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Effect of the Herbicides Terbuthylazine and Glyphosate on Photosystem II Photochemistry of Young Olive (*Olea europaea*) Plants

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ABSTRACT: The purpose of this study was to understand the effect produced by the addition of the herbicides terbuthylazine (N^2-*tert*-butyl-6-chloro-N^4-ethyl-1,3,5-triazine-2,4-diamine) and glyphosate (*N*-(phosphonomethyl)glycine) on photosystem II photochemistry of young plants of *Olea europaea* L. under greenhouse conditions. The effect of soil amendment with an organic residue from olive oil production was also assessed. Terbuthylazine reduced the efficiency of photosystem II photochemistry of plants due to chronic photoinhibition, and this effect was counterbalanced by soil amendment with the organic waste, whereas the photosystem II photochemistry of olive plants was not affected by glyphosate or by glyphosate and organic waste addition. In this study, we have shown that the soil application of terbuthylazine is a source of indirect phytotoxicity for olive plants. We have also observed that the olive plants were not affected by higher amounts of glyphosate in the soil.

KEYWORDS: herbicide, *Olea europaea*, organic waste, chlorophyll fluorescence, photosynthesis

INTRODUCTION

The use of pesticides, especially herbicides, has brought to the agricultural sector great advantages during the last 50 years, but equally the negative aspects of their use are also recognized. Herbicides can damage nontarget plants such as the crop itself, and great economic losses can be realized.[1] Furthermore, the repetitive use of these compounds can exceed the buffering function of soil compartments becoming great sources of contamination of surface and groundwater.[2−5]

Despite their efficiency on target organisms, herbicides generate nonspecific phytotoxicity.[6] The reduction in the photosynthetic efficiency of plants as a consequence of stressing environmental factors such as herbicides has been observed in several studies.[7−9] Light energy absorbed by chlorophyll molecules in a leaf can undergo one of the three fates: it can be used to drive photosynthesis (photochemistry), excess energy can be dissipated as heat or it can be re-emitted as light-chlorophyll fluorescence. Hence, changes in the efficiency of photochemistry and heat dissipation can be obtained by measuring chlorophyll fluorescence of photosystem II (PSII),[10] and this can be detected in situ using a pulse amplitude modulation fluorimeter.[11] Chlorophyll and gas exchange measurements are nondestructive biomarkers of sublethal plant stress in environmental monitoring or in ecological risk assessment of herbicide exposure.[9,12−16]

Our group has previously shown that diuron and simazine, herbicides used in the past in olive crops and now banned in Spain, reduce the efficiency of photosystem II photochemistry (the ultrafast and ultraefficient light-induced charge separation and stabilization steps that occur when light is absorbed by chlorophyll[17] of seedlings and adult olive trees due to chronic photoinhibition.[15,16] In both cases, we have observed that the addition of an organic waste from olive oil production counterbalances this effect. The aim of this study was to investigate the effect of the herbicides terbuthylazine and glyphosate, both currently used in olive crops in Spain, on the photosynthetic apparatus (PSII chemistry) and gas exchange of three-year old olive trees, and to evaluate if this effect is influenced by soil amendment with an organic waste from olive oil production. These herbicides have a very different mode of action. Terbuthylazine (N^2-*tert*-butyl-6-chloro-N^4-ethyl-1,3,5-triazine-2,4-diamine) is a preemergence selective herbicide applied directly to the soil and mainly absorbed by roots. It inhibits the Hill reaction and CO_2 sorption in the chlorophyllic function.[18] Glyphosate (*N*-(phosphonomethyl)glycine) is a postemergence nonselective herbicide which inhibits 5-enolpyruvylshikimic acid-3-phosphate synthase, an intermediate enzyme in aromatic amino acid synthesis, via the shikimic acid pathway.[19] It is mainly absorbed by leaves, although some root absorption has also been reported.[20−22] Beside soil surface contamination, translocation of glyphosate from leaves to plant roots has been shown to be also responsible for glyphosate residues in the soil.[23] These herbicides also have very different soil sorption behavior,[24−27] and the effect of this soil process on photosystem II photochemistry will also be evaluated.

