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Article

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Differential Tolerance of Clones of *Eucalyptus grandis* Exposed to Drift of the Herbicides Carfentrazone-Ethyl and Glyphosate

*Tolerância Diferencial em Clones de **Eucalyptus grandis** Expostos à Deriva dos Herbicidas Carfentrazone-Ethyl e Glyphosate*

ABSTRACT - Drift of the herbicides carfentrazone-ethyl and glyphosate may affect the initial growth of eucalyptus. This study aimed to assess the effect of carfentrazone-ethyl and glyphosate drift on photosynthesis, leaf morphoanatomy, and initial growth of two clones of *Eucalyptus grandis*. Two experiments were carried out in a 2 x 4 factorial scheme, in which factor 1 was represented by two clones of *E. grandis* and factor 2 by four herbicide underdoses (control, 86.4 g a.e. ha^{-1} of glyphosate, 3.0 g a.e. ha^{-1} of carfentrazone-ethyl, and the mixture of 86.4 g a.e. ha^{-1} of glyphosate + 3.0 g a.e. ha^{-1} of carfentrazone-ethyl). Herbicide application was carried out by simulating the drift in the lower third of seedling canopy. Assessments were performed 23 days after herbicide application. Both clones presented morphoanatomical changes such as erosion of epicuticular waxes and degeneration of epidermal and parenchymal cells, especially when exposed to carfentrazone-ethyl underdose or its mixture with glyphosate. The clone CLR 383 was the most affected by the tested herbicides and presented the highest injury, lowest initial growth, lowest dry matter, and highest reduction of photosynthetic rate when treated with herbicide mixture, followed by carfentrazone-ethyl and glyphosate underdoses. The initial growth, dry matter, and photosynthesis of the clone CLR 384 were not affected by the carfentrazone-ethyl underdose. Thus, both herbicides applied in isolation or in a mixture reduced the initial growth, dry matter, and photosynthesis of eucalyptus.

Keywords: eucalyptus, photosynthesis, leaf morphoanatomy, phytointoxication.

*RESUMO - A deriva dos herbicidas carfentrazone-ethyl e glyphosate pode afetar o crescimento inicial do eucalipto. Este trabalho objetivou avaliar o efeito da deriva de carfentrazone-ethyl e glyphosate sobre a fotossíntese, a morfoanatomia foliar e o crescimento inicial de dois clones de **Eucalyptus grandis**. Foram realizados dois ensaios em esquema fatorial 2 x 4, sendo o fator 1 representado por dois clones de **E. grandis**, e o fator 2, por quatro subdoses dos herbicidas (testemunha, 86,4 g e.a. ha^{-1} de glyphosate, 3,0 g e.a. ha^{-1} de carfentrazone-ethyl e a mistura de 86,4 g e.a. ha^{-1} de glyphosate + 3,0 g e.a. ha^{-1} de carfentrazone-ethyl). A aplicação dos herbicidas foi feita simulando deriva no terço inferior da copa das mudas. As avaliações foram realizadas 23 dias após aplicação. Os dois clones apresentaram alterações morfoanatômicas, como erosão de ceras epicuticulares, degeneração de células epidérmicas e parenquimáticas, sobretudo quando expostas à subdose de carfentrazone-ethyl ou de sua mistura com glyphosate. O clone CLR 383 foi o mais afetado pelos herbicidas testados e apresentou maior injúria, menor crescimento inicial, menor massa seca e maior redução na taxa fotossintética quando tratado com a mistura, seguida das subdoses de carfentrazone-ethyl e*

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glyphosate, respectivamente. O crescimento inicial em altura, a massa seca e a fotossíntese do clone CLR 384 não foram afetados pela subdose isolada de carfentrazone-ethyl. Os resultados deste trabalho permitem concluir que os dois herbicidas, aplicados de forma isolada ou em mistura, reduzem o crescimento inicial, a massa seca e a fotossíntese em plantas de eucalipto.

Palavras-chave: eucalipto, fotossíntese, morfoanatomia foliar, fitointoxicação.

INTRODUCTION

Chemical control of weeds in eucalyptus forests is a routine and necessary practice in the initial phase of forest formation. Among the five herbicides registered in Brazil (Agrofit, 2017), glyphosate is the most used mainly because of its broad spectrum of action and relatively low cost.

