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BRIEF COMMUNICATION

Different responses of young and expanded lettuce leaves to fungicide Mancozeb: chlorophyll fluorescence, lipid peroxidation, pigments and proline content

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Abstract

This work aimed to study the effects of commercial doses of the fungicide, Mancozeb, on the photosynthetic apparatus of lettuce young leaves (YL) and expanded leaves (EL). Seven days after Mancozeb application, chlorophyll *a* fluorescence, pigment contents, lipid peroxidation, and proline content were evaluated. Independently of leaf age, Mancozeb treatment reduced the efficiency of photosystem II photochemistry, increased the nonphotochemical quenching and proline content, decreased pigment contents, and induced lipid peroxidation. Moreover, EL showed a more stable photosynthetic apparatus, less prone to oxidative damages compared with YL. The parameters measured proved to be good markers for the rapid and preliminary diagnosis of fungicide toxicity.

Additional key words: anthocyanins, dithiocarbamates, photosynthetic efficiency, toxicity.

The use of fungicides has become crucial for agriculture since fungal infections cause crop yield reductions of almost 20% worldwide (Gullino *et al.* 2010). Contact fungicides act preventively, while systemic fungicides can kill the fungus and stop the dispersal of the infection within the plant (Petit *et al.* 2012). Most of the work dealing with the impact of fungicides in agriculture is focused on their efficiency against fungal pathogens or their residual accumulation in crops (Dias 2012, Petit *et al.* 2012). However, the negative impact of fungicides on photosynthesis and oxidative stress is less explored and most of the studies were conducted only with systemic fungicides. The available studies indicate reductions in net CO_2 assimilation rate (P_N) and photosynthetic efficiency upon fungicide application (*e.g.* Xia *et al.* 2006, Petit *et al.* 2008). Fungicides seem to inhibit the biosynthesis of chlorophyll (Chl) and to retard Chl integration in the photosystems (Petit *et al.* 2012). Systemic fungicides have been observed to induce oxidative injury in plants (Gopi *et al.* 2007, Jaleel *et al.* 2007) and to increase plant antioxidant enzymes activities (Calatayud *et al.* 2001, Wu *et al.* 2002, Gopi *et al.* 2007, Jaleel *et al.* 2007). Moreover, the increase of the proline content seems to be important for reactive oxygen species (ROS) detoxification in plants exposed to systemic fungicides (Gopi *et al.* 2007, Jaleel *et al.* 2007).

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Abbreviations: C – control; Chl – chlorophyll; DM – dry mass; EL – expanded leaves; F_v/F_m – maximal efficiency of PSII; MDA – malondialdehyde; NPQ – nonphotochemical quenching; PSII – photosystem II; q_P – photochemical quenching; ROS – reactive oxygen species; YL – young leaves; Φ_{PSII} – effective quantum efficiency of PSII.
Authors' contributions: M.C. Dias and C. Santos contributed equally to the planning of the experiment. M.C. Dias and P. Figueiredo performed most of the experiments in the laboratory. M.C. Dias and C. Santos performed the statistical analyses, interpreted data, and wrote most of the manuscript. A. Gil and I.F. Duarte collaborated in the statistical analyses, data interpretation, and in the manuscript revision.

Mancozeb is one of the most extensively used, contact fungicides in the world on various crops, including lettuce. This is mainly due to its efficacy against a broad spectrum of fungi and to its low acute toxicity and environmental persistence (Gullino *et al.* 2010). However, the extensive use of fungicides generates long-term accumulation of residuals in food and in the environment (Dias 2012). In general, the overuse of fungicides and pesticides has justified their recent classification among the most challenging, emerging pollutants (*e.g*. Smital *et al.* 2004). To our knowledge, few scientific reports have been published concerning the effects of Mancozeb application. Lorenz and Cothren (1989) have applied Mancozeb to wheat plants and did not observe any changes in P_N and Chl content. However, Bremer and Bünemann (1982) reported that Mancozeb induced leaf spots/lesions and leaf drop in apple trees. Untiedt and Blanke (2004) showed that the application of a Mancozeb mixture with flusilazol and oxydemeton-methyl induced a P_N reduction and an increase in dark respiration in apple trees. Given the lack of information on the Mancozeb effects, this study aimed to investigate the effect of a commercial dose on photosynthetic efficiency, pigments, malondialdehyde (MDA), and proline content in lettuce. To our knowledge, this is the first report that focused on the use of Mancozeb in such a context.

