Stress responses of tomato protoplasts to copper and paraquat

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ABSTRACT

Plants are often exposed to external biotic or abiotic agents that can damage cells. Biotic agents include molecules produced by pathogens, and abiotic agents include ultraviolet radiation, heavy metals and xenobiotic agents such as herbicides. Because of the importance of abiotic stresses and the limited knowledge of the defense responses of plants to stresses, a study was conducted using flow cytometry to evaluate endocellular events in protoplasts of two tomato species, *Lycopersicon hirsutum* and *Lycopersicon esculentum*, following exposure to 10 mM $CuCl_2$ and 1% paraquat. During the first 30 min of exposure to 10 mM $CuCl_2$ mean fluorescence intensity values in both species decreased by more than 50% compared to the untreated control. During the first 30 min of paraquat treatment, the production of reactive oxygen species, evaluated as the incidence of protoplasts in which the superoxide anion was present, was 32.9 % and 25.4%, respectively, for *L. hirsutum* and *L. esculentum.* Hyperpolarization of the mitochondria for both tomatoes species was observed during the first 2 h. The absence of early membrane damage and the markedly detrimental effects of the treatments on the mitochondria suggest that additional mechanisms which lead to cell death may be involved in these processes, and that these mechanisms may be associated with apoptosis.
Key words: *Lycopersicon,* abiotic elicitation, apoptosis, programmed cell death.

RESUMO

Respostas dos protoplastos do tomate ao estresse gerado pelo cobre e o paraquat

As plantas estão expostas a agentes externos bióticos e abióticos que podem produzir dano nas celulas. Os agentes bióticos incluem moléculas produzidas por patógenos e os agentes abióticos incluem radiação ultravioleta, metais pesados e os agentes xenobióticos como os herbicidas. Devido à importancia do estresse abiotico e o limitado conhecimiento sob as respostas de defesa das plantas ao estresse, este estudo foi feito usando a citometría de fluxo para avaliar os eventos endocelulares de duas espécies de tomate: *Lycopersicon hirsutum* e *Lycopersicon esculentum* depois da exposição a 10 mM $CuCl_2$ e 1% do paraquat. Durante os primeiros 30 min da exposição ao $CuCl_2$ os valores da intensidade média da fluorescência em ambas as espécies diminuiu mais do 50% quando comparado com os controles não tratados. No caso do tratamento com o paraquat, depois dos primeiros 30 minutos a produção das espécies reativas do oxigênio, (avaliada pela presença do ânion superóxido) na espécie *L. hirsutum* e *L. esculentum* foi de 32,9% e 25,4%, respectivamente. A hiperpolarização das mitocôndrias das duas espécies do tomate foi observada durante as 2 primeiras horas. A ausência de dano no início ao nível da membrana e os efeitos adversos na mitocôndria causados pelos tratamentos sugerem que poda haver mecanismos adicionais que levam à morte celular, e que estes mecanismos podem estar associados à apoptose.
Palavras-chave: *Lycopersicon,* apoptose, eliciação abiótica, morte celular programada.

INTRODUCTION

Previous literature has reported that exposure of plants to heavy metals (Mithofer et al., 2004; Yakimova et al., 2007*)* and xenobiotics causes stress and cell death in plants (Álvarez & Espinosa, 2004). The hypersensitivity response (HR) is a frequently described plant defense reaction and has been considered as a type of suicide of cells surrounding the site of pathogen attack, thus preventing a subsequent invasion of biotrophic pathogens and obligate parasites such as viruses (Lamb & Dixon, 1997; Pennell & Lamb, 1997; Lam et al., 1999; Zhang et al., 2004; Hofius, 2007). In recent years, interest in the HR mechanism has increased among plant researchers because it shares similarities with apoptosis in animals (Danon et al., 2000; Hofius, 2007). Apoptosis occurs in animal cell populations in various states of embryonic development and is seen in diverse pathologies as a response to environmental stress (Godbole et al., 2003). It has been proven that different forms of stress can lead to cell death, and although differences in this process are known among plant and animal cells, there are also many similarities, suggesting that a large portion of the genetic characteristics and biochemical responses responsible for

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Abbreviations used: HR, hypersensitivity response; MFI, mean fluorescence intensity; PCD, programmed cell death; ROS, reactive oxygen species.

this process in plants have been evolutionarily conserved (Godbole et al*.,* 2003; Van Doorn, 2005).

