when comparing all treatments (n = 4, P <0.05, analysis of variance, preplanned contrast using Tukey's test of significance) to control plants grown under PAR. # indicates significant difference (n = 4, P <0.05, analysis of variance, preplanned contrast using Tukey's test of significance) when comparing all SSR treatments to control plants grown under SSR.

Lemna gibba. We found that ROS were elevated when plants were exposed to copper or SSR. ROS levels were further elevated when copper was combined with SSR. We observed that both copper and SSR induce the accumulation of the same enzymes involved in antioxidant pathways. It was also found that copper treatment alone mimics the UV-induced formation of phenylpropanoids (e.g. flavonoids). Finally, plants exposed to ROS scavengers along with copper and/or SSR exhibited alleviation of both toxicity and induction of stress responses.

Results

Formation of reactive oxygen species in the presence of copper and SSR

2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) can be taken up by living tissue, such as *L. gibba*, and is oxidized by ROS to form a highly fluorescent compound, 2′,7′-dichlorofluorescein (DCF) (Fig. 1) (Behl et al. 1994). The level of fluorescent compound formed is dependent on the amount of ROS. Plants exposed to SSR for either 4 or 8 d exhibited increased DCF fluorescence relative to plants exposed to PAR (Fig. 1). This indicated that exposure of plants to SSR radiation resulted in formation of ROS. Addition of copper in the form of $CuSO_4$ to the above PAR or SSR treatments further enhanced the level of DCF fluorescence (Fig. 1). DCF fluores-

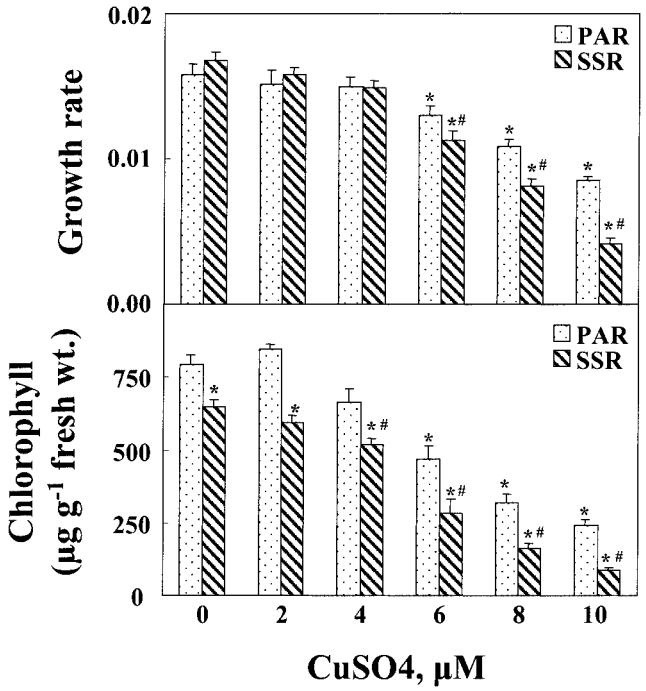

Fig. 2 Effect of copper on growth and chlorophyll content of *L. gibba* under either PAR or SSR. Plants were grown in nutrient medium supplemented with different concentrations of $CuSO_4$ ranging from 0 to 10 μM. Plants were grown for 8 d and the fronds were counted to assess the growth. Total chlorophyll was measured in DMF extracts as described in the text. * indicates significant difference when comparing all treatments (n = 6, P <0.05, analysis of variance, preplanned contrast using Tukey's test of significance) to control plants grown under PAR. # indicates significant difference (n = 6, P <0.05, analysis of variance, preplanned contrast using Tukey's test of significance) when comparing all SSR treatments to control plants grown under SSR.

cence increased with increasing concentrations of copper indicating that copper also promoted the formation of ROS (Fig. 1). DCF fluorescence was always higher in plants treated with copper plus SSR (Fig. 1), indicating that in plants exposed to the combination of copper and SSR containing UV radiation, there was greater formation of ROS. Similar levels of ROS were formed at 4 d and 8 d (Fig. 1), indicating that ROS were at steady state levels.

Effect of copper on growth and chlorophyll content

At the whole organism level, we observed that copper under both PAR and SSR caused a decrease in the growth rate and chlorophyll content of the plants (Fig. 2). The decrease in the growth and chlorophyll content was not significant at copper concentrations <4 μM. Above 4 μM copper concentration, both growth and chlorophyll content decreased significantly under PAR as well as SSR (Fig. 2). Copper plus SSR caused greater toxicity to plants than copper plus PAR. At copper concentrations ≥6 μM, both growth and chlorophyll content decreased to a greater extent under SSR than PAR (Fig. 2). The decrease in growth under all treatments was also accompanied by leaf necrosis, a possible indicator of cell death.