MATERIALS AND METHODS

Herbicides, Soil and Organic Waste. High purity (99%) standard terbuthylazine and glyphosate (98%) were used in sorption studies under laboratory conditions. Both herbicides were purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany). Terbuthylazine is a colorless powder with a water solubility of 8.5 mg L^{-1} at 20 °C, and glyphosate is an odorless white crystal very soluble in water (10 g L^{-1} at 20 °C) and practically insoluble in common organic solvents.[28]

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Table 1. Physicochemical Properties of Unamended (S) and Amended Soils at 5% Rate (S + 5% OW) and 10% Rate (S + 10% OW)

	soil		
parameter	S	S + 5% OW	S + 10% OW
organic carbon (%)	1.9	3.7	5.4
clay (%)	18.2	18.2	18.2
silt (%)	22.2	22.2	22.2
sand (%)	59.5	59.5	59.5
pH	7.4	7.1	6.7

Radiolabeled glyphosate (P-Methylene-14C) with specific activity 11.4 MBq mg^{-1}, supplied by IZOTOP (Budapest, Hungary), was also used to perform sorption studies under laboratory conditions. Commercial formulations of the herbicides were used for the studies with olive plants under glasshouse conditions: glyphosate (Glialka 36; 360 g of active ingredient L^{-1}, Presmar S.L., Spain) and terbuthylazine (CUNA; 50 g of active ingredient L^{-1}, Sipcam Inagra S.A., Spain).

The top 5 cm of a sandy soil from Southern Spain was sampled, air-dried and sieved to pass a 2 mm mesh. Physicochemical properties are given in Table 1. The organic carbon (OC) content of the soil was determined by dichromate oxidation,[29] and the pH was determined in a 1:2.5 (kg L^{-1}) soil/deionized water mixture. Soil texture was determined by sedimentation.

The oil olive-mill waste (OW) used is a residue from olive oil production obtained by a two-phase centrifugation process.[30,31] The organic matter content of this residue (determined by calcination) is 81%, and the pH is 6.7 (1:2 kg L^{-1} residue/deionized water mixture).

Herbicide Sorption Studies. Duplicate samples of 5 g of unamended soil and soil amended with OW at 5% and 10% (w/w) were treated separately with 10 mL of terbuthylazine and glyphosate solutions (initial concentrations, C_i, of 1, 5, 10, and 20 μM in 0.01 M $CaCl_2$). Previously, it was determined that equilibrium was reached in less than 24 h, and that no measurable degradation occurred during this period. The suspensions were centrifuged 10 min at 8000 rpm. In the case of terbuthylazine, the equilibrium concentrations (C_e) in the supernatants were determined by high performance liquid chromatography (HPLC) under the following conditions: Nova-Pack column, 150 mm length × 3.9 mm i.d.; column packing, C18; flow rate, 1 mL min^{-1}; eluent system water + acetonitrile (1 + 1 by volume) mixture and detection at 222 nm. In the case of glyphosate, C_e was determined by liquid scintillation counting (LSC). One milliliter aliquots were removed for analysis and mixed with 5 mL of scintillation cocktail. Differences between C_i and C_e were assumed to be the amounts adsorbed (C_s = μmol kg^{-1}). Sorption isotherms were fitted to the Freundlich equation: $C_s = K_f C_e^{1/n_f}$, and sorption coefficients K_f and $1/n_f$ calculated.