As an abiotic stress factor in plants, copper was reported to cause oxidative stress, beginning with the interruption of electron transport in the photosystems, and resulting in irreversible damage to the cell (Hsu et al., 1988; Narendranath et al., 1989; Yruela et al., 1996; Ke Shi-sheng, 2007). Evaluated based on the presence of superoxide anion, Paraquat, a herbicide whose active molecule is a bybiridyl with two quaternary nitrogen molecules, is a xenobiotic stress factor that interrupts electron flow in photosystem I. Paraquat inhibits passage of electrons to ferredoxin so that the electrons are given up to oxygen in an unpaired manner by a series of oxidation-reduction reactions and as a consequence reactive oxygen species (ROS) are produced (Karp, 2006; Yu et al., 2007). Paraquat is considered toxic, not only because it interrupts photosynthesis, but because the production of ROS results in lipid peroxidation and changes in the biomolecules that shape damage in the membranes (Yu et al., 2007). Several studies reported the significance of ROS in oxidative stress, signal transduction and plant defense (Breusegem et al., 2001; Apel & Hirt, 2004; Torres & Dangl, 2005). Although the described mechanisms by which copper and paraquat cause plant stress have been generally accepted, the present study suggests that additional pathways contribute to stress and cell death and thus may be associated with apoptosis.

Evidence in animal models supports the significance of mitochondrial signaling during cell apoptosis (Lin et al., 2006). Several studies indicate that the uncoupling of electron transport by a specific elicitor, leading to decreased ATP production, causes a decrease in the mitochondrial membrane potential, and a decrease in ROS production (Lin et al., 2006). In the present study, the protoplasts of two tomato species were exposed to copper and paraquat and evaluated by flow cytometry for the production of ROS, and damage to the cell membranes and mitochondria to determine whether or not the changes are associated with apoptosis.

MATERIALS AND METHODS

Two tomato species were used: *Lycopersicon hirsutum,* (PI 251305 accession)*,* which was reported to be resistant to phytopathogens (Vallejo, 1999) and *Lycopersicon esculentum,* (L-507 accession) variety Licato (cultivable species). These plants were obtained from the System of Vegetable Germoplasm Banks of Colombia under the Colombian Agricultural Research Corporation CORPOICA.

Material Regeneration and Propagation

The tomato materials were stored seeds, which were planted in sterile Murashige and Skoog nutrient medium (Sigma Aldrich), at a ratio of 4.4 g L^{-1} medium, 1.7 mg L^{-1} gelling agent (phytagel) and 30 g L^{-1} of sucrose. To prepare

the medium, the salts and sucrose were dissolved in sterile distilled water by boiling, dispensed in 15 mL per bottle, allowed to cool, and autoclaved for 15 min at 120ºC at 220.5 PSI.

Protoplast preparation

To obtain protoplasts, mesophyll tissue was cut from leaves produced from the germinated seeds, and two hundred milligrams of the mesophyll tissue were pre-plasmolized in the dark for one hour at 25ºC in 20 mL of CPW salts (1.0 mM NaCl, 0.2 mM KCl and 0.2 mM CaSO$_4$), 9% mannitol and 3 mM 2-(N-morpholino) ethanesulfonic (MES) acid, at pH 5.8. The plasmolizing solution was then replaced with 20 mL of 0.6% cellulase and 0.1% pectinase and incubated in darkness at 25ºC for 8 h. After the enzymatic treatment, the suspension of digested leaves was washed in 20 mL of a solution of CPW salts (consistent with concentrations used previously) and 20 g L^{-1} KCl, filtered through gauze, and centrifuged at 600 rpm for 3 min. Each pellet was resuspended in 10 mL of the washing solution and centrifuged again at 600 rpm for 3 min. The protoplasts were then suspended in 9 mL of 15% of sucrose in CPW salts and 1 mL of the washing solution and centrifuged again for 7 min at 600 rpm for microscopic observations. The protoplasts obtained were then used in flow cytometry assays (Figure 1).

