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The influence of 2,4-D and dicamba on the physiology of olive seedlings

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Abstract – The objective of this work was to evaluate the influence of herbicides 2,4-D and dicamba on the physiological response of chlorophyll a in olive (*Olea europaea*) seedlings. The following eight rates of the 2,4-D (670 g a.e. ha^{-1}) and dicamba (720 g a.e. ha^{-1}) herbicides were applied: 0, 1.56, 3.13, 6.25, 12, 25, 50, and 100% of the recommended for burndown. The herbicides were applied at 80 cm above the seedlings using a CO_2 pressurized back sprayer with a 150 L ha^{-1} spray volume. Plant gas exchange was measured using a portable infrared gas analyzer. Chlorophyll a fluorescence was evaluated using a portable modulated light fluorometer. The application of both herbicides caused a decrease in the process of CO_2 assimilation in the plants, reducing net photosynthesis and stomatal conductance. The 2,4-D herbicide caused the most severe effects on the variables related to chlorophyll a fluorescence. Low values of maximum quantic yield were observed after the application of the herbicides, of which dicamba was the most harmful. Both herbicides damage the photosynthetic apparatus of olive seedlings.

Index terms: *Olea europaea*, conductance, herbicide, net photosynthesis.

A influência de 2,4-D e dicamba na fisiologia de mudas de oliveira

Resumo – O objetivo deste trabalho foi avaliar a influência dos herbicidas 2,4-D e dicamba sobre a resposta fisiológica da clorofila a em mudas de oliveira (*Olea europaea*). As seguintes oito doses dos herbicidas 2,4-D (670 g a.e. ha^{-1}) e dicamba (720 g a.e. ha^{-1}) foram aplicadas: 0, 1,56, 3,13, 6,25, 12, 25, 50 e 100% das recomendadas para dessecação. Os herbicidas foram aplicados a 80 cm acima das mudas, por meio de um pulverizador costal pressurizado a CO_2, com 150 L ha^{-1} de volume de calda. A troca gasosa das plantas foi medida por meio de um medidor portátil analisador de gases por infravermelho. A fluorescência da clorofila a foi avaliada por meio de um fluorômetro portátil de luz modulada. A aplicação de ambos os herbicidas causou diminuição no processo de assimilação de CO_2 pelas plantas, o que reduziu a fotossíntese líquida e a condutância estomática. O herbicida 2,4-D causou os efeitos mais severos nas variáveis relacionadas à fluorescência da clorofila a. Baixos valores de rendimento quântico máximo foram observados após a aplicação dos herbicidas, dos quais o dicamba foi o mais prejudicial. Ambos os herbicidas danificam o aparelho fotossintético das mudas de oliveira.

Termos para indexação: *Olea europaea*, condutância, herbicida, fotossíntese líquida.

Introduction

Fruit of olive trees [*Olea europaea* L., Oleaceae family] can be consumed pickled or as olive oil (Cavalheiro et al., 2014). In the 2020/2021 crop season, Brazil imported about 130,000 tonnes of table olives, and approximately 100,000 tonnes of olive oil (IOC, 2023). This demand is related to nutritional characteristics of the olive oil, which is rich in unsaturated fatty acids, vitamins, and phenolic compounds (El Riachy et al., 2011).

The 2,4-D and dicamba have the same mechanism of action as herbicides (Group 4 of the Herbicide Resistance Action Committee – HRAC), but they differ for chemical groups (Alves et al., 2021). These herbicides show differences for their physiological and biochemical effects on different plant species (Patton et al., 2018). The use of these auxin-mimicking herbicides in grain crops has been a problem for orchards, which is caused by drifting, since part of the applied product deviates from its main target and reaches adjacent areas (Godinho Júnior et al., 2017). This usually occurs in hot days, or low relative humidity of the air, or wind speed above 6 km h^{-1}. The drifting problem in trees is frequent, especially after applying auxin-mimicking herbicides, such as 2,4-D and dicamba (Avila Neto et al., 2022). These herbicides are used in grain crops for burndown and weeding, especially the glyphosate and 2,4-D (Robinson et al., 2012). Auxin causes growth anomalies in plants with sensitive broadleaves (Grossmann, 2010). The most common symptoms are branch epinasty and leaf cupping, which can lead to plant death.