Plant Material and Treatments. *Olea europaea* L. trees (three years old) were grown in plastic pots (26 cm length × 15 cm diameter) filled with the sandy soil and placed in a glasshouse with minimum−maximum temperatures of 21−25 °C, 40−60% relative humidity and natural daylight (minimum and maximum light flux: 200 and 1000 μmol m^{-2} s^{-1}). Five different treatments were done in triplicate pots: Pots with the organic residue added to the soil at the rate of 10 Mg ha^{-1} (OW pots), pots with terbuthylazine added to the soil at the rate of 3 kg ha^{-1} (T pots), pots with glyphosate added to the soil at the rate of 3 kg ha^{-1} (G pots) and pots with terbuthylazine and OW (T + OW pots) or glyphosate and OW (G + OW pots). Triplicate pots without herbicide or organic waste were used as controls. Herbicides were applied to the top of the soils 24 h after the application of the OW.

The length of four branches and two leaves/branch of each plant were measured and statistically analyzed, so it was possible to form homogeneous biomass triplicates for the different treatments.

Measurement of Chlorophyll Fluorescence. Chlorophyll fluorescence was measured in the olive leaves using a portable modulated fluorimeter (FMS-2, Hansatech Instrument Ltd., England) after 24 h, 15 days and 60 days of treatment in order to know the physiological response in the short and long term. Redondo-Gómez et al.[15] reported that fluorescence parameters of olive trees were affected by herbicide treatments from week one, but Redondo-Gómez et al.[16] found that these parameters are not affected until two months of treatment. Light and dark-adapted fluorescence parameters were measured at dawn (between 1 and 7 μmol m^{-2} s^{-1}) and midday (1700 μmol m^{-2} s^{-1}) in order to determine if herbicide, OW or the combination of both affected the sensitivity of plants to photoinhibition.[10] Plants were dark-adapted for 30 min, using leaf-clips designed for this purpose. The minimal fluorescence level in the dark-adapted state (F_0) was measured using a modulated pulse (<0.05 μmol m^{-2} s^{-1} for 1.8 μs) too small to induce significant physiological changes in the plant. Maximal fluorescence in this state (F_m) was measured after applying a saturating actinic light pulse of 15000 μmol m^{-2} s^{-1} for 0.7 s. Values of the variable fluorescence ($F_v = F_m - F_0$) and maximum quantum efficiency of PSII photochemistry (F_v/F_m) were calculated from F_0 and F_m. The same leaf area of each plant was used to measure light-adapted parameters. Steady state fluorescence yield (F_s) was recorded after adapting plants to ambient light conditions for 30 min. A saturating actinic light pulse of 15000 μmol m^{-2} s^{-1} for 0.7 s was then used to produce the maximum fluorescence yield ($F_{m'}$) by temporarily inhibiting PSII photochemistry.

Using fluorescence parameters determined in both light- and dark-adapted states, the following were calculated: quantum efficiency of PSII ($\Phi_{PSII} = F_{m'} - F_s/F_{m'}$), and nonphotochemical quenching (NPQ = $F_m - F_{m'}/F_{m'}$).[32]

Chronic (PI_{chr}) and dynamic (PI_{dyn}) photoinhibition was calculated according to Werner et al.[33] as follows: $PI_{chr} = [(F_v/F_m)_{max} - (F_v/F_m)_d/(F_v/F_m)_{max}] \times 100$; $PI_{dyn} = \{[(F_v/F_m)_d - (F_v/F_m)_{mid}]/(F_v/F_m)_{max}\} \times 100$; where $(F_v/F_m)_d$ and $(F_v/F_m)_{mid}$ are dawn and midday F_v/F_m values, respectively. $(F_v/F_m)_{max}$ is maximum F_v/F_m value, which was calculated as the average of dawn measurements at different times and treatments.

Measurement of Gas Exchange. Gas exchange analysis was made using an open system (LI-6400, LI-COR Inc., Lincoln, NE, USA) after 24 h, 15 days and 60 days of treatment. Net photosynthetic rate (A), stomatal conductance to CO_2 (G_s) and intercellular CO_2 concentration (C_{ic}) were determined at an ambient CO_2 concentration of 360 μmol mol^{-1}, temperature of 25/28 °C, 50 ± 5% relative humidity and a photon flux density of 1000 μmol m^{-2} s^{-1}. The values for A, G_s and C_{ic} were calculated using standard formulas from Von Caemmerer and Farquar.[34]The photosynthetic area was calculated after painting the surface of each leaf over graph paper.

