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Rapid necrosis III: implications of environmental conditions and plant growth stage on 2,4-D resistance and effect of other auxinic herbicides in Sumatran fleabane (Conyza sumatrensis).

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Abstract:	Resistant plants of Sumatran fleabane with an unusual rapid necrosis symptom after application of 2,4-D were characterized in previous studies. Field observations indicated variability in the occurrence of the rapid necrosis (RN) caused by 2,4-D, but the causes of the variation are unknown. This study aimed to investigate the effect of environmental conditions, plant growth stage, and simultaneous and sequential herbicide mixtures with other auxin mimics on the occurrence of RN caused by 2,4-D. Application at temperature of 12°C delayed the symptoms and decreased the intensity of the RN, but still resulted in plant survival to 2,4-D. The absence of light after herbicide application caused a slight delay in the symptoms, but the production of hydrogen peroxide and the size of necrosed area were not affected by the light treatments before and after 2,4-D application. Changes in plant photosynthesis through inhibiting photosystem II do not prevent the occurrence of the RN symptom. The auxinic herbicides dicamba, triclopyr, and halauxifen-methyl do not cause RN symptoms and are effective at controlling the resistant biotype when applied without 2,4-D, but the effectiveness of these herbicides was reduced when sprayed on the

	resistant biotype either together, 4 h or 24 h after 2,4-D. The RN phenotype does not occur for dicamba and triclopyr, even in advanced plant growth stages and high doses on the resistant biotype. The herbicides dicamba and triclopyr effectively controlled resistant plants, especially when sprayed at the initial growth stages. The results of this study identified environmental, plant development effects, and herbicide interactions, that interfere with the occurrence of RN symptoms caused by 2,4-D in Sumatran fleabane. These data provide insights about the mechanisms behind the RN symptoms caused by 2,4-D and are important for identifying the causes of variability of the herbicide symptomology and performance under experimental and field conditions.

Short Title: Rapid necrosis and 2,4-D resistance

Title: Rapid necrosis: Implications of environmental conditions and plant growth stage on 2,4-D resistance and effect of other auxinic herbicides in Sumatran fleabane (*Conyza sumatrensis*)

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Abstract

Resistant plants of Sumatran fleabane with an unusual rapid necrosis symptom after application of 2,4-D were characterized in previous studies. Field observations indicated variability in the occurrence of the rapid necrosis (RN) caused by 2,4-D, but the causes of the variation are unknown. This study aimed to investigate the effect of environmental conditions, plant growth stage, and simultaneous and sequential herbicide mixtures with other auxin mimics on the occurrence of RN caused by 2,4-D. Application at temperature of 12 C delayed the symptoms and decreased the intensity of the RN, but still resulted in plant survival to 2,4-D. The absence of light after herbicide application caused a slight delay in the symptoms, but the production of hydrogen peroxide and the size of necrosed area were not affected by the light treatments before and after 2,4-D application. Changes in plant photosynthesis through inhibiting photosystem II do not prevent the occurrence of the RN symptom. The auxinic herbicides dicamba, triclopyr, and halauxifen-methyl do not cause RN symptoms and are effective at controlling the resistant biotype when applied without 2,4-D, but the effectiveness of these herbicides was reduced when sprayed on the resistant biotype either together, 4 h or 24 h after 2,4-D. The RN phenotype does not occur for dicamba and triclopyr, even in advanced plant growth stages and high doses on the resistant biotype. The herbicides dicamba and triclopyr effectively controlled resistant plants, especially when sprayed at the initial growth stages. The results of this study identified environmental, plant development effects, and herbicide interactions, that interfere with the occurrence of RN symptoms caused by 2,4-D in Sumatran fleabane. These data provide insights about the mechanisms behind the RN symptoms caused by 2,4-D and are important for identifying the causes of variability of the herbicide symptomology and performance under experimental and field conditions.

Nomenclature: 2,4-D; Sumatran fleabane, *Conyza sumatrensis* (Retz.) E. Walker, ERISU

Keywords: auxinic herbicide resistance; low temperature; light effect; photosynthesis inhibitors; plant growth stage

Introduction

Species of the genus *Conyza* are important weeds due to their high abundance, easy seed dispersion, and occurrence of hybridization. These species are cosmopolitan weeds, that settle mainly in disturbed areas (Tremmel and Peterson 1983). The germination and establishment in the crop fields occur mainly during the late fall to winter, which in Brazil are fallow or cultivated with pastures, cover crops, or winter grain cereals (Vidal et al. 2007). The seeds are positive photoblastic and do not germinate in soil depths greater than 0.5 cm (Nandula et al. 2006). Generally, the *Conyza* seeds germinate between 10 to 25 C, and 20 C is regarded as optimum for germination (Zinzolker et al. 1985). The wide genetic diversity of *Conyza* species also favors the emergence of herbicide-resistant biotypes (Bajwa et al. 2016). Herbicide resistance is one of the largest agricultural problems. In Brazil, herbicide resistance is estimated to occur on 20.1 million ha, resulting in US $1,63 billion yearly losses (Adegas et al. 2017). In this country, the most important herbicide resistant weeds are *Conyza* sp., sourgrass (*Digitaria insularis (L.) Mez ex Ekman*), italian ryegrass (*Lolium perenne* L. ssp. *multiflorum* (Lam.) Husnot), goosegrass (*Eleusine indica* (L.) Gaertner), and *Echinochloa* sp. (Adegas et al. 2022; Heap 2022). Cross-resistance occurs in Sumatran fleabane, and cases of glyphosate (5-enolypyruvyl-shikimate-3-phosphate synthase - EPSPS inhibitor, HRAC code 9) and chlorimuron (acetolactate synthase - ALS inhibitor, 2) double resistance have been in Brazil since 2011, limiting the use of these two mechanisms of action (Santos et al. 2014). Following the appearance of resistance, herbicides with other mechanisms of action were used to control the resistant population, mainly 2,4-D, an auxinic herbicide (4); the photosystem I (PSI, 22) inhibitors paraquat and diquat, ammonium glufosinate, an inhibitor of the enzyme glutamine synthetase (GS, 10), and saflufenacil, an inhibitor of the enzyme protoporphyrinogen oxidase (PPO, 14). However, the intensification of the use of these herbicides has contributed to the emergence of biotypes resistant to these mechanisms of action. In fact, in Brazil, cross-