Injuries due to low rates of 2.4-D and dicamba have been reported in sensitive species such as pecan trees (Wells et al., 2019), 'Ponkan' mandarin seedlings (Brochado et al., 2022), and tomato (Warmund et al., 2022). Few studies evaluated the impacts of these herbicides on the yield and photosynthetic physiology of plants (Brochado et al., 2022). There is a report of drifting of rates as low as 10 g of active ingredient equivalent per hectare (g a.e. ha^{-1}) of dicamba and 2,4-D causing reduction of yield and seed quality in soybean, during the R2 and V5 phenological stages (Silva et al., 2018). Dicamba drifting was observed as reaching areas 250 m away, although it does not usually exceed 20 m (Soltani et al., 2020). In the state of Nebraska, in the USA, a survey found out that dicamba applications carried out in the hottest months had more than 50% chance to cause injury in nontarget areas (Werle et al., 2018).

The problem of herbicide drifting in southern Brazil is very recurrent (Hupffer et al., 2020). In the 2019/2020 crop season, plant material was collected from orchards in Rio Grande do Sul state, in order to analyze the presence of pesticides. The results showed the presence of 2,4-D (Pinto, 2020a). In the 2020/2021 crop season, the Secretaria de Agricultura, Pecuária, e Desenvolvimento Rural (SEAPDR) fined approximately 600 producers, professionals, and companies for misusing auxin-mimicking herbicides (Pinto, 2020b).

Although there are regulatory instructions on the use of auxin-mimicking herbicides (IN 05/2019) (Rio Grande do Sul, 2019), drifting has been causing problems in orchards in the state of Rio Grande do Sul, Brazil.

The objective of this work was to evaluate the influence of herbicides 2,4-D and dicamba on the physiological response of chlorophyll a in olive seedlings.

Materials and methods

The experiment was carried out in a greenhouse, at the Universidade Federal de Santa Maria (UFSM), from July 2019 to February 2020. 'Koroneiki' olive seedlings of 50 cm height were used. They were transplanted into plastic pots of 7 L containing properly sieved and fertilized Argissoil soil. The fertilization followed recommendations for the olive culture and it was performed as follows: rates equivalent to 100, 60, and 90 kg ha^{-1} of N, P, and K, respectively (3.5 g N, 2.1 g P, and 3.2 g K, per pot). Pots were kept in the greenhouse for 60 days, where plants reached average 80 cm height of and 4 mm stem diameter.

A dry soil was weighed, and saturated with water up to 100%. After three days, the saturated soil was weighed again and properly adjusted, to reach 75% of water retention capacity – WRC (Kämpf et al., 2006), using the following equation:

$$W_{75\%} = (W_{WRC} - W_{dried}) \times 0.40 + W_{dried}$$

where: $W_{75\%}$ is the pot weight at 75% of WRC; W_{WRC} is the pot weight at WRC (three days after satured soil); W_{dried} is the pot weight with dry soil.

Irrigation was carried out daily to keep the pots at 75% WRC. In order to do that, each pot was weighed

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daily and water was added until it reached the $W_{75\%}$, if necessary. Every month, urea was added to each pot at an equivalent rate of 100 kg ha^{-1} N.

The experiment was conducted in a completely randomized design with five replicates. Treatments were applied in a 2x8 factorial arrangement, with "herbicide" and "rates" as factors, respectively. The factor "herbicide" was composed of 2,4-D and dicamba. The factor "rates" tested eight levels of each herbicide at 0, 1.56, 3.13, 6.25, 12.5, 25, 50, and 100% of the recommended rates for burndown, that is, 670 g a.e. ha^{-1} of 2,4-D and 720 g a.e. ha^{-1} of dicamba. The lower rates simulate the occurrence of herbicide drifting. The experimental unit was the 7 L pots with one olive seedling each. The herbicide was applied using a CO_2 pressurized backpack sprayer connected to a 2 m bar containing four XR Teejet 110.015 tips, at 1.76 kgf cm^{-2} pressure, which resulted in 150 L ha^{-1} application. The application was made outside the greenhouse. The environment conditions at the moment of the application were: 22.2°C temperature, 67% relative humidity, and 2.5 m s^{-1} wind speed.

After the herbicide application, the pots were placed outside the greenhouse for 24 hours at 10 m distance from each other, to avoid phytotoxicity due to herbicide volatility. After the period of 24 hours, they were brought back into the greenhouse. The following variables were analyzed: gas exchange and chlorophyll a fluorescence. Fluorescence was measured at 24, 48, and 72 hours after application (HAA). Gas exchange was evaluated 30 days after application (DAA). Gas exchange was measured for the following parameters: net photosynthesis (Np); CO_2 net assimilation rate (µmol CO_2 m^{-2} s^{-1}); stomatal conductance of water vapors (Gs) (mol H_2O m^{-2} s^{-1}); CO_2 intercellular concentration (Ci) (µmol CO_2 air mol^{-1}); transpiration rate (Tr) (mol H_2O m^{-2} s^{-1}); and water use efficiency (WUE) [mol CO_2 (mol $H_2O)^{-1}$]. WUE was determined according to the ratio between Np and Tr. A LI-COR portable infrared gas analyzer (IRGA), model LI-6400 XT, was used. The measurements were carried out using the last completely expanded leaf of the main stem. The photosynthetic radiation of 2,000 µmol m^{-2} s^{-1} and CO_2 concentration of 400 µmol mol^{-1}, inside the evaluation chamber of the IRGA's equipment, were previously determined by increasing curves in which a higher photosynthetic rate was observed (data not shown).