In areas with constant glyphosate application there are several reports of weeds resistant to this molecule mainly due to the selection pressure (Neve et al., 2003; Duke and Powles, 2008; Powles, 2008; Beckie, 2011; Norsworthy et al., 2011; Green, 2012; Shaner et al., 2009). In this context, the mixture of herbicides with distinct mechanisms of action, but with a synergistic effect, increases the spectrum of action and considerably improves control efficiency (Kumar and Jha, 2015; McCullough et al. 2015; Walsh et al., 2014, 2015). Because it presents a desirable efficiency on glyphosate-resistant weeds, such as *Commelina* spp. (Werlang and Silva, 2002), the herbicide carfentrazone-ethyl has been widely used as an alternative or in mixture with glyphosate. Another important fact is that among the herbicides registered for eucalyptus, only glyphosate and carfentrazone-ethyl are accepted by certification for use in eucalyptus forests (FSC, 2016).

Glyphosate and carfentrazone-ethyl are non-selective herbicides to eucalyptus and applications should be directed on weeds, avoiding undesired contact with the crop (Rodrigues and Almeida, 2011). Even though this is the case, during the applications part of the product can be dragged by the wind and reach eucalyptus plants by drift (Tuffi-Santos et al., 2007). When it occurs, initially plants may show anatomical changes such as erosion of epicuticular waxes, degeneration of epidermal cells (Santos et al., 2015), and visually detected symptoms such as wilting, chlorosis, and necrosis (Tuffi-Santos et al., 2007), which may affect the initial growth and development of the forest.

Thus, this study aimed to assess the effect of the herbicides carfentrazone-ethyl and glyphosate drift on leaf morphoanatomy, photosynthesis, initial height growth, and dry matter accumulation in two clones of *Eucalyptus grandis*.

MATERIAL AND METHODS

Experiment setup

This study was conducted with two experiments. The first experiment was carried out in February 2015 and the second in April 2015 with the same seedlings and method. After the first experiment, plants were pruned for removing the branches that received the herbicide application. Subsequently, plants were fertilized with 100 mL per plant of Biofert Universal® fertilizer solution (NPK, 6-4-4) diluted in water (1:100). Plants remained at rest for 20 days and, when no more intoxication symptoms caused by herbicides were observed, the second experiment was performed.

A randomized block design with 10 replications in a 2 x 4 factorial scheme (two clones and four herbicide underdoses) was used in both experiments. Each experimental plot consisted of a vase containing a eucalyptus plant. Clones were two genotypes of *E. grandis* and herbicide underdoses were 0 (control), 50 g a.e. ha^{-1} of carfentrazone-ethyl, 1,440 g a.e. ha^{-1} of glyphosate, and a mixture of 50 g a.e. ha^{-1} of carfentrazone-ethyl + 1,440 g a.e. ha^{-1} of glyphosate).

Eucalyptus seedlings were cultivated and donated by Clonar Resistência a Doenças Florestais Ltda. At 60 days of age, seedlings were transplanted to 2 L vessels containing the substrate

MecPlant® enriched with 1.3 kg simple superphosphate and 600 g Osmocote® (NPK, 19-6-10) for each 100 kg substrate. During transplanting, each vessel received 100 mL mono-ammonium phosphate solution (P and N at 52 and 12%, respectively; Vale Fertilizantes S.A., Uberaba, MG, Brazil). Subsequently, seedlings were maintained in a greenhouse.

Herbicide application

In the first experiment, the application was performed at 20 days after transplanting, when seedlings were 30 to 40 cm high. In the second experiment, the application was performed in April (20 days after the end of the first experiment). Both applications were performed simulating drift in the lower third of eucalyptus canopy. A CO_2 pressurized costal sprayer was equipped with a boom containing two nozzles model TT110.02 calibrated to apply 150 L ha^{-1} spray solution. To avoid product contact with other plant parts, the upper portion of the canopy was protected by a plastic bag at the application time, allowing only the lower portion of the canopy (three branches) be exposed to the product.