Seeds of *Lactuca sativa* L. (cv. Queen of May) were germinated, then transferred to plastic pots with turf and vermiculite (2:1) and grown in a growth chamber at 20 ± 2ºC, with a 16/8-h (day/night) photoperiod and a photosynthetic photon flux density of 250 ± 20 μmol(photon) m^{-2} s^{-1}. After 4 weeks of growth, Mancozeb [manganese zinc ethylene bis(dithiocarbamate), *PESTANAL®*, *SIGMA*, Germany] was applied once as foliar spray at the concentration of 2 g L^{-1} (as commercially recommended) while the control (C) plants were sprayed with water on the foliage till run-off. Seven days after Mancozeb application, Chl fluorescence were measured in leaves that were completely developed (expanded leaves, EL) and in leaves that were not fully expanded (young leaves, YL). In addition, leaf samples were collected and frozen in liquid nitrogen for further quantification of pigments, MDA, and proline.

The Chl *a* fluorescence measurements were performed *in situ* with a portable fluorimeter *Mini-PAM* (*Walz*, Effeltrich, Germany) as described by Dias *et al.* (2013) and maximal efficiency of PSII (F_v/F_m), photochemical quenching (q_P), effective quantum efficiency of PSII (Φ_{PSII}), and nonphotochemical quenching (NPQ) were calculated as described by van Kooten and Snel (1990). Pigment contents were extracted with a acetone/Tris (50 mM pH 7.8) buffer (80:20, v:v) and measured by spectrophotometric absorption at 470, 537, 647 and 663 nm according to Sims and Gamon (2002). Anthocyanins were extracted with a methanol/HCl/water (90:1:1, v:v:v) buffer and measured by spectrophotometric absorption at 650 and 529 nm, according to Sims and Gamon (2002). Lipid peroxidation on leaves was evaluated by measuring MDA production as described by Dias *et al.* (2011). Free proline was extracted and determined as described by Khedr *et al.* (2003).

The experiment was repeated twice (two independent assays); for each experiment, 8 plants were used, 4 plants were maintained under control conditions and 4 plants were exposed to Mancozeb. Data collected from both experiments were analysed by one-way analysis of variance (*ANOVA*) using the *Sigma Stat* program for *Windows, version 3.1*. Comparisons between means were evaluated by a Post-Hoc test (*Tukey*'s) at a significant level $P = 0.05$. Data were presented as mean ± SD of two experiments.

Seven days after Mancozeb exposure, 100% of lettuce plants survived. YL exposed to Mancozeb, showed significant decreases in Chl *a*, Chl *b*, and carotenoid contents (by 27%, 25%, and 23%, respectively) in comparison with the C plants (Table 1). However, in EL, only Chl *a* decreased significantly by 18% (Table 1). No significant changes were observed in Chl *a*/*b* (Table 1). The anthocyanins content decreased upon Mancozeb exposure in both EL and YL (Table 1). EL exhibited higher contents of anthocyanins than YL. F_v/F_m was not significantly affected by Mancozeb exposure, whereas Φ_{PSII} significantly decreased in both EL and YL (Table 1). In the C plants, EL showed a higher q_P than EL exposed to Mancozeb (Table 1). The lowest NPQ was observed in YL of the C plants (Table 1). Mancozeb exposure induced a significant increase in MDA and proline content (Table 1).

Mancozeb showed the more pronounced, negative impact on Chl and carotenoids in YL. This might be due to the fact that pigment-protein complexes of the photosynthetic apparatus of YL were less stable, thus pigments might degrade more easily upon Mancozeb exposure. Moreover, pigment synthesis in YL might be also negatively affected by Mancozeb (Solymosi and Schoefs 2010), leading to a stronger reduction of their pigment content. The Chl and carotenoid contents were less affected by Mancozeb in EL, possibly because of their more stable photosynthetic apparatus with a lesser need for active biosynthetic routes. Garcia *et al.* (2002) and Saladin *et al.* (2003) also found a general decrease in pigment contents in grapevine treated with a nonsystemic fungicide (fludioxonil) and in tobacco treated with a systemic fungicide (carbendazim). Mancozeb did not affect F_v/F_m, thus indicating that irreversible photoinhibition did not occur in PSII reaction centres. In accordance, a significant rise of NPQ was observed in the Mancozeb-treated plants. These results showed that part of the excited energy in PSII antenna was dissipated as heat, in this way preventing photoihibitory damages. The evaluation of Φ_{PSII} revealed that the proportion of light absorbed by Chl associated with PSII and used in photochemistry was reduced by fungicide exposure. Moreover, fungicide application also decreased the proportion of the "open" state PSII centres (q_P) in EL. The Φ_{PSII} in these leaves declined mainly due to a closure of the reaction centres that resulted from light saturation and from the lower contents of Chl *a*. The reduction of

Table 1. Pigments, chlorophyll (Chl) *a* fluorescence parameters, malondialdehyde (MDA), and proline content in expanded and young leaves of control and Mancozeb-exposed lettuce plants. Values are means ± SD (n = 6, for proline and MDA, and n = 8 for the other parameters). *Different letters* indicate significance differences between treatments at a significant level equal to 0.05.