Statistical Analysis. Statistical analysis was carried out using Statistica v. 6.0 (Statsoft Inc., Tulsa, OK, USA). Data was analyzed using one- and two-way analysis of variance (ANOVA; F-test). Significant test results were followed by Tukey test for identification of important contrasts.

RESULTS

Sorption Studies. Terbuthylazine and glyphosate sorption isotherms are shown in Figure 1, and sorption coefficients for each herbicide on original soil (S) and soils amended with OW at 5% and 10% (w/w) (S + 5% and S + 10%) are given in Table 2. Glyphosate adsorbs on soil to a much higher extent than terbuthylazine, the K_f values of which were increased by a factor of 1.43 and 7.96, when soils were amended with 5% and 10% OW,

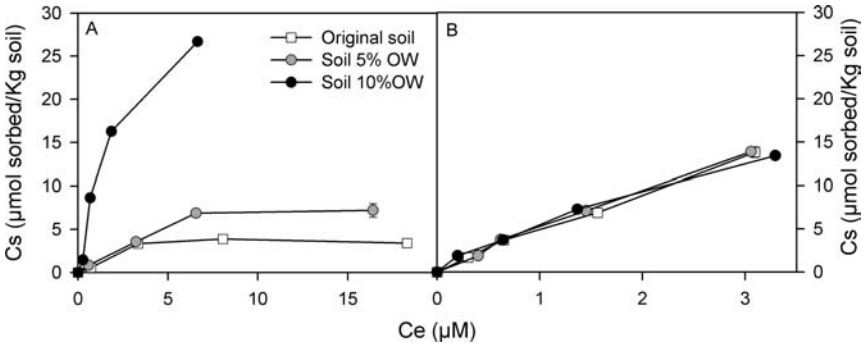

Figure 1. Terbuthylazine (A) and glyphosate (B) adsorption isotherms in soil unamended (white, A and B) and amended at 5% (gray, A and B) and 10% with OW (black, A and B).

Table 2. Terbuthylazine and Glyphosate Sorption Coefficients of Unamended (S) and Amended Soils at 5% Rate (S + 5% OW) and 10% Rate (S + 10% OW)

herbicide	soils	K_f ($mg^{1-1/n_f} kg^{-1} mL^{1/n_f}$)	$1/n_f$	R^2
terbuthylazine	S	0.964 (1.490 − 0.622)[a]	0.57 ± 0.23[b]	0.85
	S + 5% OW	1.386 (1.763 − 1.089)	0.69 ± 0.13	0.93
	S + 10% OW	7.680 (9.680 − 5.344)	0.86 ± 0.25	0.89
glyphosate	S	5.130 (5.496 − 4.798)	0.83 ± 0.09	0.99
	S + 5% OW	5.440 (5.640 − 5.288)	0.81 ± 0.04	0.99
	S + 10% OW	5.411 (5.633 − 5.190)	0.78 ± 0.05	0.99

[a] Numbers in parentheses are standard errors (SE) about the mean K_f. [b] Numbers are mean $1/n_f$ ± SE.

respectively. No significant increase in sorption upon amendment was found in the case of glyphosate.