Fluorescence emission of chlorophyll a was analyzed using a portable light-modulated fluorometer (Junior-Pam Chlorophyll Fluorometer, Heinz Walz, Germany). Leaves were previously adapted to the dark for 30 min, in order to measure the initial fluorescence (F_0). After adaptation, a pulse of saturating light (10,000 µmol m^{-2} s^{-1}) was applied for 0.6 s to determine the maximum fluorescence (Fm), the maximum quantic yield (MQY) of the photosystem II (PSII), and the variable fluorescence (Fv).

Data were analyzed for normality and homoscedasticity, using the Shapiro-Wilk's test and the O'neill-Matthews' test, respectively. A factorial analysis of variance (α=0.05) was carried out using the statistical software R (R Core Team, 2022), and the ExpDes package (Ferreira et al., 2011). Once statistical significance was found, the means were compared, using the Scott-Knott's test (α=0.05).

Results and discussions

All variables met the statistical assumptions tests. Significant relationships were observed as the following descriptions: between rates and net photosynthesis (Np), and between herbicides and Np, in the analyses performed at 24, 48, 72 HAA (Figure 1 A, B, and C); between dose and stomatal conductance of water vapors (Gs), and between herbicides and Gs, for the analysis performed at 48 HAA (Figure 1 D); between rates and internal CO_2 concentration (Ci), in the analysis performed at 24 HAA (Figure 2 F); and between herbicides and internal CO_2 concentration (Ci), in the analyses performed at 24 HAA (Figure 3A) and 48 HAA (Figure 3 B).

There were also significant relationships between rates and initial fluorescence (F_0), in the analysis at 24 HAA (Figure 4 A), and between herbicides and F_0 in the analyses at 24 HAA (Figure 3 C) and 48 HAA (Figure 3 D). Significant relationships were also observed between rates and variable fluorescence (Fv), in the analysis at 24 HAA (Figure 4 B), and between herbicides and Fv, in the analysis at 24 HAA (Figure 3 E).

Significant relationships were also found between herbicides and maximum fluorescence (Fm), in the analysis at 48 HAA (Figure 3 F); and between herbicides and maximum quantum yield (MQY) of photosystem II, in the analyses at 24 HAA (Figure 3 G) and 48 HAA (Figure 3 H).

For Np, the evaluation carried out at 24 HAA showed a general increase of the values for both herbicides, according to the increase of their dose compared to the zero dose (Figure 1 A). The application of both herbicides resulted in decreases of the Np values, in comparison with the control, in the evaluation carried out at 48 HAA, and such decreases were more pronounced for the herbicide 2,4-D (Figure 1 B). In the other evaluations, the same decrease pattern was maintained for the herbicide 2,4-D.

The evaluation carried out at 48 HAA showed that plants that received rates above 12.5% of the herbicide 2,4-D had Gs values decreased by about 60%, in comparison to the controls, with no such effect when dicamba was applied (Figure 1 D). There was a simple dose effect on the evaluation carried out at 72 HAA. Regardless of the herbicide applied, rates from 6.25% caused stress to the plants, decreasing the stomatal conductance values (Figure 4 A). The WUE showed a simple dose effect in the evaluation carried out at 24 HAA (Figure 2 E), with a decrease in its values