Anatomy and leaf micromorphology

Leaf samples were collected for light and scanning electron microscopy two days after herbicide application. Samples were fixed in Karnovsky's solution (Karnovsky, 1965) and, for a better penetration of the fixing solution, these samples were maintained in a desiccator and the air evacuated with a vacuum pump (0.1 mmHg) for 20 min.

For light microscopy analysis, samples were dehydrated with a graded series of ethanol (30, 50, 70, 90, and 100%, the latter washing repeated three times) and embedded in the acrylic resin methacrylate (Historesin, Leica). Cross-sections of 7 µm thickness were obtained on a Reichert rotary microtome, stained in toluidine blue pH 4.0 (O'Brien and McCully, 1981), and mounted on a slide and coverslip by using Entellan®. After observation, photographic documentation was performed by means of a light microscope (Zeiss Axioskop 2 model) with a coupled digital camera (model MRC3).

For scanning electron microscopy (SEM) analysis, samples were post-fixed in 1% osmium tetroxide, dehydrated in a graded series of acetone (30, 50, 70, 90, and 100%, the latter washing repeated three times) and dried to the critical point (model Balzers CPD 030). After assembly in stubs using double-sided carbon tape, samples were coated with gold in a metallizer (model Balzers SCD 050). Subsequently, this material was analyzed by scanning electron microscope (Zeiss LEO 435-VP model) and images were recorded in digital files.

Assessment of clone tolerance to herbicides

Clone tolerance to herbicide drift was assessed at 23 days after herbicide application (DAA) by phytointoxication percentage, plant height growth, shoot dry matter production, and photosynthesis. Phytointoxication assessment was carried out by means of visual observations using a 0 to 100% scale, in which zero is the absence of intoxication and 100% represents plant death (Frans et al., 1986).

At the end of experiment 2, growth was measured by the total height between the substrate base and the apex of all plants. Subsequently, plants were collected close to the substrate and stored in paper bags. After drying in a forced air ventilation oven at 65 °C, plants were weighed daily for five days until reaching a constant weight.

Photosynthesis was assessed in both tests by using a CO_2/H_2O infrared gas analyzer (LI 6400, Li-Cor, Lincoln, NE), with a controlled internal light (1,000 µmol photons m^2 s^{-1}) and CO_2 (400 µmol mol^{-1}). Three measurements were carried out on a fully expanded leaf from the apical portion of the canopy (protected from application) in each of the 10 replicate plants.

Statistical analysis

The data were submitted to analysis of variance and, when relevant, means were compared by Tukey's test at 5% significance ($p>0.05$). The data of intoxication and photosynthesis of plants

assessed in both experiments showed similar results and, therefore, were analyzed by means of the joint analysis of data (Oliveira, 1993). The quantitative variables were submitted to the Pearson correlation test.

RESULTS AND DISCUSSION

Effects of herbicide drift on leaf anatomy and micromorphology

At 2 DAA, plants previously exposed to glyphosate and carfentrazone-ethyl underdoses, applied in isolation, presented epidermal cells with eroded epicuticular waxes on the abaxial (Figure 1) and adaxial (Figure 2) surfaces. In addition to the erosion of epicuticular waxes, plants from the clone CLR 383 exposed to carfentrazone-ethyl and the mixture glyphosate + carfentrazone-ethyl drift showed a turgor loss and rupture of the cuticle of stomatal guard cells (Figure 1E and G).

In the cross-sectional view, the most severe detrimental effect assessed at 2 DAA was caused by the carfentrazone-ethyl application (Figure 3). Leaves of both clones directly exposed to the application of this herbicide either in an isolated underdose or in a mixture with glyphosate presented degraded cells in the adaxial epidermis and palisade and spongy parenchyma (Figure 3E and H).

Anatomical changes resulting from the action of the herbicide carfentrazone-ethyl are unprecedented. Detrimental effects on leaf morphology due to herbicide action, such as erosion of epicuticular waxes, cuticle rupture, and degeneration of epidermal and parenchymal cells observed here were also reported in other studies on glyphosate (Tuffi-Santos et al. 2007; Santos et al., 2015). The herbicide carfentrazone-ethyl has a faster effect when compared to glyphosate due to its mechanism of action (Silva and Silva, 2012), which is corroborated in our study from the morphoanatomical analyses.