Effect of copper and UV radiation on SOD activity

L. gibba possesses three isoforms of superoxide dismutase (SOD): Mn SOD, Cu-Zn SOD and Fe SOD (Fig. 3). The characterization of the isoforms of SOD was established using specific inhibitors, NaCN and H_2O_2 and was carried out according to Pan and Yau (1992) (data not shown). The density of each SOD isozyme was quantified using densitometry. For the clarity of presentation, the negative staining of SOD isoforms has been converted to a positive image in this study (Fig. 3). In plants, Mn SOD is in mitochondria, Cu-Zn SOD is in both the cytosol and chloroplast, and Fe SOD is present in the chloroplast stroma (Kanematsu and Asada 1994, Bowler et al. 1994, Allen et al. 1997). Treatment of *L. gibba* with increasing concentrations of copper resulted in elevated levels of Mn SOD and Cu-Zn SOD (Fig. 3). In contrast, Fe SOD decreased with increasing copper concentrations, particularly above a 4 μM copper concentration (Fig. 3). Similar to the increase in the DCF fluorescence in plants treated with SSR containing

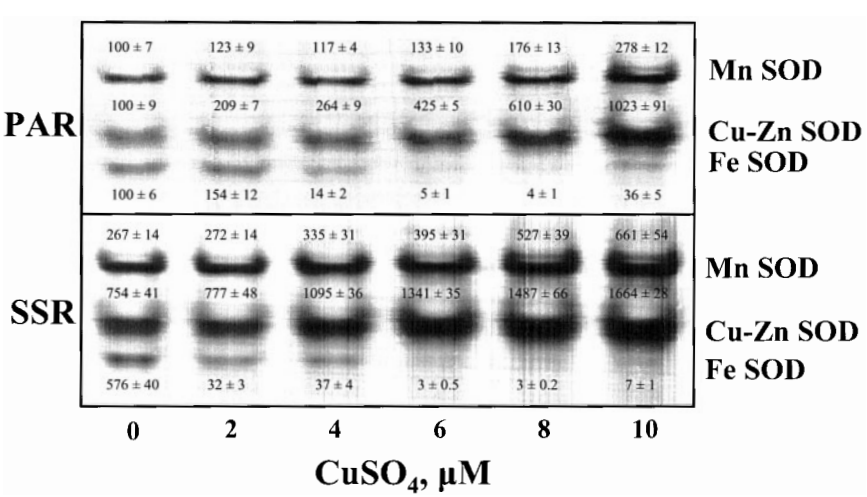

Fig. 3 SOD activity of *L. gibba* treated with different concentrations of copper. Plants were treated with 0–10 μM copper and incubated under either PAR or SSR for 8 d. Thereafter, plants were homogenized as described in Materials and Methods, and the extracts were subjected to non-denaturing PAGE and the SOD isozymes were quantified with an activity stain. Positions of three classes of SOD are indicated. Densitometric analysis was performed and relative intensity of bands with respect to PAR grown control plants is shown as the mean and standard error of the mean (n = 4).

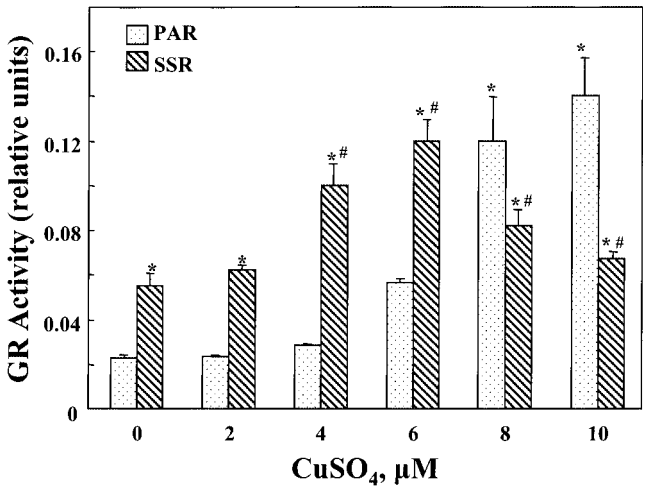

Fig. 4 Effect of copper on the glutathione reductase activity of *L. gibba* exposed to either PAR or SSR. Extraction of proteins was as in Fig. 2. * indicates significant difference when comparing all treatments (n = 4, P <0.05, analysis of variance, preplanned contrast using Tukey's test of significance) to control plants grown under PAR. # indicates significant difference (n = 4, P <0.05, analysis of variance, preplanned contrast using Tukey's test of significance) when comparing all SSR treatments to control plants grown under SSR.