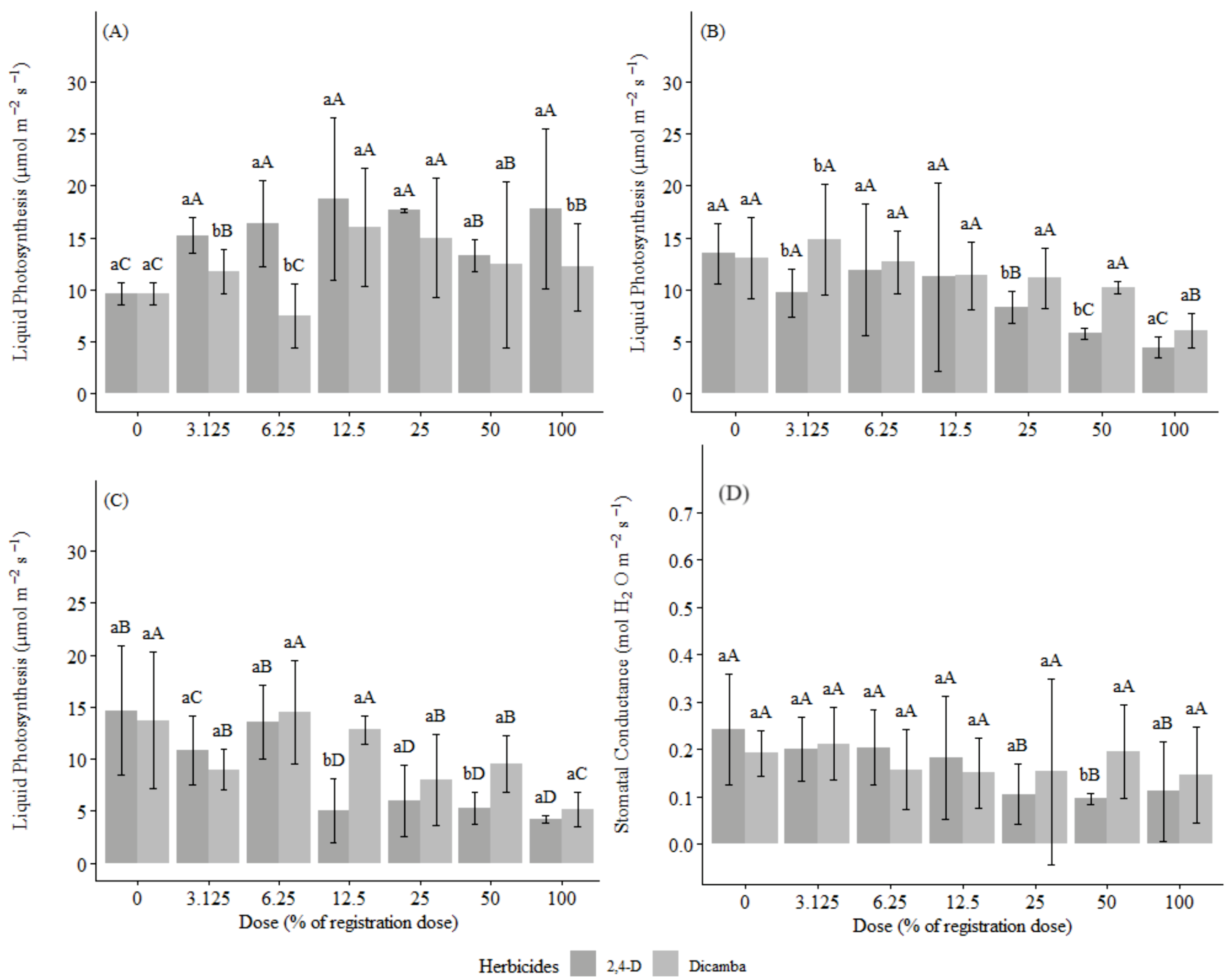

Figure 1. Net photosynthesis of olive (*Olea europaea*) seedlings measured at the following times after treatment: (A) 24, (B) 48, (C) 72 hours. Stomatal conductance measured at 48 hours after treatment (D) of 2,4-D and dicamba. Uppercase letters indicate compared rates of each herbicide, and lowercase letters indicate compared herbicides, by Scott-Knott's test, at 5% probability.