Evaluation of protoplast damage by flow cytometry

Protoplast damage induced by 10 mM CuCl$_2$ and 1% paraquat was evaluated by flow cytometry using EPICS XL (Coulter Electronics, Inc., Hialeah, FL). Between 6000 and 10,000 events (protoplasts) were evaluated. Readings were carried out every 15 min for the first 2 h of treatments (early events) and subsequently at 3, 24, 48 and 72 h (late events). In initial assays concentrations of 10, 1 and 0.1 mM CuCl$_2$ and 1, 0.2 and 0.02% paraquat were used. To evaluate alterations in mitochondrial permeability that are associated principally with apoptosis, protoplasts in their own medium were stained with 50 nM DiOC6. To detect plasma membrane damage, which takes place during necrosis and late apoptosis, protoplasts were stained with 0.1 µg/mL propidium iodide. Mitochondrial damage was defined as cell populations showing changes in the MFI of DiOC6, which depends on the differences in potentials between the mitochondrial membrane and the cytosol, causing an effect known as depolarization or hyperpolarization (Figure 2). To evaluate the production of ROS (i.e. the superoxide anion), the protoplasts with the treatments were incubated for 20 min in the dark at room temperature in 0.1 µg/mL dihydroethydine and analyzed by flow cytometry.

Statistical analysis

Linear regression analyses were performed to obtain the percentage of membrane damage, percentage of protoplast with reactive oxygen species and kinetics of the mean fluorescence intensity (MFI) of DiOC6, which
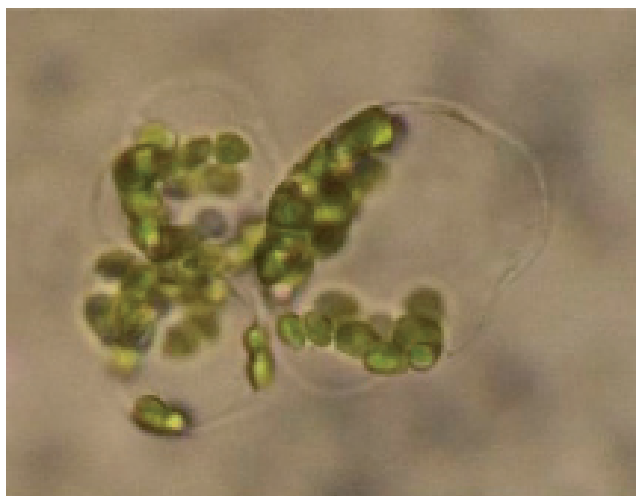
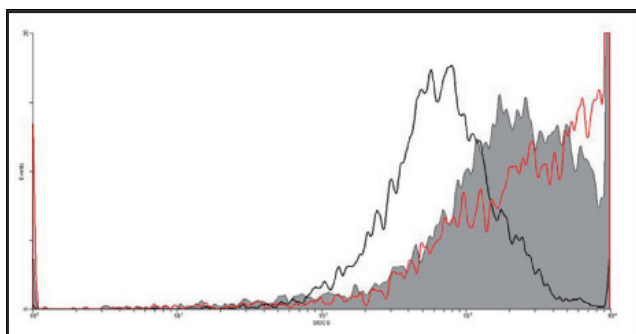**FIGURE 1 -** Photograph of three protoplasts of the species *Lycopersicon hirsutum*.