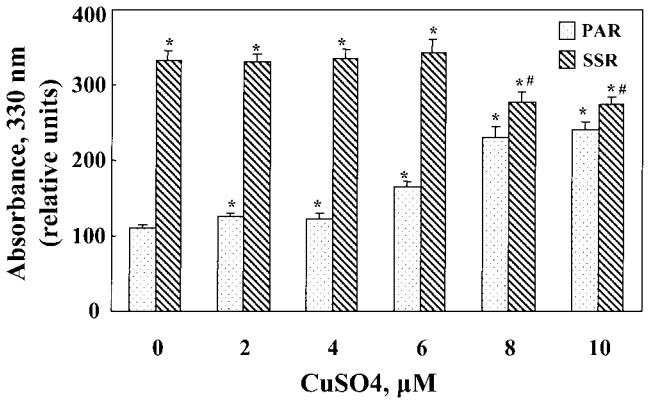

Fig. 5 Accumulation of UV-absorbing compounds in *L. gibba*. Plants were treated with 0–10 μM copper and incubated under either PAR or SSR for 8 d. Pigments were extracted with DMF as described in the text. UV absorbing compounds were quantified as absorbance at 330 nm. * indicates significant difference when comparing all treatments (n = 4, P <0.05, analysis of variance, preplanned contrast using Tukey's test of significance) to control plants grown under PAR. # indicates significant difference (n = 4, P <0.05, analysis of variance, preplanned contrast using Tukey's test of significance) when comparing all SSR treatments to control plants grown under SSR.

UV radiation (Fig. 1), the SOD activity increased in *L. gibba* exposed to SSR, relative to PAR exposure (Fig. 3). It is interesting to note that the induction of Mn SOD and Cu-Zn SOD became pronounced at a threshold concentration of 4 μM copper in both PAR and SSR, where the generation of ROS due to copper also became distinct.

Effect of copper and UV radiation on GR activity

Increasing copper concentrations in the presence of either PAR or SSR containing UV resulted in elevated levels of glutathione reductase (GR) activity in *L. gibba* (Fig. 4). However, the GR activity in plants exposed to copper in the presence of SSR rises to a peak value at 6 μM copper, and then drops as the copper concentration is increased (Fig. 4). This decrease after the peak in GR activity may simply reflect the whole organism toxicity observed above. SSR treatment alone was able to induce GR activity (Fig. 4). It should be pointed out that although the activity of GR diminishes at higher copper concentrations, it is nevertheless higher than in the control plants not treated with copper. Similar to the results for SOD, the level of GR increased in concert with the level of ROS (c.f. Fig. 1, 3, 4).

Induction of phenylpropanoid pathway components in response to copper treatment

It was observed that plants treated with SSR containing UV had greater amounts of UV-absorbing compounds than plants exposed to PAR (Fig. 5). Interestingly, copper treatment alone mimicked the UV effect in causing an increase in UV-absorbing compounds under PAR (Fig. 5). This increase in UV-absorbing compounds under PAR was dependent on copper concentration. Interestingly, only above 4 μM copper did accumulation of UV-absorbing compounds occur (Fig. 5). We note, this is the same concentration of copper that initiated ROS production and the induction of SOD and GR activities. Copper plus SSR did not exhibit significant increase in the UV-absorbing compounds over SSR or copper alone (Fig. 5). In fact, copper concentrations above 6 μM in the presence of SSR caused a slight decrease in the UV-absorbing compounds relative to SSR alone, perhaps reflecting toxicity (Fig. 5).

To analyze the nature of these UV-absorbing compounds, we collected methanol extracts from plants treated with copper in the presence or absence of SSR and partitioned the pigments by HPLC (Fig. 6). From the absorption spectral properties of different components separated by HPLC (data not shown), it was concluded that these components were mainly flavones (Marby et al. 1970, Wilson et al. 2001). Interestingly, the copper-alone and SSR-alone treatments each caused accumulation of virtually the same population of phenylpropanoid compounds, and this accumulation was higher than in the control plants.

Similar to the A330 and HPLC data, chalcone synthase (CHS), the enzyme that catalyzes the committal step in flavonoid biosynthesis was induced by copper treatment under PAR (Fig. 6). SSR alone also resulted in the up regulation of CHS. However, copper at higher concentrations with SSR caused a decrease in the CHS relative to SSR alone (Fig. 6).