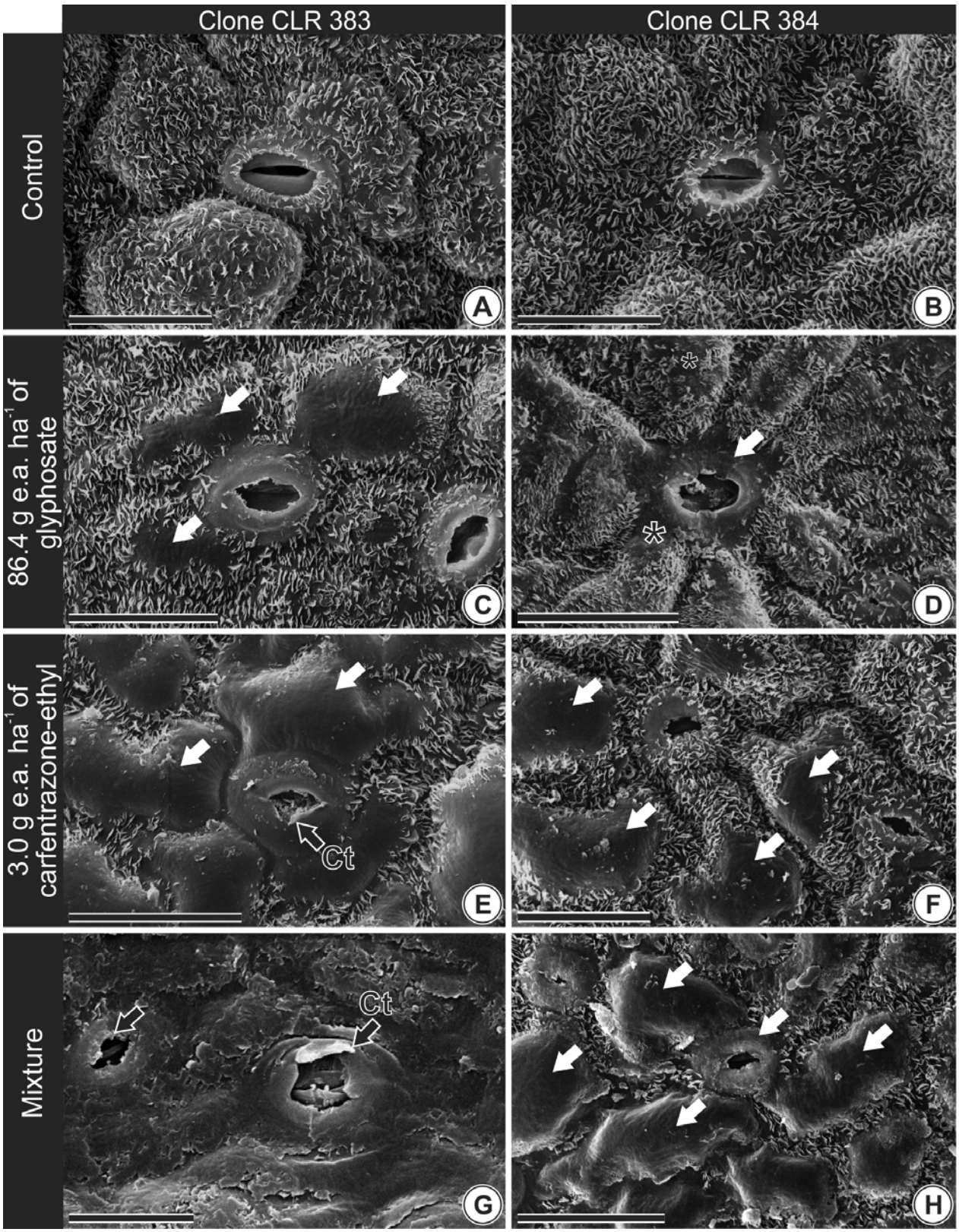
Tolerance of clones to herbicides and correlation analysis between studied variables

A significant interaction was observed between clone and herbicide for photosynthesis, phytointoxication percentage, total height, and shoot dry matter (Tables 1 and 2). Plants from the clone CLR 383 exposed to underdose applications of glyphosate, carfentrazone-ethyl, and the mixture of both herbicides presented the lowest values of photosynthetic rate (Table 1). A similar result was observed for plants from the clone CLR 384, except for those receiving the isolated application of carfentrazone-ethyl underdose (Table 1).