resistance was identified in Sumatran fleabane to paraquat, chlorimuron, and glyphosate in 2016 (Albrecht et al. 2020), and to 2,4-D, paraquat, diuron, glyphosate and saflufenacil, in 2017 (Pinho et al. 2019).

A unique case of resistance to the herbicide 2,4 D with an unusual resistance mechanism was identified in a biotype of Sumatran fleabane from the state of Paraná, Brazil in 2015. Rapid necrosis (RN) symptoms begin about 2 h after herbicide spraying and later the plants regrow from the axillary buds, resulting in a resistance factor of 18.6 compared with a susceptible biotype (Queiroz et al. 2020). Recently, a second study on this case of resistance identified that the RN mechanism may be related to changes in auxin transport or in the Transport Inhibitor Response 1 (TIR1) receptor, and it is not related to the 2,4-D detoxification by glutathione-S-transferase or cytochrome P450 monooxygenase enzymes (Queiroz et al. 2022). Furthermore, the oxidative stress related to RN was responsive to temperature and was not light-dependent in Sumatran fleabane resistant plants that also showed rapid photosynthetic damage (Leal et al. 2022). There is no report of other species showing similar resistance to auxinic herbicides in the literature (Figueiredo et al. 2022; Peterson et al. 2016). However, a similar phenotype has been reported in giant ragweed (*Ambrosia trifida* L.) resistant to glyphosate in the USA (Brabham et al. 2011). This mechanism has been proposed to increase the production of hydrogen peroxide and it is influenced by temperature and light (Harre et al. 2018a; Moretti et al. 2017). In the resistant biotype of giant ragweed, the RN limited the action of other herbicides and caused antagonism between glyphosate and the herbicides atrazine, cloransulam, dicamba, lactofen, and topramezone (Harre et al. 2018b). Despite their similarity, the 2,4-D RN-resistant biotype of Sumatran fleabane does not develop the RN symptoms in response to glyphosate (Queiroz et al. 2020).

A previous study identified that the RN caused by 2,4-D in Sumatran fleabane was influenced by temperature, indicating the possible involvement of metabolic and/or transporter

proteins (Leal et al. 2022). There are only a few studies about the influence of the temperature on the 2,4-D efficacy in plants of the genus *Conyza* even in susceptible biotypes (Montgomery et al. 2017; Silva et al. 2021). A study in horseweed [*Conyza canadensis* (L.) Cronq.] identified higher control efficiency of 2,4-D at noon (11 to 13:30 h, 16-26 C) than in the early morning (6 to 6:30, 6 to 13 C) (Montgomery et al. 2017). In general, low temperatures reduce the efficacy of auxinic herbicides due to a reduction in herbicide uptake and translocation (Richardson 1977).

The occurrence of rapid necrosis has been reported as a variable in field conditions. Anecdotal evidence related to temperature and light has been associated with the low effect of the herbicide 2,4-D and with the intensity of the rapid necrosis. A previous study indicated that under low light (29 μmol m^{-2} s^{-1}) the H_2O_2 production was reduced in Sumatran fleabane, and the onset of RN symptoms was delayed in comparison to high light conditions (848 μmol m^{-2} s^{-1}) (Queiroz et al. 2020). A similar response was observed in another 2,4-D resistant biotype of Sumatran fleabane, which showed similar levels of H_2O_2 under dark and under light (520 μmol m^{-2} s^{-1}) conditions, and it was higher in the resistant biotype than in the susceptible biotype (Leal et al. 2022). Another factor affecting the onset of rapid necrosis is the plant growth stage in the timing of herbicide spraying which is variable in field conditions. Due to the increasing occurrence of plants with rapid necrosis caused by 2,4-D, there is a necessity for more information on the effect of mixtures of 2,4-D and other auxinic herbicides to control resistant biotypes. In addition, alternative herbicides can also be applied after the visualization of the rapid necrosis, and the efficacy of such applications is also unknown. The aim of this study was to investigate the effect of environmental conditions, plant growth stage, and simultaneous and sequential herbicide mixtures on the occurrence of rapid necrosis caused by 2,4-D in Sumatran fleabane.