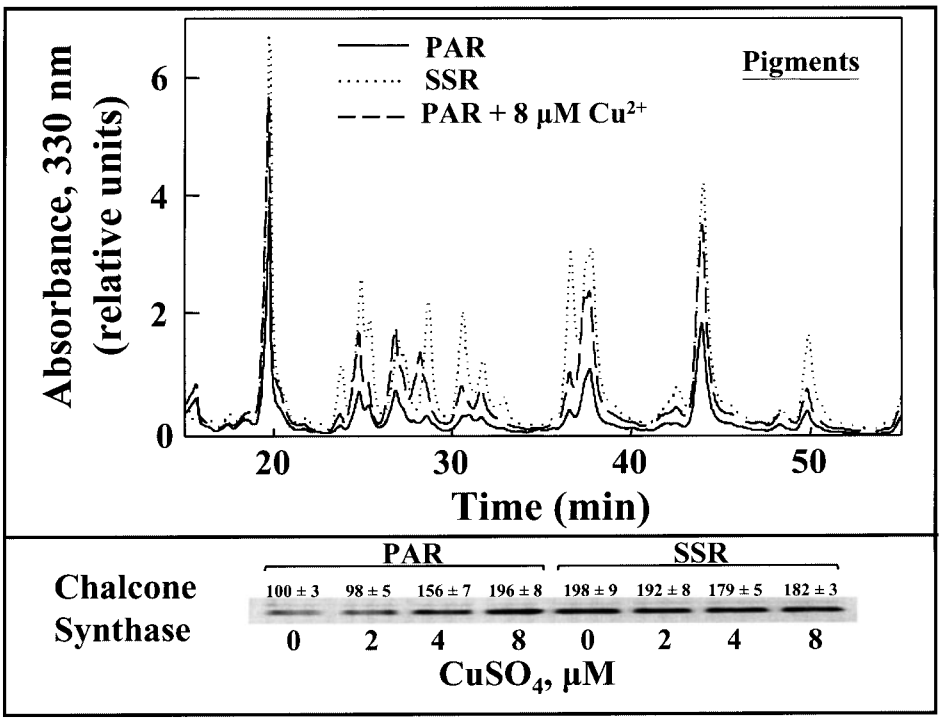

Fig. 6 (A) Accumulation of different UV-absorbing compounds in *L. gibba* treated with copper. Plants were grown under SSR or PAR plus copper up to a concentration of 8 μM for 8 d. Plants were homogenized in 80% aqueous methanol and the homogenates were centrifuged to collect the clear methanolic supernatants. The methanolic extracts were resolved by HPLC into different UV-absorbing components as described in the text. (B) Induction of chalcone synthase in *L. gibba* treated with 0, 2, 4 and 8 μM copper and grown under either PAR or SSR for 8 d. Plants were homogenized as described in Materials and Methods and protein extracts were subjected to SDS-PAGE. The resolved proteins were transferred onto a Nylon membrane and probed with antiserum against the chalcone synthase enzyme. Densitometric analysis was performed and relative intensity of bands with respect to PAR grown control plants is shown as the mean and standard error of the mean (n = 3).

Alleviation of toxicity and induction of stress responses by ROS scavengers

To further examine if ROS could be involved in the signaling of stress responses by both copper and UV radiation, plants were exposed to the ROS scavengers, dimethyl thiourea (DMTU) and GSH. DMTU is known to scavenge both hydroxyl radical and H_2O_2, while GSH is known to detoxify a variety of ROS including H_2O_2. Both DMTU- and GSH-treated plants exhibited diminished formation of ROS when exposed to copper and/or SSR (Fig. 7A). We also monitored for the protective effects of these ROS scavengers against toxicity as assayed using both growth and chlorophyll content (Fig. 7B, C). As well, accumulation of UV absorbing compounds and induction of GR was monitored as a stress response induced by both copper and SSR. Interestingly, both DMTU and GSH alleviated the toxicity responses caused by copper and/or SSR (Fig. 7B, C). The accumulation of UV absorbing compounds by both copper and SSR was suppressed when plants were exposed to DMTU and GSH (Fig. 7D). Similarly, DMTU and GSH diminished the induction of GR by copper and UV radiation (Fig. 7E). Overall, these results showed that DMTU and GSH alleviated the above-mentioned stress responses elicited by copper and SSR.

Discussion

Metals and UV radiation are two environmental stressors that can impact on plants simultaneously. Although there is information on the individual detrimental effects of copper and UV on plants, information on the combined effects of metals and UV is scarce. One of the main effects of metals and UV on plants is oxidative stress, implying the involvement of ROS. However, there is very little information on the role of ROS inducing common responses in plants by different stressors. Therefore, we initiated this study to examine if two stressors associated with ROS production could be involved in the elicitation of similar responses. An important outcome of this study is that both copper and SSR containing UV radiation induced the same responses in concert with ROS production. Further, these responses were alleviated when formation of ROS was suppressed by DMTU and GSH. Thus, ROS can be implicated in putative common signaling pathways of these two different stressors.