Plants from both clones presented higher phytointoxication when exposed to the mixture of herbicide underdoses (Table 2). In all treatments with herbicide underdoses, the clone CLR 383 showed a higher intoxication percentage when compared to the clone CLR 384 (Table 2). Plants from the clone CLR 383 presented a lower height and dry matter when submitted to the drift of carfentrazone-ethyl and glyphosate mixture (Table 2). However, only the isolated glyphosate underdose and the mixture of both herbicides affected the amount of dry matter in the clone CLR 384 (Table 2). The clone CLR 383 presented the lowest height and dry matter production with the application of carfentrazone-ethyl underdoses, in isolation or in a mixture with glyphosate, when compared to the clone CLR 384 (Table 2).

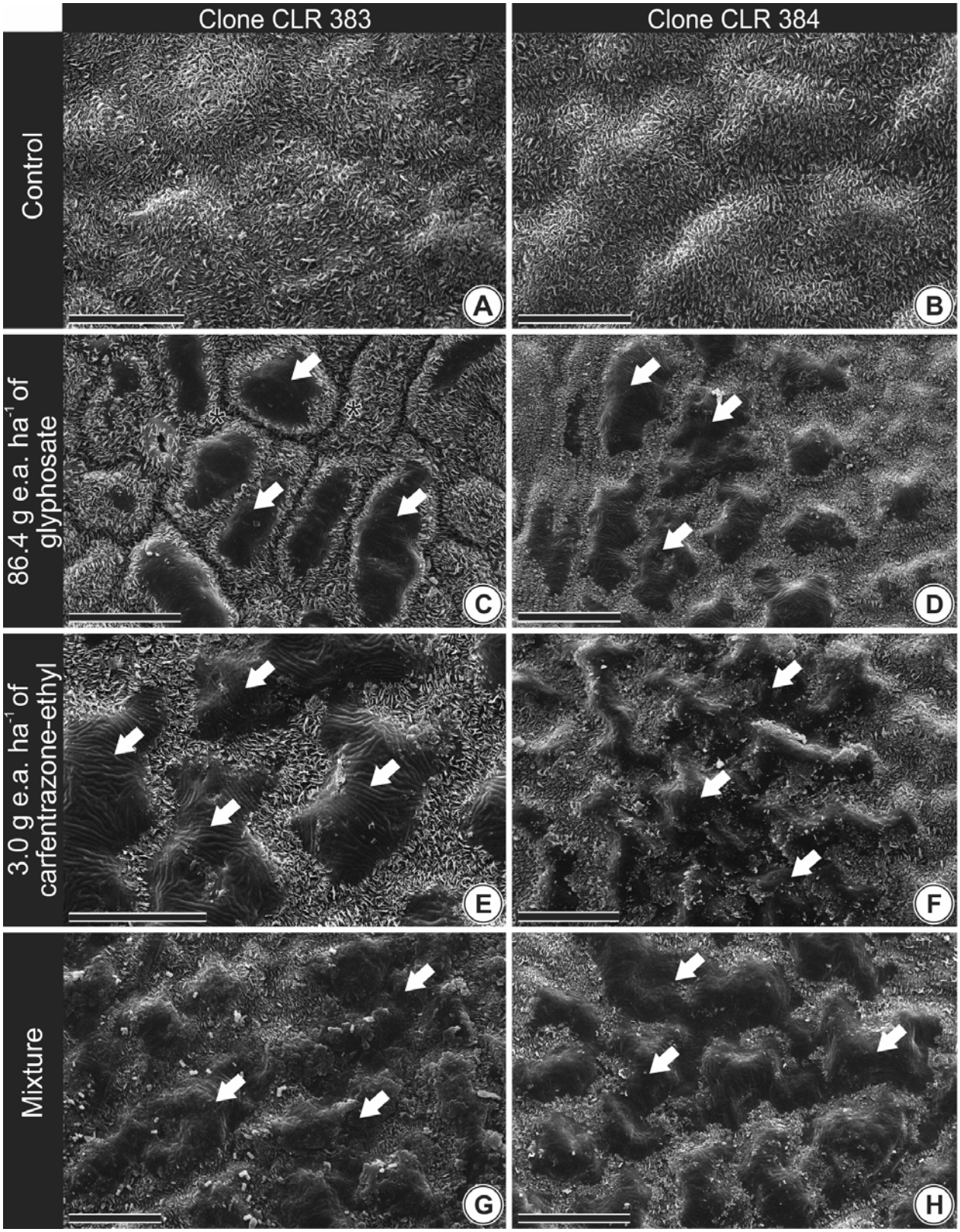
All variables studied to determine the tolerance of clones to herbicides are correlated with each other (Table 3). Phytointoxication caused by herbicide underdoses in both clones negatively affected total height, shoot dry matter accumulation, and photosynthesis (Table 3). However, as expected, photosynthesis presented a positive correlation with growth in height and shoot dry matter accumulation (Table 3).