FIGURE 2 - Mean fluorescence intensity (MFI) of DiOC6 stain in protoplasts of *L. hirsutum* during the first fifteen minutes of treatment with 10mM $CuCl_2$ and 1% paraquat. Grey histogram (untreated control), empty histogram (treatment with copper), red histogram (treatment with paraquat).

signified either depolarization or hyperpolarization of the internal mitochondria membrane. These variables were analyzed separately during the first 2 h (early events) and between 3 and 72 h (late events). This model was used to fulfill the aim of the study, which was to determine effects of treatments on cell integrity.

RESULTS

Responses of protoplasts to copper

No increase was observed in incidence of protoplasts with membrane damage for *L. hirsutum* or *L. esculentum* during 2 h of exposure to 10 mM $CuCl_2$. During this period, MFI values for untreated protoplasts of both tomato species were similar , but MFI values of the $CuCl_2$ -treated protoplasts decreased by more than 50%. The treated protoplasts showed a decreased ability to capture DiOC6

compared to the untreated protoplasts (Figure 3, Table 1). None of the $CuCl_2$ treatments induced the oxidation of dihydroethydine in the protoplasts of either tomato species during the first 30 min, indicating that the mitochondria were damaged before ROS were produced. The mitochondrial responses observed during the first 2 h of exposure to $CuCl_2$, specifically depolarization, continued for 3-72 h after the treatments in protoplasts of both tomato species (Figure 4, Table 1).

Response of *L. hirsutum* and *L. esculentum* protoplasts to paraquat

During the first 2 h of paraquat treatment, when measurements were made every 15 min, an increase in the capture of DiOC6 was observed, which was interpreted as membrane hyperpolarization. The fluctuations observed in the MFI of the untreated controls were small and the linear regression models for the periods of evaluation were not significant and had near-zero slopes. In *L. esculentum* a similar phenomenon was observed, but hyperpolarization levels were slightly lower (Figure 5, Table 1). A sudden high production of reactive oxygen species is an important aspect of cell damage. In contrast to the copper treatment, paraquat caused significant production of ROS. During the first 30 min of paraquat treatment, the production of ROS, evaluated as the incidence of superoxide anion in the protoplasts, was 32.9% and 25.4%, respectively for *L. hirsutum* and *L. esculentum*. (Table 2).

The responses of the protoplasts measured during the first 3 h of treatments indicated in general that the integrity of the protoplast plasma membrane was maintained for both species. During late events the membrane of *L. hirsutum* was approximately 17% damaged with a slope of 0.84 and a p-value of 0.05, which was greater than the untreated control. *L. esculentum* showed a slope of 8.7, which was also greater than the untreated control (Table 3).

On the other hand, the hyperpolarization or depolarization of the mitochondrial membranes caused by the paraquat treatment that was observed during the period of early events continued throughout the late events. *L. hirsutum* showed a greater hyperpolarization at 3 h, after which it behaved similarly to the untreated control. *L. esculentum* behaved similarly to *L. hirsutum* (Figure 6). During the late events, dihydroethydine oxidation levels were above 50% for the two species treated with paraquat, and a positive slope of 1 was observed for *L. hirsutum* in comparison to a negative slope for the untreated control (Table 3). Although this research did not reveal any significant regression models for *L. esculentum*, 49.9% of the protoplasts exhibited ROS production, showing a slope 10 times greater than that of the untreated control (Table 3).