Chlorophyll Fluorescence. Maximum quantum efficiency of PSII photochemistry (F_v/F_m), quantum efficiency of PSII (Φ_{PSII}) and nonphotochemical quenching (NPQ) for plants from control, OW, T, G, T + OW and G + OW pots 24 h, 15 days and 60 days after herbicide application are shown in Figure 2. No differences between treatments were observed for F_v/F_m values at midday 24 h or 15 days after herbicide treatment, but 60 days after treatment with terbuthylazine plants recorded the lowest F_v/F_m values at midday (0.60 respect 0.80 for the control; ANOVA, $P < 0.0001$; Figure 2A−C). This trend persisted at dawn, as Figure 3 shows (ANOVA, $P < 0.0001$). Data corresponding of F_v/F_m at dawn after 24 and 15 days of treatment are not shown since no significant differences were observed (ANOVA, $P > 0.05$). Pots treated with terbuthylazine and glyphosate showed lower Φ_{PSII} values 15 days after herbicide treatment (Figure 2E), although significant differences were not recorded (ANOVA, $P > 0.05$). Nonetheless, Φ_{PSII} for T-treated pots was significantly lower than for the control after 60 days (ANOVA, $P > 0.01$; Figure 2F). A similar trend was recorded for NPQ (Figure 2G−I). Chronic and dynamic damage for the different treatments is shown in Figure 4. T-treated pots showed the highest chronic photoinhibition, but, when terbuthylazine was amended with OW (T + OW), chronic damage was markedly lower. In contrast, G and G + OW treatments showed chronic photoinhibition similar to that of the control.

Gas Exchange. Plants from T-treated pots recorded lower values of net photosynthetic rate (A) than the control 15 days after herbicide treatment (ANOVA, $P < 0.05$); and the lowest values after 60 days (ANOVA, $P < 0.0001$) (Figure 5B,C). These differences were not observed when OW was added. Although stomatal conductance (G_s) of plants from T-treated pots was significantly lower than that of the control 15 days after herbicide treatment, it became similar to that of the control at the end of the experiment. The addition of OW increased G_s values in both cases (Figure 5B,C). Addition of terbuthylazine reduced intercellular CO_2 concentration (C_{ic}) after 15 days, while no significant differences were found in the case of T + OW. After 60 days, an increase was observed in both treatments (Figure 5H,I).

For G-treated pots no significant differences were found for A, G_s and C_{ic} values during the experiment, but in G+OW-treated pots a decrease in both G_s and C_{ic} values after 15 days was found, effect which disappeared at the end of the study.

DISCUSSION

The lower values of F_v/F_m for terbuthylazine treated plants at midday after 60 days of experiment (Figure 2C) are due to photoinhibition of olive plants at high light flux, which gives rise to a lower proportion of open reaction centers,[10] as observed in other studies with herbicides.[8] This photoinhibition is caused by damage in photosynthetic components, as of chlorophyll molecules of photosystem II, and this effect can be short-term and reversible (dynamic photoinhibition) or long-term and irreversible, persisting at a dawn measurement (chronic photoinhibition).[10,33] As no recovery was found at dawn in the case of pots treated with terbuthylazine (Figure 3), we can conclude that these plants expressed a chronic photoinhibition at long-term (Figure 4), which can be explained by the mode of action of terbuthylazine, which inhibits the flux of electrons in PSII in plants.[18] It could be expected that T + OW treatment also demonstrated a negative effect due to the addition of the herbicide, but no lower values in this parameter were found. The addition of OW to soil greatly increases terbuthylazine sorption (Table 2; Figure 1A), which has been shown to be influenced by an increase in organic matter.[26,35−37] This increase in sorption makes terbuthylazine less available to be absorbed by plants, reducing or avoiding the phytotoxic effect of the herbicide. Similar results were found by Redondo-Gómez et al.[15,16] in studies with olive plants and other triazine herbicides. These results suggest that, although terbuthylazine addition to soils could affect negatively photochemistry of olive plant, this effect could be reduced by the use of OW as alperujo in soils. The addition of this waste to the soil does not affect herbicide biological activity[38] and improves soil psychochemical properties.[30] Furthermore, it is easily accessible since it is generated close to olive crops.