The results found here bring new information for the herbicide carfentrazone-ethyl regarding microscopic changes in leaf anatomy, as well as its effect on eucalyptus photosynthesis. Although no study in the literature has cited a photosynthesis reduction due to the action of carfentrazone-ethyl, it is known that this herbicide inhibits the action of the protoporphyrinogen oxidase IX (PPO) enzyme (Silva and Silva, 2012). Thus, carfentrazone-ethyl can indirectly affect photosynthesis since PPO enzyme is essential in chlorophyll biosynthesis



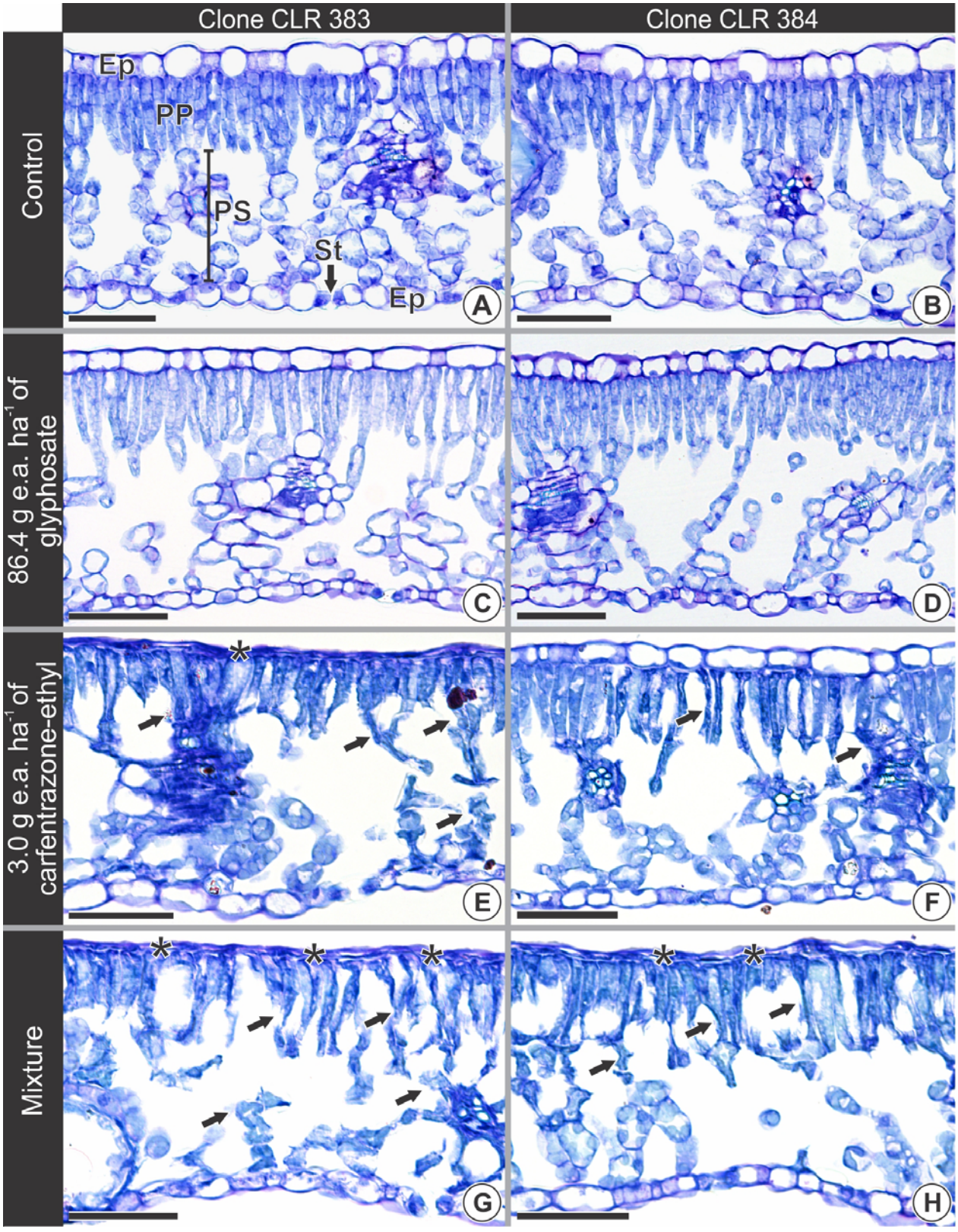
(A–B) Cells of the stomatal complex with turgor and epicuticular waxes with a characteristic conformation of the species. (C–H) Eroded epicuticular waxes (white arrows). (E) and (G) Wall degeneration and cuticle rupture (Ct) in the stomatal guard cells (black arrows). Mixture = 86.4 g a.e. ha^{-1} glyphosate + 3.0 g a.e. ha^{-1} carfentrazone-ethyl. Bars = 25 µm.