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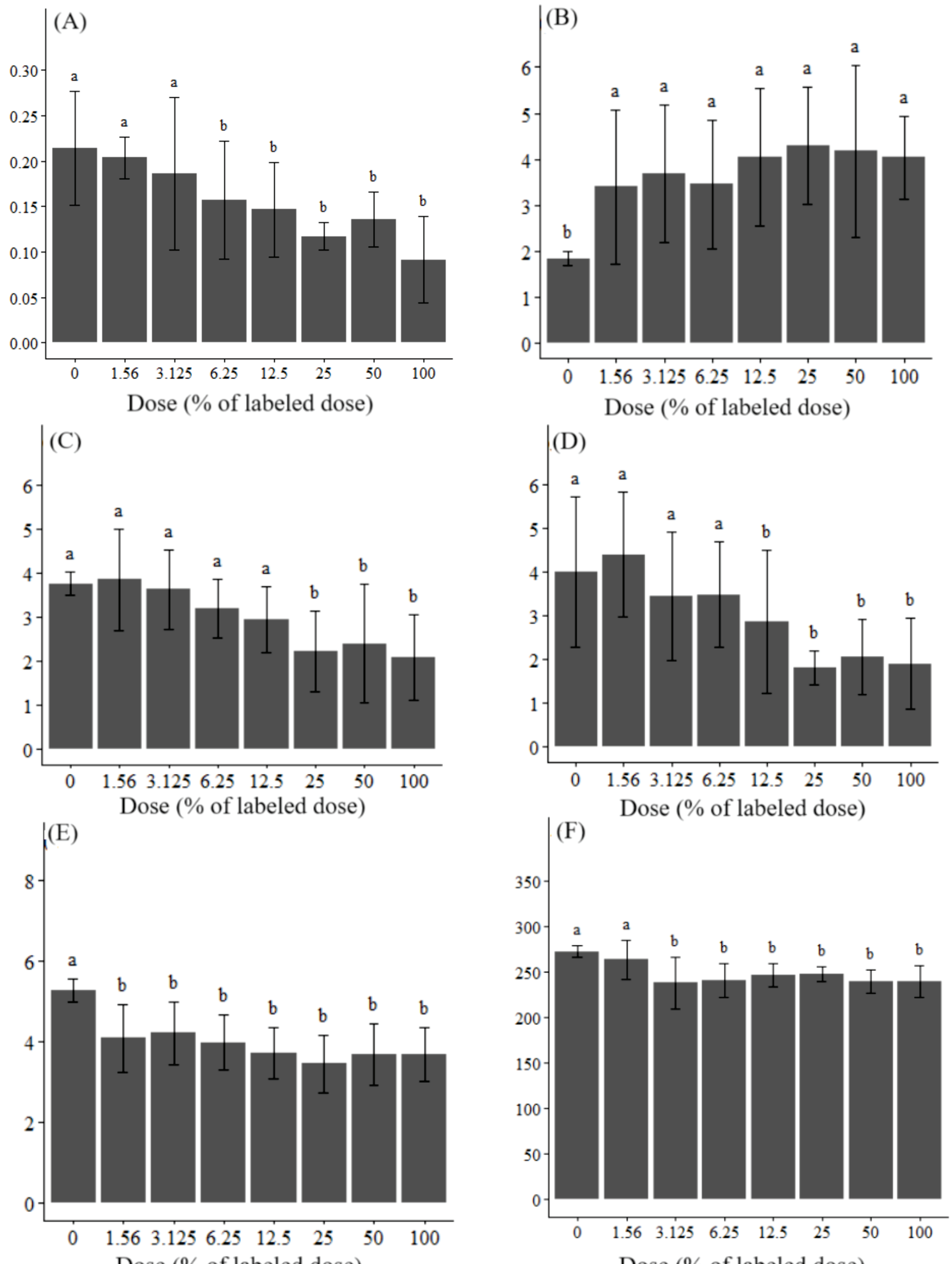

Figure 2. Simple effect of 2,4-D and dicamba doses on olive (*Olea europaea*) seedlings, after application time: on the stomatal conductance, at 72 hours (A); on transpiration rate, at 24, 48 and 72 (B, C, and D); on the water use eficciency, at 24 h (E); and on the internal CO_2 concentration at 24 h (F). Lowercase letters compared rates of herbicides, by Scott-Knott's test, at 5% probability.

from the dose of 1.56%, which was proportional to the increase in the dose of herbicides.

The analysis performed at 24 HAA showed a simple dose and herbicide effects for the Ci variable, for which the values decreased by 30% in relation to the others from the dose of 3.13% (Figures 2 F and 3 A). Herbicide 2,4-D showed lower values in comparison with dicamba, and the same fact occurred in the evaluation carried out at 48 HAA (Figure 3 B). For variables related to chlorophyll a fluorescence, dicamba had a slight increase of the values for F_0, showing that the plants suffered more significant stresses than those subjected to 2,4-D (Figure 3 C and D).

The same behavior was verified for Fv, which showed a simple dose and herbicide effects only in the evaluation carried out at 24 HAA, when it was possible to observe that all rates applied displayed no variation in the values of Fv (Figures 3 E and 4 B). Likewise, for Fm, for which only the evaluation carried out at 48 HAA showed a simple herbicide effect, it was evident that dicamba was responsible for causing a more significant stress effect on the plants in the