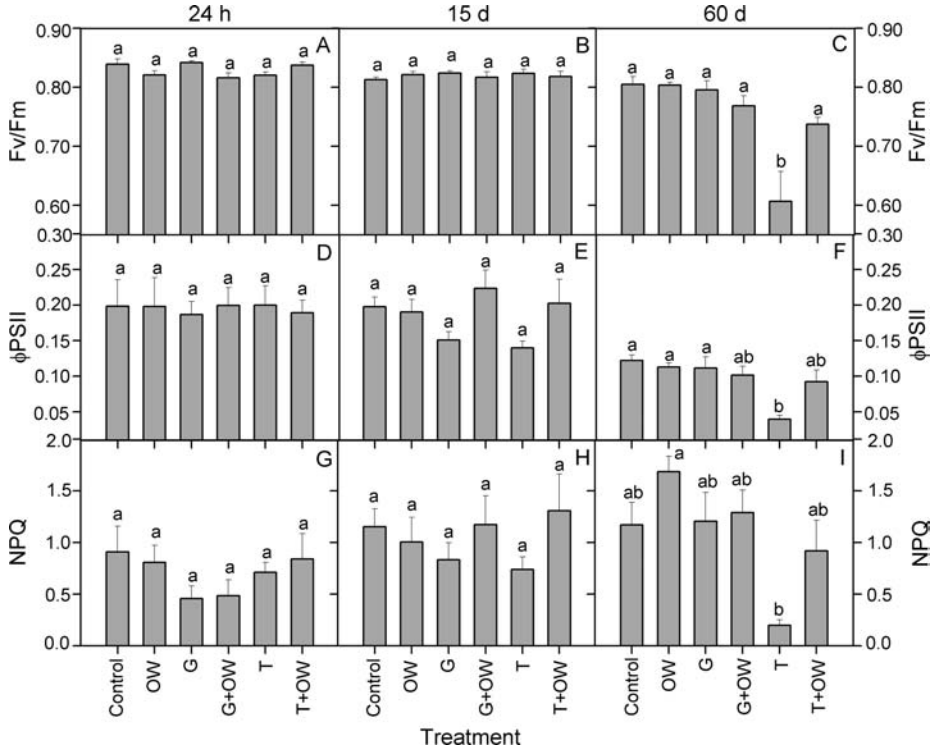

Figure 2. Maximum quantum efficiency of PSII photochemistry, F_v/F_m (A−C), quantum efficiency of PSII, Φ_{PSII} (D−F), and nonphotochemical quenching, NPQ (G−I) at midday in *Olea europaea* treated with solid olive-mill organic waste (OW), glyphosate (G), and both of them (G + OW), terbuthylazine (T) and T + OW after 24 h (A, D, G); 15 days (B, E, H); and 60 days (C, F, I). Values represent mean ± SE, $n = 9$. Different letters indicate means that are significantly different from each other (Tukey test, $P < 0.05$).

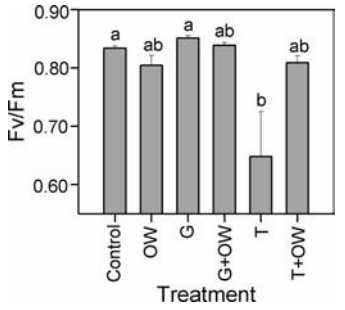

Figure 3. Maximum quantum efficiency of PSII photochemistry (F_v/F_m) at dawn in *Olea europaea* treated with solid olive-mill organic waste (OW), glyphosate (G), and both of them (G + OW), terbuthylazine (T) and T + OW after 60 days. Values represent mean ± SE, $n = 9$. Different letters indicate means that are significantly different from each other (Tukey test, $P < 0.05$).