We found using DCF fluorescence, that exposure of plants to copper or SSR caused increased formation of ROS relative to control plants grown in PAR. If there were excess ROS formed within plant cells, one would expect impairment in sev-

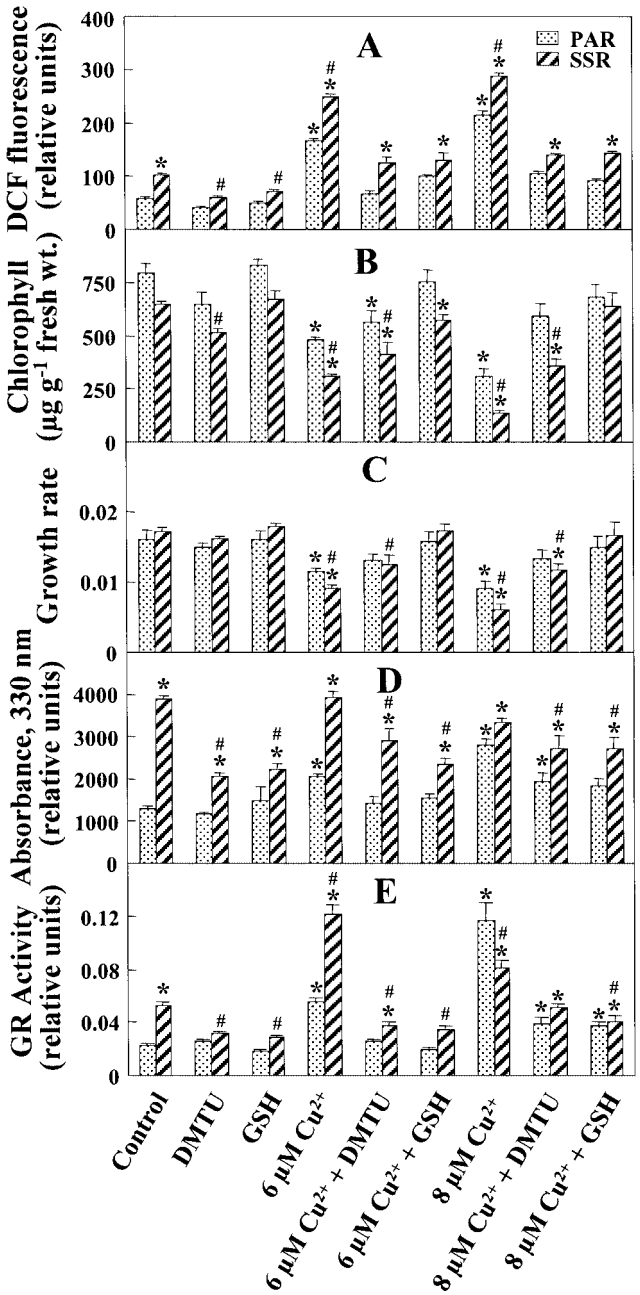

Fig. 7 Effect of the ROS scavengers DMTU and GSH on the (A) formation of ROS, (B) chlorophyll content, (C) growth, (D) UV-absorbing compounds and (E) GR in *Lemna gibba*. Plants were grown under PAR or SSR plus 6 or 8 μM copper for 8 d. In addition, where indicated 5 mM DMTU or 0.25 mM GSH were supplemented to the growth medium. Assays for ROS formation, growth, chlorophyll content, GR and UV-absorbing compounds were done as described in Fig. 1, 2, 4 and 5. * indicates significant difference when comparing all treatments ($n = 4$, $P < 0.05$, analysis of variance, preplanned contrast using Tukey's test of significance) to control plants grown under PAR. # indicates significant difference ($n = 4$, $P < 0.05$, analysis of variance, preplanned contrast using Tukey's test of significance) when comparing all SSR treatments to control plants grown under SSR.

eral metabolic processes that could eventually lead to cell death (Levine et al. 1994, Jabs et al. 1996, Desikan et al. 1998). Indeed, copper in the presence of both PAR and SSR caused a decrease in the growth and chlorophyll content of *L. gibba* implying whole organism toxicity. Interestingly, the damaging effects of copper were more pronounced under SSR than under PAR, which was consistent with the greater formation of ROS. In addition, the ROS scavengers, DMTU and GSH, lowered the formation of ROS and alleviated the diminishment of growth and chlorophyll content. Thus, ROS could be implicated in the copper-induced toxicity.

Biological systems induce a variety of scavenging systems to detoxify ROS (Bowler et al. 1994, Dixon and Paiva 1995, Jansen et al. 1996b, Allen et al. 1997, Dixon et al. 1998, Asada 1999, Grace and Logan 2000). SOD is an example of an enzyme induced in plants under stress to detoxify superoxide radicals (Bowler et al. 1994, Allen et al. 1997, Kliebenstein et al. 1998, Asada 1999). In fact, in this study when plants were treated with copper, we observed a significant increase in the activity of Cu-Zn SOD and Mn SOD isozymes under both PAR and SSR. Mitochondria possess Mn SOD, while chloroplasts contain Cu-Zn SOD (Kanematsu and Asada 1994, Bowler et al. 1994, Allen et al. 1997). Thus, it is possible that enhanced production of superoxide radicals occurs in both mitochondria and chloroplasts in response to copper treatments. This seems logical as these organelles possess major electron transport systems, and therefore, via redox cycling, copper could pass electrons to molecular oxygen to generate superoxide (Kumar et al. 1978, Babu et al. 2001). SSR without copper also induced Mn SOD and Cu-Zn SOD. We note that both copper and SSR generated increased ROS production relative to PAR alone, and SOD activity began to rise with the onset of this elevated ROS production. Thus, it is possible that ROS signal the induction of SOD. In contrast to Cu-Zn SOD and Mn SOD, Fe SOD that is associated with chloroplast stroma decreased with increasing concentrations of copper. The decrease in Fe SOD could be because excess copper competes with Fe and causes diminished uptake of Fe (Kurepa et al. 1997, Patsikka et al. 2002). In addition, the differences in the upstream regions of Fe SOD genes with those of Cu-Zn SOD and Mn SOD suggest that there could be differential regulation of gene expression (Alscher et al. 2002).