Material and Methods

Plant material and data analysis

The resistant biotype MARPR9-RN (biotype RN) was collected in the city of Maripá, Paraná, Brazil (24.55°S, 53.72°W) and the susceptible biotype LONDS4-S (biotype S) was collected in Londrina, Paraná, Brazil (23.33°S, 51.21°W). Both biotypes were described in Queiroz et al. (2020). Resistant plants were bagged and selfed for two generations after selection with 804 g ae ha^{-1} 2,4-D (DMA® 806 BR SL, DMA® 806 BR SL, Corteva Agrisciences, São Paulo, SP, Brazil; labeled use rate of 1005 g ae ha^{-1} for Sumatran fleabane control) in a greenhouse to produce the seeds (Queiroz et al. 2020). Sowing was carried out in plastic trays measuring 15 cm by 10 cm, filled with substrate. The trays were maintained in a greenhouse at 28 ± 5 C and daily irrigated to promote seed germination. One seedling at the stage of four immature leaves was transplanted into individual 200 mL plastic pots previously filled with substrate, maintained in a greenhouse, and irrigated daily. All the studies were conducted twice in a completely randomized design with four replicates. The statistical software R v.4.2.1 was used for data analysis (R Core Team 2022). Data were submitted to the non-parametric tests of Shapiro-Wilk and histogram to verify the normal distribution and transformed as necessary. After that, data were submitted to ANOVA, and when significant ($p \leq 0.05$) the means were compared by Tukey´s HSD test ($p \leq 0.05$) using the *Expdes.pt* package (Ferreira et al. 2021). Herbicide dose-response curves were adjusted using the three-parameter nonlinear log-logistic model using the *drc* package (Ritz et al. 2015). Data from two replicates of each experiment were submitted to Bartlett's test for homogeneity of variance using the car package, and when considered homogeneous, the data were analyzed together. All the repeated experiments were similar, and the replications of each experiment were analyzed together.

Dose-response evaluation of seven auxinic herbicides

153 The study evaluated the occurrence of rapid necrosis and plant in response to increasing
154 doses of auxinic herbicides. Resistant and susceptible plants at 10-15 cm of height (8-10 leaves)
155 were sprayed with the herbicides dicamba (Clarity® SL, BASF, Durham, NC, USA) at 15, 30,
156 60, 120, 240, 480, 960 and 1920 g ae ha^{-1}; halauxifen-methyl (Arylex™ SC, Dow AgroSciences
157 Industrial) at 0.2, 0.4, 0.9, 1.8, 3.5, 7, 14 and 28 g ae ha^{-1}; triclopyr (Garlon 480 BR® EC, Dow
158 AgroSciences Industrial) at 23, 45, 90, 180, 360, 720, 1440 and 2880 g ae ha^{-1}; fluroxypyr
159 (Starane® EC, Dow AgroSciences Industrial) at 9, 19, 37, 75, 150, 300, 599 and 1199 g ae ha^{-1};
160 florpyrauxifen-benzyl (Loyant™ SL, Dow AgroSciences, Indianapolis, IN, USA) at 0.2, 0.5,
161 0.9, 1.9, 3.8, 7.5, 15 and 30 g ae ha^{-1}; picloram (Padron® SL, Dow AgroSciences Industrial) at
162 8, 15, 30, 60, 120, 240, 480 and 960 g ae ha^{-1}. For the herbicide 2,4-D the rates for susceptible
163 biotype were 25, 50, 101, 201, 402, 804, 1608, and 3216 g ae ha^{-1} and for the resistant biotype
164 were 101, 201, 402, 804, 1608, 3216, 6432, and 12864 g ae ha^{-1}. The considered labeled rate
165 for Sumatran fleabane control was 560 g ae ha^{-1} of dicamba, 7 g ae ha^{-1} of halauxifen-methyl,
166 and 1005 g ae ha^{-1} of 2,4-D. The dose for the other herbicides was selected based on the
167 recommendation for similar species because there is no label recommendation for *Conyza*
168 species. The label rates considered were 720 g ae ha^{-1} of triclopyr, 300 g ae ha^{-1} of fluroxypyr,
169 7.5 g ae ha^{-1} of florpyrauxifen-benzyl, and 360 g ae ha^{-1} for picloram. Plants were sprayed in a
170 spray chamber (Generation III Research Sprayer, DeVries Manufacturing, Hollandale, MN)
171 calibrated at 262 kPa delivered by a TJ8002E nozzle, resulting in an output volume equivalent
172 to 200 L ha^{-1}. Plant injury was evaluated by a visual percentage scale rating the RN in the
173 biotype RN and the occurrence of epinasty in the susceptible biotype at 35 d after treatment
174 (DAT), where 0% corresponded to the absence of symptoms and 100% to total plant control.
175
176 *Effect of the rapid necrosis on the effect of other auxinic herbicides*

Plants of the biotypes RN and S at 10 to 15 cm of height (8 to 10 leaves) were sprayed with the herbicides 2,4-D at 670 g ae ha^{-1} alone and in a simultaneous mixture with dicamba at 480 g ae ha^{-1}, halauxifen-methyl at 7 g ae ha^{-1}, or triclopyr at 720 g ae ha^{-1}. These herbicides were also applied 4 and 24 h after 2,4-D spraying. The occurrence of RN was evaluated at 3 DAT and plant injury at 35 DAT as described above. Data were submitted to ANOVA, $p \leq 0.05$, and means were compared by Tukey´s test ($p \leq 0.05$). Analysis of the effect of interactions between herbicides was performed using the Colby method (Colby 1967), which compares the effect of control of herbicides in mixture with the effect of the herbicides used alone, and reveals additive, synergistic or antagonistic responses. Synergism occurs when the observed effect is higher than the expected effect of the mixture, antagonism occurs when the observed effect is less than expected, and the additive response occurs when the observed effect is equal to the expected. Expected and observed values were compared using the t-test ($p<0.05$).