Parameters	Expanded leaves		Young leaves	
	Control	Mancozeb	Control	Mancozeb
Chl *a* [μg g^{-1}(FM)]	438 ± 14.4^{a}	357 ± 16.8^{c}	491 ± 49.2^{b}	356 ± 20.5^{c}
Chl *b* [μg g^{-1}(FM)]	172 ± 30.9^{ab}	154 ± 26.2^{ab}	181 ± 10.6^{a}	136 ± 17.2^{b}
Chl *a*/*b*	2.46 ± 0.39^{a}	2.43 ± 0.45^{a}	2.59 ± 0.28^{a}	2.27 ± 0.52^{a}
Carotenoids [μg g^{-1}(FM)]	187 ± 35.6^{ab}	170 ± 38.1^{b}	220 ± 26.2^{a}	171 ± 9.8^{b}
Anthocyanins [μg g^{-1}(FM)]	312 ± 3.0^{a}	290 ± 5.0^{b}	272 ± 2.0^{c}	251 ± 5.0^{d}
F_v/F_m	0.83 ± 0.02^{ab}	0.80 ± 0.03^{b}	0.85 ± 0.02^{a}	0.82 ± 0.03^{ab}
Φ_{PSII}	0.63 ± 0.04^{a}	0.44 ± 0.04^{b}	0.62 ± 0.04^{a}	0.48 ± 0.04^{b}
qP	1.24 ± 0.12^{a}	0.95 ± 0.15^{b}	1.09 ± 0.23^{ab}	1.08 ± 0.04^{ab}
NPQ	2.8 ± 0.10^{a}	3.20 ± 0.29^{a}	2.20 ± 0.33^{b}	2.90 ± 0.47^{a}
MDA [nmol g^{-1}(FM)]	14.2 ± 3.8^{c}	23.0 ± 1.7^{b}	19.2 ± 3.2^{cb}	32.3 ± 5.4^{a}
Proline [nmol g^{-1}(FM)]	12.3 ± 2.1^{b}	16.5 ± 1.7^{a}	6.5 ± 1.1^{c}	10.1 ± 2.0^{b}

Φ_{PSII} in the YL seemed to be related to the decline in Chl *a*. Petit *et al.* (2008) reported the reduction of Φ_{PSII} in grapevine after application of fludioxonil. Xia *et al.* (2006) found that the exposure of cucumber to systemic fungicides decreased Φ_{PSII} and F_v/F_m, due to the reduction in q_P.

The elevated MDA content in lettuce leaves suggested that Mancozeb induced oxidative damage, either by indirect ROS production or by inhibition of antioxidative enzymes. Furthermore, YL seemed to be more prone to oxidative damages. The lesser oxidative damages found in EL could be due to the high proline contents. Proline has been described to play an important role in membrane structure protection and also as a ROS scavenger (Gopi *et al.* 2007). The increase of proline contents was reported by Gopi *et al.* (2007) and Jaleel *et al.* (2007) in carrots and in Madagascar periwinkle plants treated with triazole fungicides. However, contrary to our results, these authors found a reduction in MDA content in fungicide-exposed plants. These differences might be attributed to the fact that these studies were conducted in different species, using other fungicides and possibly in leaves in different developing stages. We should also take into account that fungicide effects might also depend on treatment duration.

Several studies pinpointed that anthocyanins protect the photosynthetic apparatus from photoinhibition by absorbing light before it reaches PSII and they can also act as an efficient ROS quencher (Zhang *et al.* 2010). However, our present work showed that anthocyanins did not play a crucial role in lettuce stress protection.

Mancozeb reduced Φ_{PSII}, affected pigment contents, and induced oxidative damages. The general increase of NPQ in Mancozeb-exposed leaves might contribute to a higher photoprotection, avoiding photoinhibitory damages. The results demonstrated that EL were: (*1*) less affected by Mancozeb, possibly because of a more stable photosynthetic apparatus; and (*2*) less prone to oxidative damage, probably due to a greater capacity to undertake key metabolic adjustments to maintain a higher antioxidant protection. The parameters used in this work proved to be good markers for a rapid and preliminary diagnosis of fungicide toxicity, even when recommended doses were used. Further investigation should focus on the effects of Mancozeb on the cell metabolic pathways that are additionally targeted by this pesticide.

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