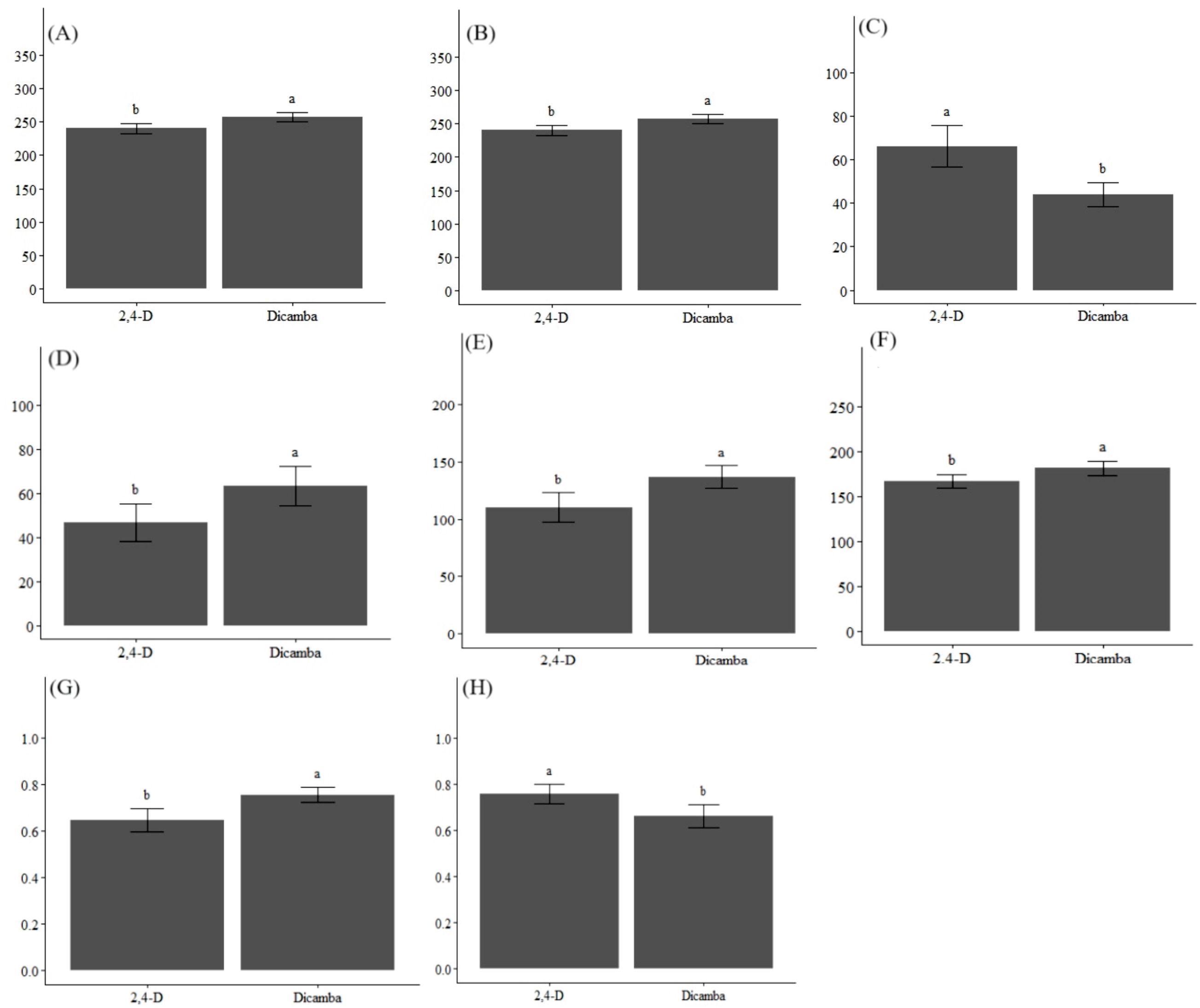

Figure 3. Simple effect of 2,4-D and dicamba application, on the following variables of olive (*Olea europaea*) seedlings: internal CO_2 concentration, measured at 24 (A) and 48 hours (B); initial fluorescence, at 24 (C) and 48 hours (D); fluorescence, measured at 24 hours (E); maximum fluorescence, measured at 48 hours (F); and maximum quantum yield of the PSII, measured at 24 (G) and 48 hours (H). Lowercase letters compared the herbicides, by Scott-Knott's test, at 5% probability.