Effect of temperature on the occurrence of rapid necrosis

The first experiment evaluated the time course of the rapid necrosis symptom at low temperature. Initially, plants of the resistant, and the susceptible biotypes were grown in a greenhouse at a temperature of 25 ± 5 to C. Four days before spraying the plants were transferred to a growth chamber (Percival, Boone, IA) at 12 C and 13-h photoperiod (300 µmol m^{-2} s^{-1}). Plants at 10 to 15 cm of height (8 to 10 leaves) were sprayed with 804 g ae ha^{-1} of 2,4-D. Four 12mm-diameter leaf discs were collected from the fifth leaf of four plants at different times after 2,4-D spraying and kept at 10 C. A hydrogen peroxide assay was performed using the 3,3'-diaminobenzidine (DAB) staining method (Thordal-Christensen et al. 1997). The presence of H_2O_2 was visualized by color change (brown) where DAB polymerized with this

compound. The staining associated with H_2O_2 was determined in the Image J program (National Institutes of Health, Bethesda, MD).

The second experiment evaluated the effect of 2,4-D doses and temperatures on the occurrence of RN symptoms. Factor A was the biotypes S and RN. Factor B comprised the temperatures of 12 and 30 C, and factor C was the 2,4-D doses of 50.25; 201; 402; 804, and 1608 g ae ha^{-1}. After spraying half of the plants were kept in a growth chamber (Percival) at 12°C and 13 h photoperiod and the other half was kept in a growth chamber (ATC40, Conviron) at 30°C and 13 h of photoperiod, both with light intensity of 300 μmol m^{-2} s^{-1}. Plant visual injury on a percentage scale was evaluated for RN in the resistant biotype and epinasty in the susceptible biotype at 1 and 21 DAT.

Effect of changes in photosynthesis on the occurrence of rapid necrosis

Plants of the biotype RN at 5 cm in height (4 to 5 leaves) were grown in nutrient solution. Treatments consisted of the herbicide 2,4-D singly or preceded by the application of the photosystem II inhibitor herbicides atrazine (Aclamado BR® SC, Ouro Fino Química S.A, Uberaba, MG, Brazil) and diuron (Diox® SC, Ouro Fino Química S.A) at 100, 500, 1000, 2500, 5000, and 10000 μM and maintained for nine hours. Then, the nutrient solution was renewed, and the herbicide 2,4-D was applied to the nutrient solution at the concentration of 2000 μM and maintained for six hours. After this period, the solution was renewed again. The evaluation of symptoms was performed at 1 DAT using a percentage visual scale, in which 0% corresponds to the absence of injury and 100% to plant death. The resistant plants were evaluated for rapid necrosis and the susceptible plants for the epinasty symptoms. The time for onset of rapid necrosis symptoms after herbicide application was also evaluated at intervals of 15 minutes until 5 h after herbicide spraying.

A second study evaluated the biotype RN submitted to different periods of light. Plants were initially grown in a greenhouse at 25 ± 5 C. When the plants were at 10 to 15 cm of height (8 to 10 leaves), they were transferred to a growth chamber with a temperature of 25 C and absence of light for zero, one, two, and three days before the herbicide treatment. After that, four drops of 2,4-D herbicide were applied with a micropipette at a concentration of 4.02 g ae L^{-1} per leaf sampled. Half of the plants remained in the absence of light and the other half were transferred to a growing chamber with a temperature of 25°C and 400 μmol m^{-2} s^{-1} of light after herbicide application. The evaluation was performed on 11 mm diameter leaf discs collected from the herbicide application site 90 min after application. For each treatment, four leaf discs were collected, and each disc consisted of a repetition. The leaf disc was incubated in a solution with DAB (1 mg mL^{-1}, pH 3.8) at room temperature for eight hours. The staining associated with H_2O_2 was determined for each disc in the Image J program as described earlier. In addition, the plants were photographed at the onset of the symptoms and at 5 h later. The necrotic area of four leaves per treatment was measured using the Image J program. Each experimental unit consisted of a leaf disc obtained from an individual leaf where the herbicide was applied. The time for onset of rapid necrosis symptoms after herbicide application was also recorded for each leaf collected for the necrosis area measurement. An auxiliary green light was used to evaluate the onset of symptoms in plants kept in the dark.

Effect of plant growth stage on the occurrence of rapid necrosis

Factor A was the biotypes S and RN. Factor B corresponded to the plant growth stage 1, corresponding to 5 to 8 cm of height and 10 to 12 leaves plants (S1), stage 2 for 30-45 cm plants with 22-25 leaves (S2), and stage 3 for plants with 45-60 cm and 30-40 leaves (S3). The factor C was herbicides doses of 2,4-D at 50.25; 201; 402; 804; 1608 and 3216 g ae ha^{-1}; dicamba at

30; 120; 240; 480; 960 e 1920 g ae ha^{-1} and triclopyr at 45; 180; 360; 720; 1440 and 2880 g ae ha^{-1}. Visible plant injury on a percentage scale was evaluated at 49 DAT.