GSH is an important antioxidant metabolite, and is known to quench several radical-mediated processes (Allen et al. 1997, Dixon et al. 1998, May et al. 1998, Asada 1999). The enzyme GR catalyzes the formation of GSH from the oxidized form of glutathione. The induction of SOD and GR by copper and/or SSR treatment may indicate an acclimation response of the plants. Again, the up-regulation of GR occurs under copper and/or SSR conditions when the onset of ROS is observed. Furthermore, the loss of GR activity under higher concentrations of copper plus SSR could be due to the toxicity caused by excessive formation of ROS. That is the amount of GR induced under these conditions was not enough to detoxify excess ROS

and hence results in stress. In fact, when GSH was supplied to plants externally, there was an alleviation of toxicity and diminished ROS production. It should be mentioned that the higher levels of ROS observed under copper treatments is not simply due to the loss of GR activity. This is because the diminished GR activity by higher copper concentrations under SSR is still higher than the control levels. In fact, the higher levels of GR induced by copper could actually mean the amount of ROS produced is underestimated, as the production of GSH by GR would scavenge ROS. Similar to GR, induction of other ROS detoxifying enzymes like ascorbate peroxidase could also contribute to an underestimation of the amount of ROS generated. Thus, the higher levels of ROS observed under increasing concentrations of copper if anything underestimate the actual ROS flux.

A well-known acclimation response of UV-B in plants is the induction of flavonoids and other UV-absorbing compounds. Strikingly, plants exposed to copper without UV radiation exhibited an induction of UV-absorbing compounds. This accumulation of UV-absorbing compounds increased with increasing concentrations of copper when plants were exposed to PAR. We also observed that the enzyme CHS, which catalyzes the committal step in flavonoid biosynthesis, was upregulated by both copper plus PAR and SSR alone. It should be noted that the induction of flavonoids was significantly higher under SSR than under PAR plus UV-A (data not shown). Thus, for all practical purposes, the induction of flavonoids under SSR could be attributed to UV-B radiation (Jansen et al. 1996b, Jansen et al. 1998, Wilson et al. 1995, Wilson et al. 1998). Interestingly, the copper plus PAR and SSR-alone treatments each caused accumulation of the virtually same population of phenylpropanoid compounds, and this accumulation was higher than in the control plants. It should also be noted that higher copper concentrations under SSR cause the lowering of flavonoids/UV-absorbing compounds. Nevertheless, the levels of flavonoids under all SSR treatments were higher than plants grown under PAR. Because higher copper concentrations under SSR cause enhanced toxicity at the whole organism level, we attribute the diminishment in the accumulation of flavonoids under these conditions to decreased physiological efficiency of plants. In addition, it is possible that UV and copper generate differential amounts of different species of ROS. Thus, the saturation of flavonoid accumulation under SSR plus copper treatments could be due to the generation of similar levels of a particular type of ROS that is more effective in signaling flavonoid accumulation. Therefore, while the lower flavonoid levels at higher copper concentrations under SSR are probably due to stress, their higher levels when compared to PAR grown plants can still be a response due to ROS signaling.

If flavonoids induced by UV-B function as UV-screens in plants (Caldwell et al. 1983, Mazza et al. 2000, Wilson et al. 2001), one might wonder why copper induces the same population of flavonoids. It is interesting to note that flavonoids and other UV-absorbing compounds have been reported to act as scavengers of ROS (Grace and Logan 2000, Caldwell 2001). Some flavonoids containing the hydroxyl groups have been shown to chelate heavy metals and detoxify ROS (Brown et al. 1998). This is consistent with a potential dual role of flavonoids acting as antioxidants and UV screens.