Figure 1 - Effect of herbicide drift on the abaxial leaf surface two days after application in clones of *Eucalyptus grandis* (scanning electron microscopy).



(A–B) Epidermal cells with turgor and epicuticular waxes with a characteristic conformation of the species. (C–H) Eroded epicuticular waxes (white arrows). Mixture = 86.4 g a.e. ha^{-1} glyphosate + 3.0 g a.e. ha^{-1} carfentrazone-ethyl. Bars = 25 µm.

Figure 2 - Effect of herbicide drift on the adaxial leaf surface two days after application in clones of *Eucalyptus grandis* (scanning electron microscopy).



(A–D) Cells with conformation and turgor characteristic of the species. (E–H) Degraded epidermal cells (asterisks); parenchyma cells with turgor loss (arrows). Abbreviations: Ep – epidermis; PP – palisade parenchyma; PS – spongy parenchyma; St stoma. Mixture = 86.4 g a.e. ha^{-1} glyphosate + 3.0 g a.e. ha^{-1} carfentrazone-ethyl. Bars = 25 µm.

Figure 3 - Effect of herbicide drift on the leaf anatomy of clones of *Eucalyptus grandis* two days after application.

(Tanaka et al., 2011; Kobayashi et al., 2014). In addition, microscopic evidence, such as the degradation of palisade parenchyma cells due to the action of this herbicide, may be closely linked to a lower photosynthesis since the affected cells constitute the main photosynthetic tissue (Taiz and Zeiger, 2013).

The herbicide glyphosate also indirectly affects photosynthesis by inhibiting the biosynthesis of carotenoids, chlorophylls, fatty acids, and amino acids (Fedtke and Duke, 2005), as well as secondary metabolism products essential for photosynthesis, such as quinones (Dewick, 1998). The reduced photosynthetic rate, as observed in our study, has also been reported in the literature on plant species exposed to glyphosate (Ding et al., 2011; Yanniccari et al., 2012; Agostinetto et al., 2016; Gomes et al., 2016; Radwan and Fayez, 2016).

Table 1 - Effect of the drift of glyphosate (G), carfentrazone-ethyl (CE) or their mixture (G+CE) on photosynthesis (A) in two clones of *Eucalyptus grandis* at 23 days after application

Treatment	A (µmol m^{-2} s^{-1})	
	CLR 383	CLR 384
Control	13.2±0.6Aa	14.5±0.5Aa
G	9.6±0.5Ab	9.9±0.7Ab
CE	6.8±0.4Bc	12.8±0.5Aa
G+CE	6.5±0.7Bc	9.4±0.8Ab

Means ± standard error of the mean followed by the same uppercase letters in the columns and lowercase letters in the rows do not differ significantly from each other by the Tukey's test (p≤0.05).