Results and Discussion

Dose-response evaluation of auxinic herbicides

In a previous study, the resistant biotype showed RN symptoms to 2,4-D and MCPA herbicides, both classified as phenoxy herbicides, and only showed epinasty symptoms to other auxinic herbicides applied at labeled use rates (Queiroz et al. 2020). However, some field observations have identified the occurrence of RN in overlapping herbicide applications in some populations. In the present study, the effect of several auxinic herbicides was evaluated using dose-response curves. The symptoms of RN were observed only in the biotype RN in response to the 2,4-D herbicide. The other auxinic herbicides dicamba, halauxifen-methyl, triclopyr, fluroxypyr, florpyrauxifen-benzyl, and picloram, even applied at high rates in the dose-response assay promoted only the typical symptom of epinasty and controlled both RN and susceptible biotypes (Figures 1A to 1F). The 2,4-D herbicide controlled susceptible plants with the dose of 804 g ae ha^{-1}, but the resistant biotype showed only 40% control at that dose (Figure 1G). The resistance factor (RF) for 2,4-D at 3 DAT was 0.66, because the susceptible plants were evaluated for epinasty and the resistant plants for the rapid necrosis symptoms, which were equivalent in some doses. At 35 DAT the RF was 7.39 for 2,4-D (Table 1).

Auxinic herbicides are an important group of selective herbicides used to control dicot weeds (Peterson et al. 2016). Resistance to these herbicides limits the options for controlling *Conyza* species, in which herbicide resistance has already been reported to other five mechanisms of action (inhibitors of photosystems I and II, EPSPS, ALS, and PPO inhibitors)

(Santos et al. 2014; Pinho et al. 2019). The results obtained in this study are important to confirm the efficacy of other six auxinic herbicides in the control of the biotype RN.

Rapid necrosis caused by 2,4-D results in antagonism to other auxinic herbicides

The herbicides dicamba, halauxifen-methyl, and triclopyr were applied singly, mixed with, or 4 h after the application of 2,4-D to evaluate the effect of RN on the efficacy of these alternative herbicides. The absence of antagonism was observed in the evaluation at 3 DAT of the simultaneous or sequential application of 2,4-D and either dicamba, halauxifen-methyl, or triclopyr in the biotype RN (Table 2). However, after plant regrowth, the herbicide injury at 35 DAT indicated an antagonism between 2,4-D and these three herbicides for controlling biotype RN when these herbicides were used either in association with or 4 h after 2,4-D (Table 2). After 2,4-D spraying, the R-RN plants developed the symptoms of RN, with partial leaf wilt and necrotic spots that expand over time (Queiroz et al. 2020). It is possible that the leaf necrosis could have reduced the herbicide absorption and mobility from the leaf to the meristems when used in a simultaneous or sequential application. Similar antagonism was found in giant ragweed resistant to glyphosate by RN for five herbicides with different mechanisms of action (Harre et al. 2018b). The prevention of herbicide resistance is dependent on rotation and mixtures of different herbicide mechanisms of action, especially for the increasing problem of resistance in *Conyza* species (Cantu et al. 2021), but the occurrence of antagonism in the RN plants jeopardizes this strategy and increases the herbicide resistance problem. In addition, herbicide resistance management also requires other nonchemical weed control and agronomic measures that contribute to resistance prevention.

Commented [JKN1]: Provide common name.

Low temperature delays the occurrence of RN

298 At a temperature of 25 C or higher, necrosis symptoms were detected in the biotype RN
299 about 2 h after spraying, and leaf desiccation after 1 DAT (Queiroz et al. 2020). Considering
300 the field observation of variable occurrence of RN, we postulated that low temperature might
301 modulate the effect of 2,4-D. Therefore, we evaluated the effect of 2,4-D at the temperature of
302 12°C. In this situation, the RN symptoms were detected only after 1 DAT and were less intense
303 in comparison with the application at 30 C (Figures 2A, B, and C). The typical 2,4-D symptoms
304 of epinasty were also less intense in the susceptible biotype at 12 C in comparison with 30 C
305 (Figures 2A and B). At 21 DAT, the injury caused by 2,4-D was higher in the susceptible
306 biotype in comparison with biotype RN, characterizing the occurrence of resistance to 2,4-D
307 (Figure 2D). In this evaluation, the application of 2,4-D at the temperature of 12 C for biotype
308 RN resulted in lower plant injury in comparison to 30 C (Figure 2D). The obtained results agree
309 with the standard Q10 (temperature quotient) principle in biology, which indicates that for most
310 biochemical reactions the rate of reaction changes by a factor of 2 for every 10 degree C change
311 in temperature.

312 Not only was the necrosis delayed at low temperature, but ROS accumulation was also
313 delayed. Previous results indicated that the onset of ROS accumulation was about 15 min after
314 2,4-D spraying at the temperature of 25 C and high light (848 μmol m^{-2} s^{-1}) and results in about
315 62% of the leaf area stained with DAB (Queiroz et al. 2020). In the present study carried out at
316 12 C, the onset was 60 to 120 min after spraying and ROS formation was maximum at 240 min
317 covering 63% of leaf area (Figure 2E). A similar change has been reported in giant ragweed
318 with the resistance to glyphosate by RN, in which the amount of H_2O_2 at 30 C was more than
319 twice that obtained at 10 C, after 2.5 h of spraying (Harre et al. 2018a). Also, in that study, the
320 temperature affected the occurrence of antagonism with other systemic herbicides. The control
321 of giant ragweed resistant to glyphosate with the association of the herbicides atrazine,
322 cloransulam, dicamba, and topramazone was 12 to 21% less effective than expected at 30 C

and 11 to 16% less efficient at 10 C. Overall, the antagonism was up to 10% greater at 30 C than at 10 C (Harre et al. 2018a).