It is striking that copper and SSR induce biosynthesis of the same population of flavonoids and the same ROS scavenging enzymes. It seems unlikely that one would observe such similar responses without a common signaling pathway. In this context, until now, UV-B photoreceptors acting as signal inducers for flavonoid biosynthesis have not been conclusively identified in plants. Therefore, one important question that this research addresses is the nature of the UV-B photoreceptor for the induction of flavonoids and other UV acclimation mechanisms. One could assume that there is a specific copper receptor and a specific UV receptor, and by distinct signaling pathways, they could activate the same processes. However, if one instead posits that the signaling is done by ROS, a much simpler explanation can be considered. That is, both copper and UV have the ability to generate ROS by independent mechanisms, but converging into a common ROS pathway. Copper would do so by redox cycling and UV would do so by photosensitization generating singlet oxygen (Kumar et al. 1978, Aruoma et al. 1991, Babu et al. 2001, He and Häder 2002). That being the case, there would not necessarily be a specific receptor in either case. Instead, the cell is simply detecting the elevated ROS levels that these two stressors can generate. Consistent with this conclusion, DMTU and GSH concomitantly lowered ROS, induction of GR and flavonoid levels. This may also explain why other stresses that can generate ROS (e.g. pathogens, drought, heat, ozone and nutrient deficiency) also induce flavonoid accumulation and other acclimation processes similar to those observed here (Caldwell et al. 1983, Chen et al. 1993, Levine et al. 1994, Dixon and Paiva 1995, Allan and Fluhr 1997, Lamb and Dixon 1997, Booker and Miller 1998, Van Camp et al. 1998, Bowler and Fluhr 2000, Mazza et al. 2000, A-H-Mackerness et al. 2001, Orozco-Cardenas et al. 2001, Wilson et al. 2001). However, under excessive ROS formation, the signaling of flavonoid induction and/or other stress responses (for instance GR activity) by ROS could be derailed. This could be attributed to the ROS-mediated toxicity at the whole organism level as observed under higher copper concentrations plus SSR treatments. Based on the above discussion, UV-B radiation does not need a specific UV-B photoreceptor for signaling. Instead, any chromophore that can induce ROS formation via photosensitization due to UV-B exposure would become a UV-B photoreceptor.

Materials and Methods

Plant growth and chemical treatment

Stocks of *L. gibba* L.#G-3 were cultured axenically on half-strength Hutner's medium under 100 μmol m^{-2} s^{-1} of continuous cool-white fluorescent irradiation (Huang et al. 1993). For experimental work, unless stated otherwise, plants were grown for 8d in the pres-

ence of either 100 μmol m^{-2} s^{-1} of PAR or 100 μmol m^{-2} s^{-1} of SSR [100 μmol m^{-2} s^{-1} of PAR (400–700 nm); 10 μmol m^{-2} s^{-1} of UV-A (320–400 nm); 1 μmol m^{-2} s^{-1} UV-B (290–320 nm)] (Huang et al. 1993). The SSR has a PAR : UV-A : UV-B ratio similar to that of natural sunlight. The SSR is only at about 5% of solar PAR and therefore, contains approximately 5% of environmental UV-B. In this study, UV was only applied as a mixture of UV-B and UV-A. However, in previous studies it has been shown that the acclimation responses presented here are much stronger under UV-B than UV-A (Wilson et al. 1995, Jansen et al. 1996b, Jansen et al. 1998, Wilson et al. 1998). For the copper treatments, the nutrient medium was supplemented with 0–10 μM $CuSO_4$. Copper was added to the media by dilution from a stock solution of 2 mM $CuSO_4$ in reverse osmosis (RO) purified H_2O. For the DMTU treatments, the nutrient medium was supplemented with 5 mM DMTU from a stock solution of 1 M DMTU in RO-purified H_2O. For the GSH treatments, the nutrient medium was supplemented with 0.25 mM GSH from a stock solution of 100 mM GSH in RO-purified H_2O.

Measurement of ROS

After *L. gibba* plants were placed in media with different concentrations of $CuSO_4$, the plants were divided into two groups. One group was incubated under PAR and the other under SSR for either 4 or 8 d. Each group had a control that was not treated with copper. Production of ROS in *L. gibba* was measured using H_2DCFDA (Molecular Probes, Eugene, OR) (Behl et al. 1994). After the 4- and 8-day treatments, plants were washed three times with half-strength Hutner's medium and treated with 5 μM H_2DCFDA for 30 min at 25°C. H_2DCFDA is a non-fluorescent compound that can be readily taken up by cells. Once inside cells, the acetate is cleaved by endogenous esterases. The acetate-free, reduced form of 2′,7′-dichlorodihydrofluorescein (H_2DCF) is trapped inside the cells and can be oxidized by ROS, particularly the hydroperoxides, to form a highly fluorescent compound, DCF. DCF fluorescence was measured on intact plants and the fluorescence was normalized to the total soluble protein. To confirm the efficiency of the measurement, DCF fluorescence was also measured using plant homogenates normalized to the total soluble protein. Because we found no significant differences in the DCF fluorescence measured with either intact plants or plant homogenates, we chose to use intact plants for measurements. DCF fluorescence was also determined on a fresh weight basis and similar results were achieved. It must be mentioned that using sub-optimal concentrations of H_2DCFDA on both control and copper affected tissues produced insignificant differences in the amounts of ROS detected. However, using a concentration of 5 μM H_2DCFDA that is higher than optimal levels showed higher amounts of ROS in copper treated plants. Thus, both control and copper-affected tissues appear to have taken H_2DCFDA to more or less similar levels. The DCF fluorescence was measured using an excitation wavelength of 485 nm and an emission wavelength of 530 nm. All the fluorescence data were collected using a fluorescence plate reader (Cytofluor 2350, Millipore, Mississauga, ON, Canada).