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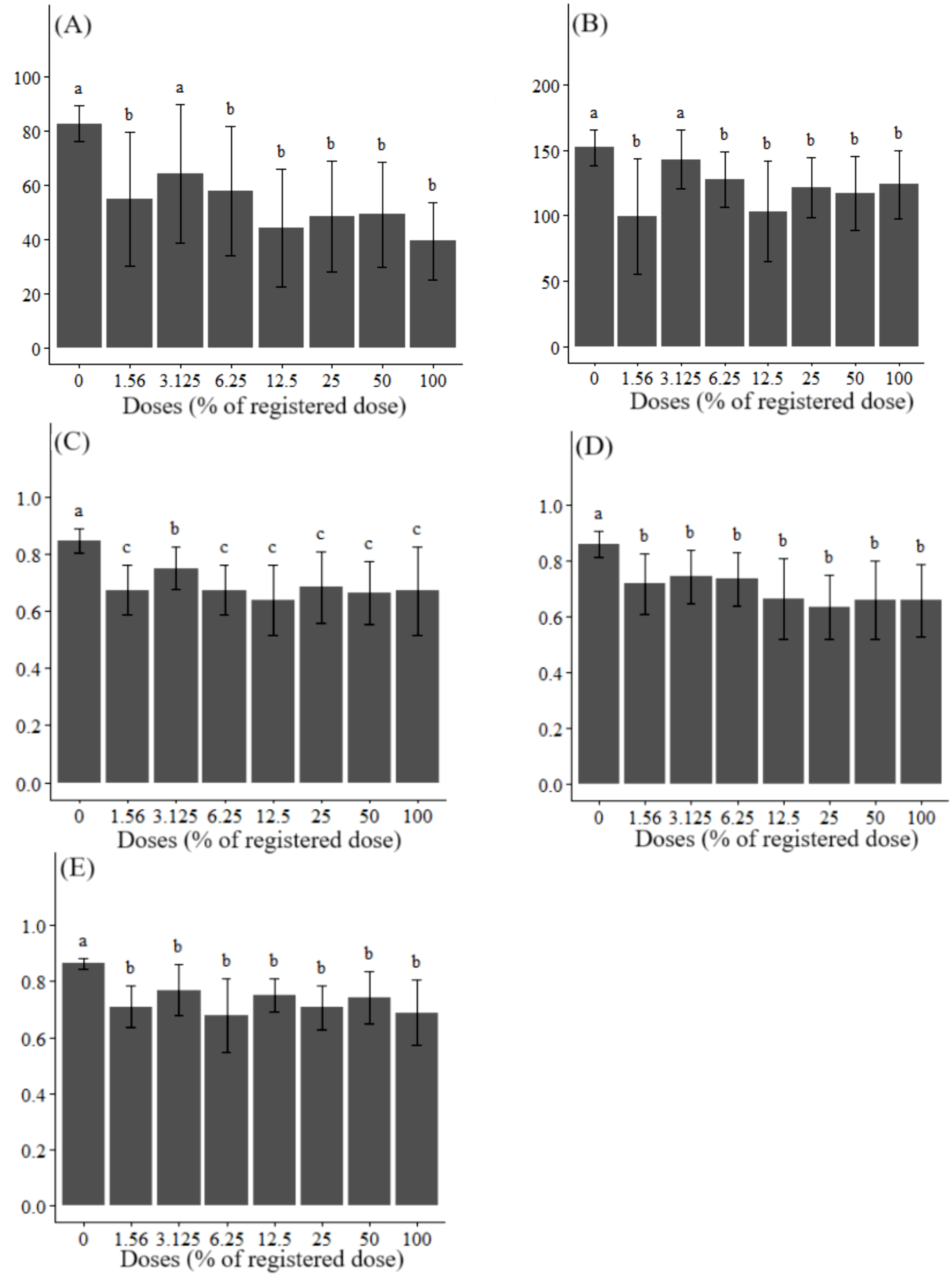

Figure 4. Simple effect of 2,4-D and dicamba application on the following variables of olive (*Olea europaea*) seedlings: initial fluorescence measured at 48 hours after treatment (A); fluorescence measured at 24 hours after treatment (B); and maximum quantum yield of the photosystem II, measured at 24, 48 and 72 hours after treatment (C, D, and E). Lowercase letters compared rates of the herbicides, by Scott-Knott's test, at 5% probability.

variables analyzed (Figure 3 F), regardless of the dose, as already seen for F_0.

In the analysis at 24 HAA, 2,4-D was responsible for the lowest values. At 48 HAA, dicamba obtained the lowest value of the relationship. At 72 HAA, both herbicides had the same value of MQY, leading to the conclusion that plants were under stress regardless of the herbicide applied. Photosynthesis and vegetative growth are linked, and when photosynthesis is inhibited under adverse conditions, such as damage caused by herbicide, the vegetative growth slows significantly. Stomata are the main channels of gas exchange between leaves and the environment, and the reduction in Gs due to the application of herbicides contributes to the loss of photosynthesis in olive plants.