Effect of light and photosynthesis inhibitors on the occurrence of rapid necrosis

The effect of light and photosynthesis inhibitors could provide insights about the mechanisms behind the symptoms of the RN caused by 2,4-D and regarding the variability of the RN intensity under field conditions. The application of photosystem II inhibitors, atrazine, and diuron in nutrient solution did not delay the onset (Figure 3A) or decrease the intensity (Figure 3B) of RN at any of the concentrations evaluated in relation to the application of the herbicide 2,4-D alone. These results indicate that alterations in plant photosynthesis through photosystem II inhibition do not prevent the occurrence of symptoms related to the resistance in the biotype RN.

In the second study related to the effect on photosynthesis, treatments were evaluated in the presence and absence of light. Plants maintained in the absence of light after the application of the 2,4-D herbicide started the RN symptoms from 134 to 144 min after treatment, and in plants exposed to 400 μmol m^{-2} s^{-1} light after application, the RN symptoms began earlier at 95 to 109 min after treatment (Figure 4A). The periods of one to three days of dark treatment before herbicide spraying resulted in similar results for RN.

Regarding the effect on oxidative stress, no difference was observed in the production of H_2O_2 in leaf discs evaluated at 90 min after herbicide application, independent of the light regime (Figure 4B). Similar results were observed for the area of RN at 5 h after treatment when the RN symptom was consolidated (Figure 4C). These results indicate that the absence of light after herbicide application causes a slight delay in the RN symptoms, but the production of hydrogen peroxide and the size of necrosed area were not affected by the light treatments neither before nor after 2,4-D application.

Similar results were observed in previous studies, wherein low light (29 μmol m^{-2} s^{-1}) delayed the onset of symptoms of RN compared with higher light (848 μmol m^{-2} s^{-1}) (Queiroz et al. 2020). However, the production of H_2O_2 was not dependent on the presence of light in this species and the symptoms also occurred in the dark condition. Another study evaluated the consequences on photosynthesis after 2,4-D application on an RN biotype of Sumatran fleabane (Leal et al. 2022). The photosynthetic performance was reduced by 20% in 1 h after the application of the 2,4-D herbicide, showing lower performance of the electron transport chain. After 4 h of treatment, these metabolic alterations were also observed in the susceptible biotype. Photosynthetic damage was rapidly observed in the resistant compared with the susceptible biotype due to the differential physiological response to 2,4-D (Leal et al. 2022). These symptoms are probably related to the increase of ROS and the effect of the necrosis of the leaf tissue on photosynthesis.

The resistance mechanism of RN is affected by light intensity and temperature. In low light there is a delay of 3 h for the symptom onset and the amount of H_2O_2 accumulated is also reduced in comparison to high light (Queiroz et al. 2020). Here, we report that low temperature (12°C) causes a stronger effect; the symptoms were only manifested 1 DAT and were subtler, H_2O_2 began to accumulate between 60 and 120 min. As discussed before, these interactions between environmental conditions and the RN mechanism have been reported also for the resistance to glyphosate found in giant ragweed (Moretti et al. 2017; Harre et al. 2018a). These findings are important because, in the field, where the environment is more variable than in greenhouse or growth chamber experiments, these interactions may confound the diagnostic of RN and influence the efficacy of other herbicides when applied in association with 2,4-D.

Effect of plant growth stage

Plant regrowth at 49 DAT characterizes plant survival after the occurrence of RN in the biotype RN as determined in previous studies (Queiroz et al. 2020). Resistant plants were effectively controlled only with 2,4-D doses higher than 1340 g ae ha^{-1} applied in plants at stage 1 (5 to 8 cm and 10 to 12 leaves). At this stage, the susceptible plants were controlled with the dose of 50.25 g ae ha^{-1} (Figure 5A). The RF for biotype RN in comparison with biotype S was 7.9, 82.5, and 24.8 for applications to stages 1, 2, and 3, respectively (Table 3). Despite the lower RF in stage 1 in comparison with stages 2 and 3, the occurrence of plant regrowth at 49 DAT was observed in treatments with the recommended dose of 804 g ae ha^{-1}.

The RN symptoms were not observed for dicamba and triclopyr treatments in resistant plants, regardless of the dose used and growth stage at application. These plants showed similar symptoms of epinasty to the biotype S for both herbicides, after one day of application (data not shown). The efficacy of dicamba and triclopyr between the biotypes RN and S was similar for application at stages 1 and 2 (Figures 5B and 5C). However, at stage 3, control of the biotype RN was inferior to the biotype S at doses higher than 120 g ae ha^{-1} of dicamba and for the doses of 45 to 360 g ae ha^{-1} of triclopyr (Figures 5B and 5C). The recommended stage of application is stage 1 (5 to 8 cm and 10 to 12 leaves). The other two evaluated stages (30 to 45 cm and 22 to 25 leaves; 45 to 60 cm and 30 to 40 leaves) represent situations where late burndown applications are necessary, which frequently occurs in farm situations. Although the auxinic herbicides are not recommended for application on plant stages 2 and 3 this is evidence of decreased efficacy of dicamba and triclopyr for the 2,4-D resistant plants caused by RN.