Dose-Response Assays

An inverse dose-response effect for the membrane damage variable was observed in both species because the
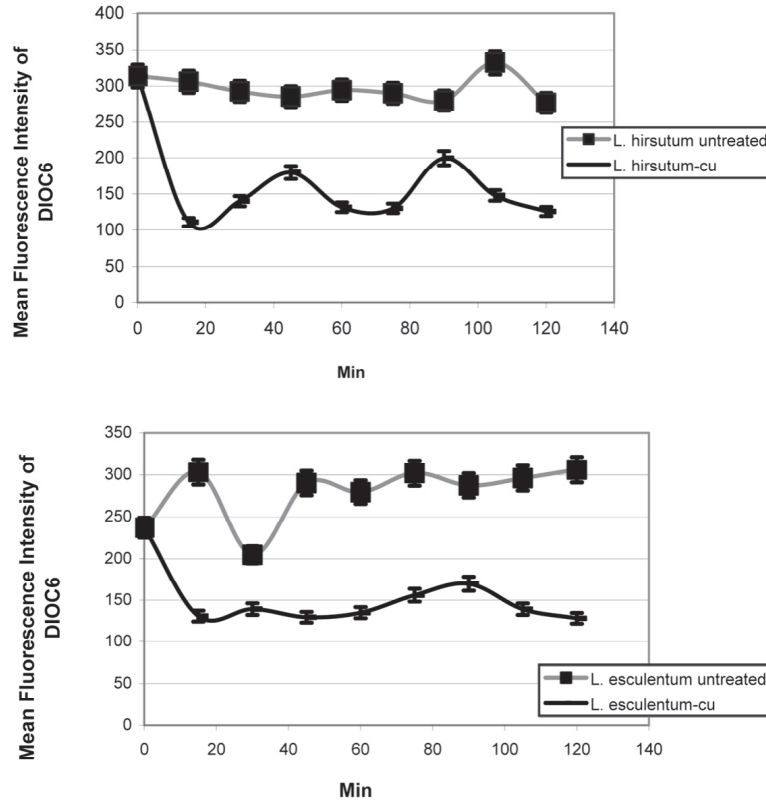
FIGURE 3 - Mean fluorescence intensity (MFI) of DiOC6 stain in protoplasts of *L. hirsutum* and *L. esculentum* during the first 2 h of treatment with 10mM CuCl$_2$ compared to that in untreated control protoplasts.

TABLE 1 - Regression models for the mean fluorescence intensity (MFl) of protoplasts of *Lycopersicon hirsutum* and *Lycopersicon esculentum* during the first 2 h and 72 h of treatment with 10 mM CuCl$_2$ and during 2 h of 1% paraquat treatment

| Treatment	Regression model	p-value
During 2 h of treatment with 10 mM CuCl$_2$		
L. hirsutum untreated	MFI= 307.406 - 0.278262*min	0.002
L. hirsutum copper treatment	MFI= sqrt(66454.5 - 5128.14*sqrt(min))	0.05
L. esculentum untreated	MFI= exp(5.5373 + 0.0168308*sqrt(min))	0.03
L. esculentum copper treatment	MFI= sqrt(40816 8 - 2387 77*sqrt(min))	0 05
During 72 h of treatment with 10 mM CuCl$_2$		
L. hirsutum untreated	MFI= sqrt(99258.4 - 5335.55*sqrt(hour))	0.55
L. hirsutum copper treatment	MFI= sqrt(8414.06 + 80345.8/hour)	0.002
L. esculentum untreated	MFI= 1/(0.00334466 + 0.00454673/hour)	0.25
L. esculentum copper treatment	MFI= sqrt(12981.7 + 7.39108*hour^2)	0.12
During 2 h of treatment with 1% paraquat		
L. hirsutum untreated	MFI= 307.406 - 0.278262*min	0.002
L. hirsutum paraquat treatment	MFI= 391 962 + 1 25267*min	0.16
L. esculentum untreated	MFI= 249 607 + 0 479333*min	0.11
L. esculentum paraquat treatment	MFI= 228.458 + 1.50978*min	0.007
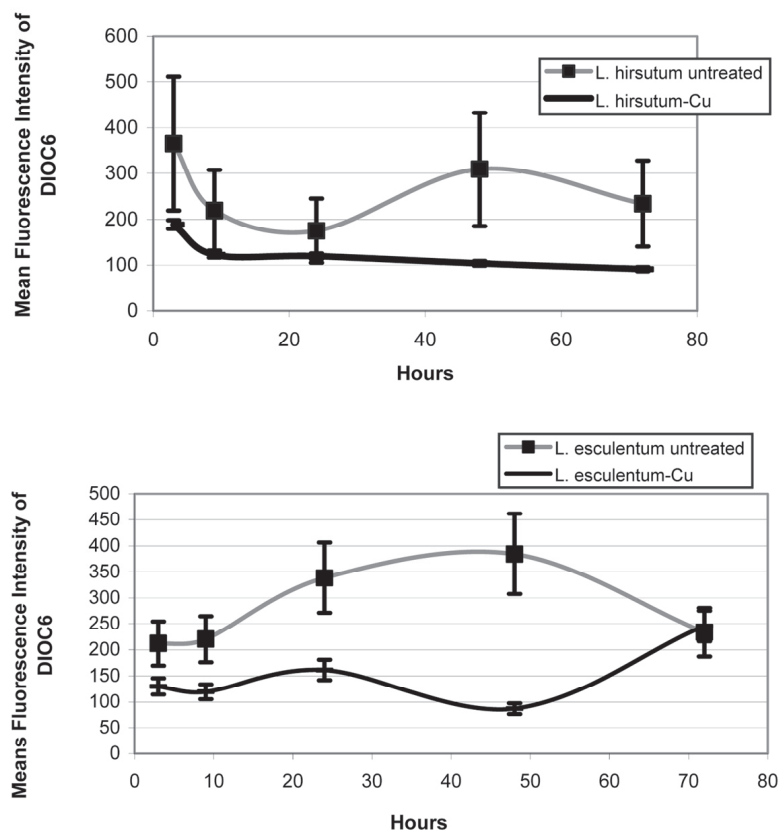
FIGURE 4 - Mean fluorescence intensity (MFI) of DiOC6 stain in protoplasts of *L. hirsutum* and *L. esculentum* during the first 72 h of treatment with 10mM CuCl$_2$ compared to that in untreated control protoplasts.