Whole organism toxicity

Eight *L. gibba* fronds (two plants) were placed in nutrient medium supplemented with 0–10 μM $CuSO_4$. The plants were grown for 8 d under 100 μmol m^{-1} s^{-1} of PAR or SSR. The medium with chemicals was replenished by static renewal every 2 d (Greenberg et al. 1992). At the end of 8 d, the fronds were counted to assess the growth (Greenberg et al. 1992).

Assay of superoxide dismutase activity

SOD activity was measured by non-denaturing polyacrylamide gel electrophoresis (Beauchamp and Fridovich 1971). Crude extracts from *L. gibba* were prepared for SOD analysis according to Shaaltiel and Gressel (1986). Plants were homogenized in 0.1 M potassium phosphate buffer (pH 7.8) at 4°C. The homogenate was centrifuged at 3000×*g* and the supernatant was stored at –70°C until further analysis. Thawed homogenates were fractionated on non-denaturing 10% polyacrylamide gels at 120 V for 3 h at 4°C. The gels were then incubated in dark at room temperature in 80 ml of RO water containing 0.016 g nitroblue tetrazolium (Sigma, MO, U.S.A.) for 20 min with gentle shaking. The gels were transferred to 80 ml of 0.05 M potassium phosphate buffer (pH 7.8) containing 1.0 mg riboflavin (Sigma, MO, U.S.A.) and 100 μl of TEMED (Sigma, MO, U.S.A.). The gels were incubated in the dark at room temperature with gentle shaking for 15 min. The gels were rinsed with distilled water, transferred in to 100 ml of 0.05 M potassium buffer (pH 7.8) containing 15 mg EDTA and exposed to cool white fluorescent light at room temperature. The SOD activity on the gels appears as a negative staining. However, the negative staining has been presented in this manuscript as a positive image for the clarity of presentation. Identification of the SOD isozymes was done by inhibitor studies as described by Pan and Yau (1992). The density of each SOD isozyme was also quantified by densitometry.

Assay of glutathione reductase activity

Extracts from *L. gibba* were prepared as described for the SOD activity assay. GR activity was measured spectrophotometrically as described by Smith et al. (1988). The assay medium contained 0.1 M potassium phosphate buffer (pH 7.8), 1 mM EDTA, 3 mM 5,5′-dithiobis-2-nitrobenzoic acid (DTNB), 2 mM NADPH and 20 mM oxidized glutathione (GSSG). GR activity was measured spectrophotometrically as a rise in absorbance at 412 nm due to the reduction of DTNB.

Pigment analysis

Pigment analysis in *L. gibba* fronds was done as described by Jansen et al. (1996b). Fronds (~10 mg) were placed in either 1 ml of dimethylformamide (DMF) or 1 ml 80% aqueous methanol to extract the pigments. Chlorophyll quantification in the DMF extracts was done according to Moran (1982). UV-absorbing components (e.g. flavonoids) in the methanol extracts were analyzed on a Hypersil ODS reverse phase HPLC column (250×4.6 mm, 5 μm) (Supelco Canada, Oakville, Ontario, Canada) using a linear gradient of 0–60% acetonitrile in water (pH 3.0) according to Wilson et al. (1998). Pigments levels were determined on a fresh weight basis.

Detection of chalcone synthase

Total protein was isolated from *L. gibba* by homogenizing two fronds in 100 μl of ice-cold 200 mM Tris, pH 7.8, 0.5 mM DTT, and 10 mg ml^{-1} polyvinylpolypyrrolidone. The homogenate was centrifuged in a microfuge to recover soluble proteins in the supernatant. The protein concentration was estimated by a protein assay kit (Bio-Rad). Samples containing 40 μg of protein were separated by SDS-PAGE in a 12% polyacrylamide gel. SDS-PAGE resolved proteins were electroblotted to nitrocellulose. The nitrocellulose filters were blocked with 5% skim milk and probed with polyclonal antibodies raised against CHS (Burbulis et al. 1996) that were obtained from Dr. Marvin Edelman (Weizmann Institute of Science, Rehovot, Israel). The antibody-CHS complexes were detected with peroxidase-conjugated rabbit anti-goat IgY (ICN Biomedicals, Inc. Costa Mesa, CA, U.S.A.). The chemiluminescence produced by the secondary antibody (enhanced chemiluminescence, Amersham) was detected by radiographic film.

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