Several studies indicate satisfactory control of *Conyza* plants with the herbicide dicamba, even in advanced stages of growth (Kruger et al. 2010). For 2,4-D, however, the plant stage is important for *Conyza* control (Oliveira Neto et al. 2010; Walker et al. 2012). When evaluating hairy fleabane [*Conyza bonariensis* (L.) Cronq.] plants at rosettes of 5 cm and 10 to 15 cm in diameter to the effect of 2,4-D at 940 and 1250 g ae ha^{-1}, Walker et al. (2012) found 36% lower

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control effectiveness for taller plants compared to the 5-cm diam stage, despite the dose increase in late application. However, in a study with glyphosate-resistant horseweed, there was no growth stage effect with 2,4-D when 560 g ae ha^{-1} of the herbicide was applied to plants with heights of 0 to 7;7 to 15; 15 to 30, and 30 to 45 cm (Kruger et al. 2010). The environmental conditions may complicate further the effect of the plant growth stage on the control of *Conyza* species by auxinic herbicides. The current study indicates that RN-resistant plants treated at early growth stage, such as 1 (5 to 8 cm and 10 to 12 leaves) are more affected by 2,4-D than at later herbicide applications. The herbicides dicamba and triclopyr are less effective on plants of the biotype RN treated at growth stage 3 (45-60 cm and 30-40 leaves).

In conclusion, the auxinic herbicides dicamba, triclopyr, and halauxifen-methyl do not cause RN symptoms and are effective at controlling the RN 2,4-D resistant biotype when applied without 2,4-D use. However, the effectiveness of these herbicides was reduced when sprayed on the resistant biotype either together, 4 h or 24 h after 2,4-D herbicide. The temperature at spraying time modulates the occurrence of RN. Application at 12 C delays the symptoms and decreases its intensity, but still results in plant survival after 2,4-D application. The absence of light after herbicide application causes a slight delay in the RN symptoms, but the production of hydrogen peroxide and the size of necrotic area are not affected by the light treatments either before or after 2,4-D application. The RN phenotypic expression does not occur for the herbicides dicamba and triclopyr, even in advanced plant growth stages of application and high doses. The RN-resistant plants treated at the early plant stages of 5-8 cm and 10 to 12 leaves are more affected by 2,4-D than at later herbicide applications. The herbicides dicamba and triclopyr are less effective on older plants of the biotype RN (45 to 60 cm and 30 to 40 leaves). This study identified that environmental, plant growth stage effects, and herbicide interactions can interfere with the occurrence of RN caused by 2,4-D in Sumatran

fleabane and are important for identifying the causes of variability in herbicide symptomology and performance under experimental and field conditions. Future research through transcriptome and genetic mapping will be important to characterize the mechanism of resistance related to rapid necrosis caused by 2,4-D in Sumatran fleabane.

Acknowledgments

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Conflicts of Interest

No conflicts of interest have been declared.

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523 230
524

525 **Table 1** Log-logistic equation parameters and resistance factors for herbicide control at 35 days
526 after treatment (DAT) for Sumatran fleabane biotypes RN (2,4-D rapid necrosis resistant) and
527 S (2,4-D susceptible), for seven auxinic herbicides and at 03 DAT after application of 2,4-D.

Herbicide	Biotype	Evaluation	*b*	*c*	*d*	*e* (ED_{50})	ED_{80}	RF
				%		g ae ha^{-1}		
dicamba	S	35 DAT	-1.20*	3.10^{ns}	100	34.46*	109.25*	
	RN		-0.91*	3.37^{ns}	100	42.05*	194.06*	1.22^{ns}
halauxifen-methyl	S	35 DAT	-0.78*	-0.49^{ns}	100	0.15*	0.87*	
	RN		-0.42*	0.85^{ns}	100	0.61*	13.89*	4.13^{ns}
triclopyr	S	35 DAT	-0.97*	0.53^{ns}	100	25.03*	104.87*	
	RN		-0.99*	1.16^{ns}	100	36.37*	147.29*	1.45^{ns}
fluroxypyr	S	35 DAT	-1.42*	4.48^{ns}	100	41.15*	109.49*	
	RN		-1.37*	1.52^{ns}	100	37.16*	102.38*	0.90^{ns}
florpyrauxifen-benzyl	S	35 DAT	-0.93*	3.08^{ns}	100	1.57*	7.01*	
	RN		-0.98*	0.04^{ns}	100	1.45*	5.97*	0.92^{ns}
picloram	S	35 DAT	-1.36*	0.10^{ns}	100	7.87*	21.80*	
	RN		-1.07*	0.10^{ns}	100	8.94*	32.74*	1.14^{ns}
2,4-D	S	35 DAT	-2.52*	3.98^{ns}	100	108.41*	188.05*	
	RN		-4.11*	28.24*	100	801.00*	3483.60*	7.39*
2,4-D	S	03 DAT	-0.38*	-1.93^{ns}	100	1214.51*	46593.1*	
	RN		-0.46*	0.77^{ns}	100	799.63*	11652.0*	0.66^{ns}

528

529 Difference * statistically significant or ^{ns} not statistically significant for parameter b (curve
530 slope) with 0; parameter c (lower limit) with 0; parameter d (upper limit) with 100; parameter
531 e (effective dose for 50 % control) between S and RN biotypes; RF (resistance factor) with 1.

532

Table 2 Rapid necrosis (%) at 3 days after treatment (DAT), injury (%) at 35 DAT, expected effect (Exp.) of the association, and the result of the interaction (Int.) of the 2,4-D herbicide mixed with dicamba, halauxifen-methyl or triclopyr, according to the method proposed by Colby (1967) on the Sumatran fleabane biotype RN (2,4-D rapid necrosis resistant).