lowest level of damage and the smallest slope occurred at the highest dosage of 10 mM CuCl$_2$. There was no clear dose-response effect for mitochondrial damage because although there was an effect for the MFI, the depolarization or hyperpolarization effect as a function of concentration was unknown. As in the other experiments, the regression models for ROS production were generally not significant. In the dose-response assays for paraquat treatments, none of the models of the membrane damage variable were significant for either species. Mitochondrial damage was more clearly affected by paraquat dosage in *L. hirsutum,* which showed a gradient of hyperpolarization in accordance with increasing concentration. *L. esculentum* did not show a clear dose-response effect, but at 48 h the highest dosage showed the greatest degree of hyperpolarization. Dihydroethydine oxidation did not bring about a clear dose-response effect; however, a significant production of these oxidative species was observed.

DISCUSSION

The literature has reported that the exposure of plants to heavy metals, specifically to copper, affects the photosystems and also provokes an oxidative explosion that

leads to cell damage that is potentially irreversible (Hsu & Lee, 1988; Narendranath et al., 1989; Yruela et al., 1996; Ke Shi-sheng, 2007). It should be noted that copper did not cause an oxidative burst as is normally suggested in other studies involving heavy metals in which the enzymatic activity associated with the Halliwell-Asada cycle has been evaluated (Gallego et al., 1996; Sang-Hoon et al., 2007; Akbulut & Çakır, 2010). These results suggest that ROS do not play a role in copper-induced cell damage in the two species, either in direct damage or as molecules that participate in chemical signaling. These results differ from those found in other reports. However, due to experimental differences, it is not possible to directly compare the other studies to the present one.