The analysis of dicamba at 72 HAA showed a decreased Np for plants that received rates above 25.00% (Figure 1 C). Decrease of Np about 51% was also observed when this herbicide was applied to wheat plants, in comparison with the control (Agostinetto et al., 2016). It seems that olive trees are susceptible to damage caused by the 2,4-D herbicide. In addition, the application of herbicides results in low Gs (Agostinetto et al., 2016), since they promote stomatal closure, resulting in lower O_2 and CO_2 flow to the cells, consequently decreasing photosynthesis. However, at 30 DAA, the plants that received 2,4-D herbicide showed a decrease of Gs, when using the 25.00% dose. At the other doses, there was an increase in the Gs value, indicating that the plants recovered after this evaluation period. In a similar study, dicamba caused a greater damage than 2,4-D on the photosynthetic apparatus of Brazilian peppertree (*Schinus terebinthifolius* Raddi), mainly on the net assimilation rate of CO_2, stomatal conductance, intercellular CO_2 concentration, transpiration rate, carboxylation efficiency of Rubisco, and on the fluorescence emission of chlorophyll a (Avila Neto et al., 2022). Dicamba also had the most phytotoxicity effect on non-herbicide tolerant soybean, on a simulated drifting trial (Brochado et al., 2023). As for broccoli (*Brassica oleracea* L.), low rates of 2,4-D (16.8 g ha^{-1}) reduced 50% of total yield and showed more prominent phytotoxicity than dicamba (Mohseni-Moghadam & Doohan, 2015). These results suggest the potential of damage of low rates of auxin-mimicking herbicides in olive trees.

A decrease in the values from the dose of 12.50% of the herbicides is evidenced for Tr, in the evaluations carried out at 48 and 72 HAA (Figure 2), showing that the plants decreased their transpiration after stomatal closure, since both variables are closely linked (Alves et al., 2019). Thus, the higher is the dose of the herbicide applied, the lower will be the Tr obtained. Auxin-mimicking herbicides take three weeks on average, to cause the death of weeds. However, even if some plants did not die, sensitive plants exposed to them, in the present work, underwent negative effects after 30 days of herbicide application. This can be explained by the fact that these herbicides act on the plant physiology, leading to stomatal closure and consequent decrease of the plant metabolism, even after a long time of application (Grossmann, 2010).

That water use efficiency is directly related to stomatal closure and higher values for this parameter are found while the stomata are open (Araldi et al., 2012). In case the stomata are closed, the plants decrease the WUE, decreasing the amount of transpired water and, consequently, the production of dry matter (Machado et al., 2010). The internal concentration of CO_2 is closely linked to the stomata, since, when closed (Ferraz et al., 2012), they decrease the intake of CO_2, decreasing its values, subsequently interfering with the photosynthetic rate of the plants. The initial fluorescence indicates the energy absorbed by the antenna-complex not transmitted to photosynthetic pigments (Rascher et al., 2000). The values of F_0 are not always constant, since they can increase if some damage occurs to the PSII reaction centers (Ferreira et al., 2015). Therefore, in the present work, the F_0 low values indicate that the herbicide applications caused no damage to the PSII reaction centers, at 48 HAA.

It was possible to observe a decrease for the values of MQY from the dose of 1.56% compared with the control (Figure 4 C, D, and E). When this happens, will be a reduction in the amount of energy used by the plant to perform photosynthesis (Catunda et al., 2005). Emissions of chlorophyll a are related to the state of PSII, which plays a vital role in plant photosynthesis (Buonasera et al., 2011). Low values of the potential quantum efficiency of PSII indicate damage to the photosynthetic apparatus of plants (Marques et al., 2020), which occurred in the present work, from the application of herbicides 2,4-D and dicamba on olive seedlings. Thus, the decrease of stomatal conductance directly affected Ci, WUE, and Tr (Figure 1), consequently decreasing Np (Cui et al., 2020), as seen in this work, when simulating herbicide drifting.

Rates from 12.50% for both herbicides significantly affected the stomata conductance, net CO_2 assimilation rate, transpiration rate, and initial fluorescence, and all in the initial analysis at 24 HAA. Dicamba influenced all other variables, except for WUE at 48 and 72 HAA, and intercellular CO_2 concentration at 30 DAA. These results confirm the above mentioned, since, while the herbicide 2,4-D affected the variables related to gas exchange, the herbicide dicamba affected the variables related to PSII. In addition, it was evident that the plants were under stress, concerning the variables referring to chlorophyll a fluorescence, since the MQY showed lower values after applying the herbicides.

Conclusions

1. The drifting of auxin-mimicking herbicides causes damage to the photosynthetic apparatus of plants, as indicated by the low values of net photosynthesis, stomatal conductance of water vapors, and CO_2 intercellular concentration.
2. Herbicide 2,4-D decreases the gas exchange (stomatal conductance of water vapors, water use efficiency, transpiration rate, CO_2 intercellular concentration, and net photosynthesis), and dicamba decreases the chlorophyll a fluorescence (initial fluorescence, variable fluorescence, maximum fluorescence, and maximum quantic yield).

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