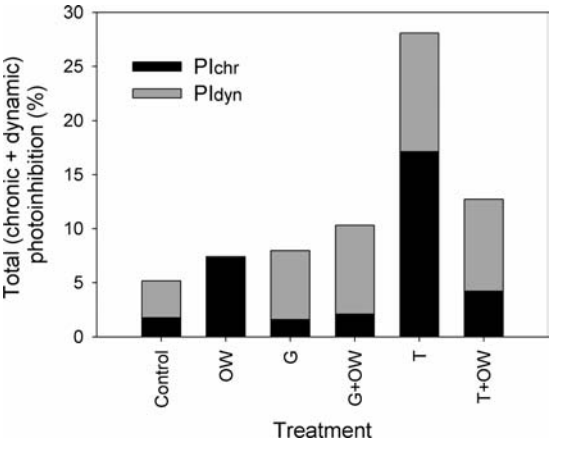

Figure 4. Chronic (filled bars) and dynamic (shaded bars) photoinhibition of adult leaves of *Olea europaea* treated with solid olive-mill organic waste (OW), glyphosate (G), and both of them (G + OW), terbuthylazine (T) and T + OW after 60 days of treatment.

In the case of glyphosate, which competes for the same sorption sites in soil as phosphorus,[39] adsorption occurs on the mineral phase of the soils (mainly on variable clay minerals), being strongly adsorbed by these soil components and becoming almost immobile in the soil profile.[24,25] The increase in soil organic matter upon amendment with the organic residue OW did not affect glyphosate sorption (Table 2; Figure 1B). However, the higher values of K_f for glyphosate in the original unamended soil compared to terbuthylazine indicates that this strong sorption, together with the low amount adsorbed by roots,[20−22] makes glyphosate unavailable to be absorbed by plants, and, consequently, no effects on photochemistry of olives plants are observed (Figure 2). Also the mode of action of this herbicide accounts for the results observed. Although glyphosate has been shown to inhibit δ-aminolevulinic acid synthesis in other crops blocking the synthesis of chlorophyll and other porphyrins,[40] its main mode of action is to inhibit of the enzyme 5-enolpyruvylshikimate-3phosphate synthase, which is not directly related to photosynthesis.[19]

Dissipation of energy as heat (NPQ) is a protecting mechanism in plants, which usually increases when F_v/F_m and Φ_{PSII}