Observations of the protoplasts of the two tomato species indicated that 10mM CuCl$_2$ quickly destabilized the electron transport chain on the mitochondria, but did not affect the integrity of the plasma membrane. All of the experiments, including the dose-response assays, revealed that the mitochondria were the main site of cell damage, an effect that was present in both species but that probably occurs more quickly in *L. esculentum*. The observed cell damage suggested a mechanism that may be associated with apoptosis. The results suggest that abiotic stresses that have
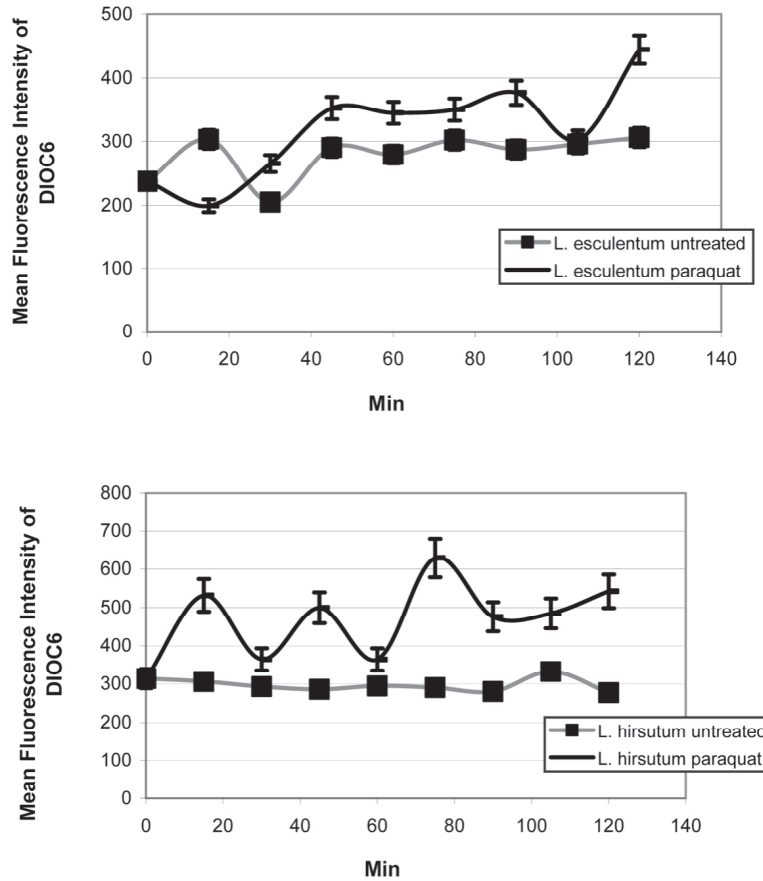
FIGURE 5 - Mean fluorescence intensity (MFI) of DiOC6 stain in protoplasts of *L. hirsutum* and *L. esculentum* during the first 2 h of treatment with 1% paraquat compared to that in untreated control protoplasts.

TABLE 2 - ROS production expressed as percentage of protoplasts with the presence of superoxide anion in *L. hisutum* and *L. esculentum* after the first 30 min of treatment with 1% paraquat

Treatment	Percentage of protoplasts with ROS presence	Coefficient of variation
L. hirsutum untreated	1.9	0.38
L. hirsutum paraquat treatment	32.9	2.18
L. esculentum untreated	2.0	0.57
L. esculentum paraquat treatment	25.4	2.18

no co-evolutionary relationships can induce the expression of mechanisms closely linked to an immune response.

The results of the paraquat treatments confirm that oxidative stress begins as an early event, but is not exclusive to photosystem I. The mitochondria also undergo early-stage alteration, which makes sense considering the similarity between the electron transport chain systems of chloroplasts and mitochondria. The high dihydroethydine oxidation observed suggests that ROS production plays a role in cell death, not only during late events but also during early events, and is a possible initiator of destructive processes or a participator in chemical signaling.