Table 2 - Effect of the drift of glyphosate (G), carfentrazone-ethyl (CE) or their mixture (G+CE) on phytointoxication percentage, height, and dry matter in two clones of *Eucalyptus grandis* at 23 days after application

Treatment	Phytointoxication (%)		Height (cm)		Dry matter (g/plant)	
	CLR 383	CLR 384	CLR 383	CLR 384	CLR 383	CLR 384
Control	0±0Aa	0±0Aa	69.8±0.8Aa	71.8±1.1Aa	27.7±0.7Aa	30.3±0.9Aa
G	15.5±1.3Ab	17±0.8Ab	58.5±1.0Ab	55.4±0.8Ab	23.1±0.5Ab	23.4±0.8Ab
CE	23.8±0.8Bc	10.3±0.7Aa	47.9±1.5Bc	68.3±1.0Aa	19.7±0.5Bc	28.5±0.9Aa
G+CE	29±0.8Bc	22.5±0.8Ab	45.1±0.7Bc	52.5±0.7Ab	19.3±0.6Bc	22.7±0.6Ab

Means ± standard error of the mean followed by the same uppercase letters in the columns and lowercase letters in the rows do not differ significantly from each other by the Tukey's test (p≤0.05).

Both clones of *E. grandis* showed a differential tolerance to the tested herbicides. Plants from the clone CLR 383 exposed to drift of the isolated underdose of carfentrazone-ethyl or to the mixture underdose showed a higher intoxication percentage and reduction in photosynthesis and, consequently, a lower growth in height and dry matter accumulation when compared to plants from the clone CLR 384. Our results showed that a reduction in photosynthesis is directly correlated with a lower growth in height and dry matter accumulation in plants exposed to herbicides, which consequently affects the growth and development of eucalyptus plants. Other studies report differential tolerance to the herbicide glyphosate among genotypes of the same species or *Eucalyptus* hybrids. When exposed to glyphosate drift, the clone of *E. urophylla* is more tolerant when compared to clones of *E. grandis* or hybrids of *E. urophylla* x *E. grandis* (Tuffi-Santos et al., 2007). Carvalho et al. (2015) also confirm that hybrid clones of *E. urophylla* x *E. grandis* respond differently to glyphosate drift. Tuffi-Santos et al. (2009) worked with clones of *E. grandis* and found that although the factor clone has not influenced intoxication percentage and shoot dry matter accumulation, it significantly affected plant height when submitted to glyphosate. For the herbicide carfentrazone-ethyl, studies are still incipient. Tuffi-Santos et al. (2006) reported its phytotoxic effect on plants of *E. urophylla*. Tiburcio et al. (2012) worked with fomesafen, an herbicide with a mechanism of action similar to that observed for carfentrazone-ethyl, and also observed a decreased shoot dry matter in two hybrids of *E. urophylla* x *E. grandis*.

Table 3 - Correlation analysis between the studied variables to determine the tolerance of clones to herbicides

Clone CLR 383			
	Height	Dry matter	Photosynthesis
Phytointoxication	-0.96**	-0.90**	-0.94**
Photosynthesis	0.96**	0.98**	-
Clone CLR 384			
	Height	Dry matter	Photosynthesis
Phytointoxication	-0.68**	-0.65**	-0.70**
Photosynthesis	0.92**	0.97**	-

Pearson correlation coefficient values (r). **Significant (p≤0.01) by the t-test.

These results allow us to conclude that the drift of the herbicides carfentrazone-ethyl and glyphosate causes an erosion of epicuticular waxes, epidermal degeneration, and cuticle rupture, especially when applied in a mixture. The herbicide carfentrazone-ethyl causes degeneration of parenchyma cells. Both clones presented a differential tolerance to the herbicides. In the clone CLR 383, both herbicides caused a reduction of photosynthesis, height, and dry matter accumulation, especially when applied in a mixture. However, only glyphosate affected photosynthesis and growth in the clone CLR 384.

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