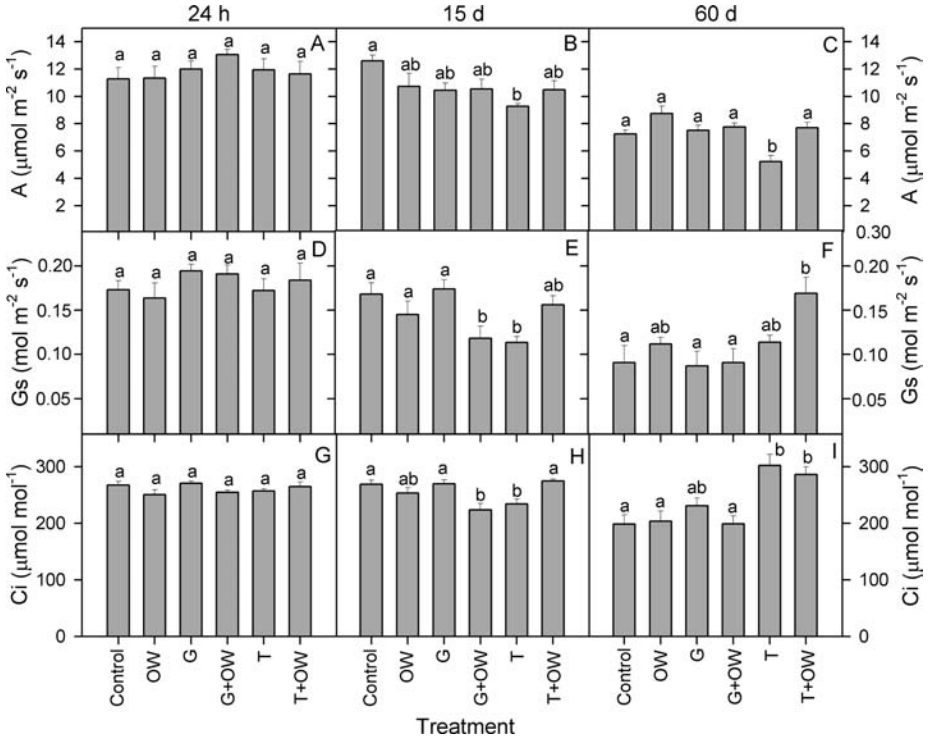

Figure 5. Net photosynthetic rate, A (A−C), stomatal conductance, G_s (D−F), and intercellular CO_2 concentration, C_{ic} (G−I) in *Olea europaea* treated with solid olive-mill organic waste (OW), glyphosate (G), and both of them (G + OW), terbuthylazine (T) and T + OW after 24 h (A, D, G); 15 days (B, E, H); and 60 days (C, F, I). Values represent mean ± SE, $n = 9$. Different letters indicate means that are significantly different from each other (Tukey test, $P < 0.05$).

decrease.[10] This was not observed in this study for terbuthylazine treated plants 60 days after treatment. This could be a consequence of the chronic damage that plants from this treatment suffered. These results do not agree with previous work with olive plants,[15,16] while they do agree with the low NPQ values found by Macinnis-Ng and Ralph,[7] in studies with diuron on the seagrass *Zoostera capricorni*.

Young olive trees showed an early expression of phytotoxic effect 15 days after terbuthylazine treatment, which became statistically significant 60 days after treatment. A significant reduction in values of Φ_{PSII} after 60 days of treatment with terbuthylazine was observed in T pots when compared to plants from the control pots (Figure 2E,F). Redondo-Gómez et al.[16] found that F_v/F_m and Φ_{PSII} were affected after one week of treatment of young olive plants with diuron and simazine, while in a similar study with adult trees, this effect was not observed until 2 months after herbicide application (Redondo-Gómez et al.),[15] as occurred in our study. The addition of OW to terbuthylazine treated plants counterbalanced the reduction of F_v/F_m and Φ_{PSII} parameters since, as described above, this OW makes terbuthylazine less available to be absorbed by plants.

In relation to gas exchange parameters, there was a very clear effect in olives treated with terbuthylazine but not in plants treated with glyphosate. The decline of net photosynthetic rate (A) may be attributed to stomatal and/or nonstomatal limitations.[41] If this reduction is produced by nonstomatal limitations, biochemical changes causing inhibition of chlorophyll synthesis would be observed. This cannot be proved in our study since no pigment studies were performed. In the case of stomatal limitations of A by G_s reduction, a decrease in intercellular concentration of CO_2 would be observed, which occurred 15 days after terbuthylazine treatment, but not at the end of the 60 day experiment. Our hypothesis is that stomatal limitations occur shortly after herbicide treatment affecting the synthesis of chlorophyll molecules after longer exposure.[41] Values for photosynthesis rate confirmed that the addition of OW reduces the negative effect generated by terbuthylazine exposure, due to the increase of sorption, since no significant decrease was found after 60 days in T + OW pots.

The use of safe and effective pesticides for crops, without developing indirect effects, is crucial for agronomists and farmers. In this study, we show that soil application of terbuthylazine is a source of indirect phytotoxicity for olive plants, reducing its photosynthetic efficiency, which was counterbalanced by the addition of OW to soils, increasing greatly the sorption of the herbicide to the soil particles and becoming less available to interact and be absorbed by the plant. Results obtained can be useful in preparing management strategies concerning herbicide use in olive crops.

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ABBREVIATIONS USED

A, net photosynthetic rate; C_e, equilibrium concentration; C_i, initial concentration; C_{ic}, intercellular CO_2 concentration; C_s, amount sorbed; F_0, minimal fluorescence level in the dark-adapted state; F_m, maximal fluorescence level in the dark-adapted state; F_v/F_m, maximum quantum efficiency of PSII photochemistry; Φ_{PSII}, quantum efficiency of PSII; G_s, stomatal conductance; K_f, sorption coefficient; NPQ, nonphotochemical quenching

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