The dose-response assays confirmed that at the concentrations used in this study, copper did not affect membrane integrity, but may have a membrane stabilizing effect in the protoplasts of *L. hirsutum* and *L. esculentum*. The ROS production experiments suggest that the effect of copper on the cell has nothing to do with membrane damage or the production of ROS, but rather it causes mitochondrial damage by destabilizing the internal mitochondrial membrane. Although an adequate dose-response relationship

TABLE 3 - Membrane damage and production of reactive oxygen species in protoplasts of *Lycopersicon hirsutum* and *Lycopersicon esculentum* during 72 h of treatment with 1% paraquat

Damage	Treatment	Intercept	Slope	p-value Model y= a + bx
Percentage of protoplasts with membrane damage	*L. hirsutum* untreated	16.55	0.32	0.06
	L. hirsutum-Paraquat	9 42	0 84	0.05
	L. esculentum untreated	3.95	0.55	-
	L. esculentum- Paraquat	-23 7	8 7	-
Percentage of protoplasts with ROS presence in the form of superoxide anion	*L. hirsutum* untreated	27 18	0 31	0.11
	L. hirsutum-Paraquat	14 65	1 004	0.08
	L. esculentum untreated	6.7	0.05	0.32
	L. esculentum- Paraquat	38.5	0.5	0.24
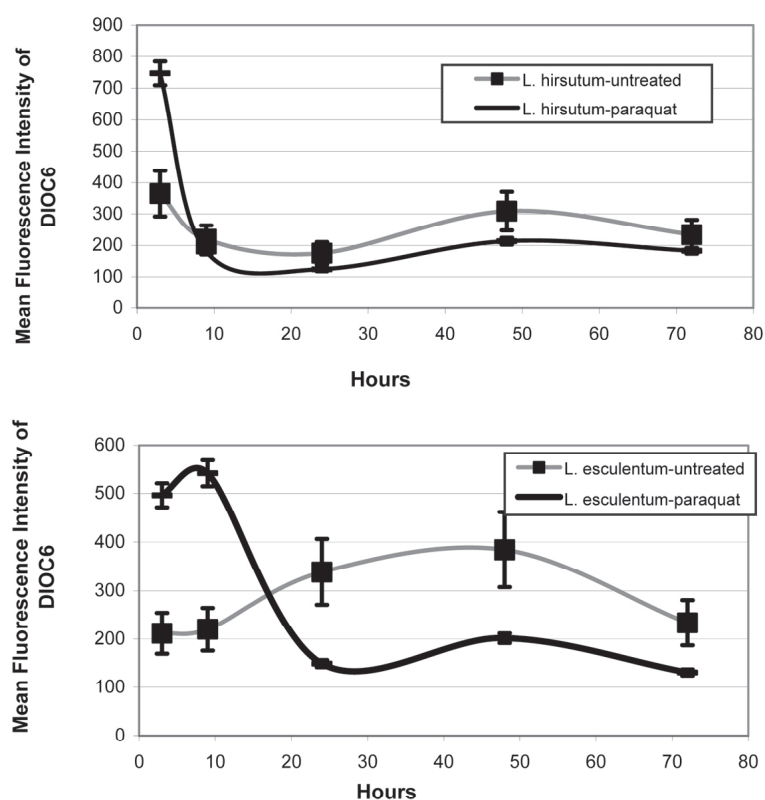
FIGURE 6 - Mean fluorescence intensity (MFI) of DiOC6 stain in protoplasts of *L. hirsutum* and *L. esculentum* during the 72 h of treatment with 1% paraquat compared to that in untreated control protoplasts.

could not be made for the paraquat treatments, significant membrane damage was observed. It is probable that the timing of the observations during the paraquat assays did not coincide with the initiation of membrane damage, and therefore a correlation with the dosage could not be made.

The tomato species used in this study exhibited differential behavior following the treatments. For example, the species *L. hirsutum* developed mitochondrial damage faster than *L. esculentum* during the copper treatment.

Moreover, a higher production of ROS and a greater change in mitochondrial membrane potential occurred as both early and late events during the paraquat treatment in *L. hirsutum*. These results showed that the two tomato species differed in their responses to copper and to paraquat. It would be interesting to do more studies targeted to evaluate the effect of stress induced by copper and Paraquat in tomato species on the differential expression of the hormonal balance (for example in the production of jasmonic acid, salicylic acid

and other organic acids) and ROS production, and how that differential expression is related to the apoptosis process as a defense response.

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