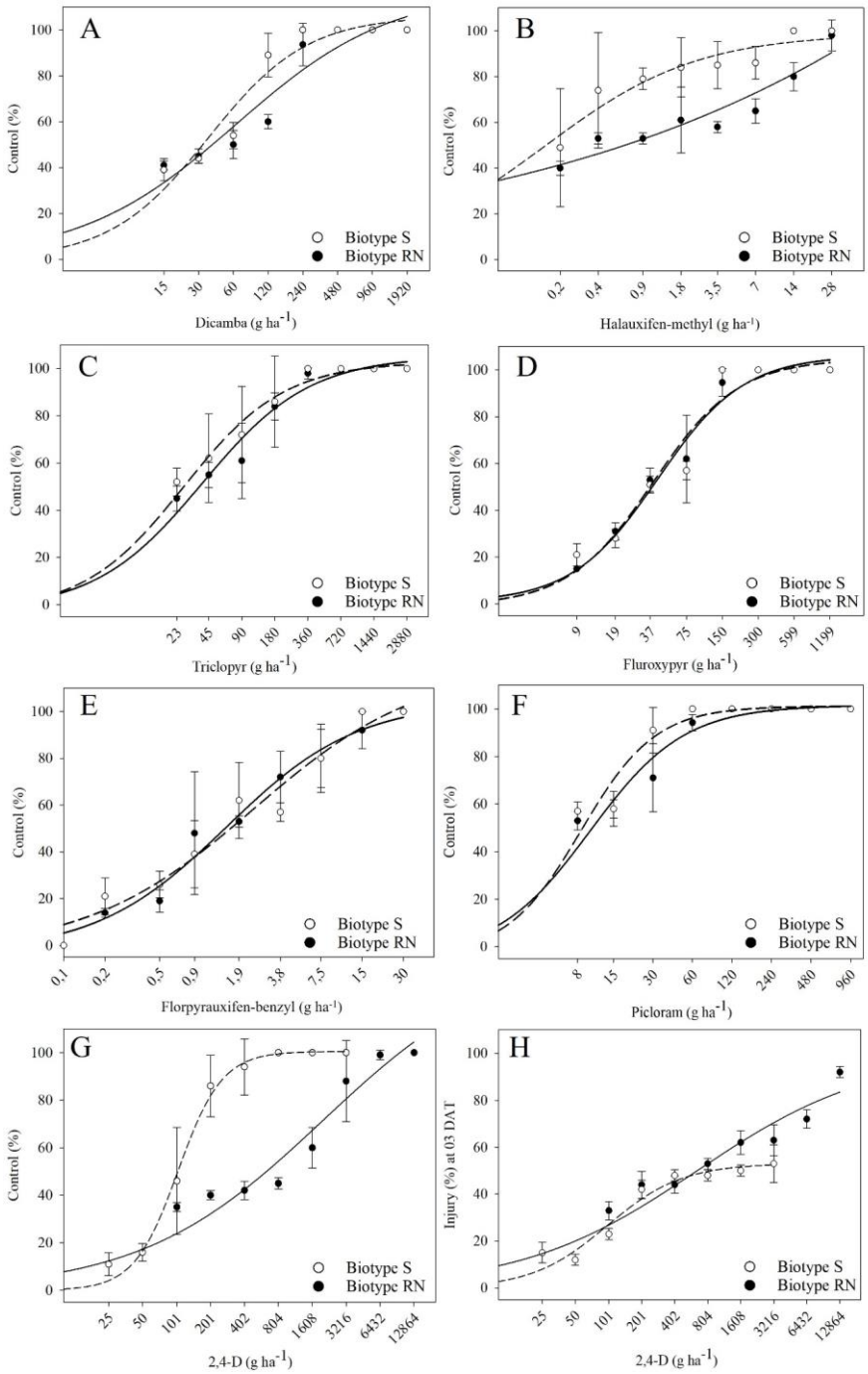
Treatment	*Rapid necrosis - 03DAT*		Colby		*Injury - 35 DAT*		Colby	
			Exp.	Int.			Exp.	Int.
	%				*%*			
Untreated control	0.0	d	-		0.0	g	-	
2,4-D	38,1	c	-		46.2	ef	-	
Dicamba	0.0	d	-		76.9	bcd	-	
Halauxifen-methyl	0.0	d	-		47.5	ef	-	
Triclopyr	3.6	d	-		99.4	a	-	
2,4-D + dicamba	40.0	c	38.1^{ns}	additive	76.3	bcd	88.6^{ns}	additive
2,4-D + halauxifen-methyl	41.9	c	38.1^{ns}	additive	57.5	def	71.5*	antagonism
2,4-D + triclopyr	52.5	a	40.3*	synergism	86.3	abc	99.6*	antagonism
Dicamba 4 h after 2,4-D	40.6	c	38.1^{ns}	additive	67.5	cd	88.6*	antagonism
Halauxifen-methyl 4 h after 2,4-D	42.5	c	38.1^{ns}	additive	57.5	def	71.5*	antagonism
Triclopyr 4 h after 2,4-D	49.4	ab	40.3*	synergism	91.3	ab	99.6^{ns}	additive
Dicamba 24 h after 2,4-D	38.1	c	38.1^{ns}	additive	70.0	cd	88.6*	antagonism
Halauxifen-methyl 24 h after 2,4-D	43.1	bc	38.1^{ns}	additive	66.3	cd	71.5^{ns}	additive
Triclopyr 24 h after 2,4-D	40.6	c	40.3^{ns}	additive	86.3	abc	99.6*	antagonism

Lower letters compare means among treatments inside each column by Tukey´s HSD test ($p<0.05$). *Significant difference between the observed and expected values by t-test ($p<0.05$); ^{ns} Non-significant difference between the observed and expected values by t-test ($p<0.05$).

Table 3 Log-logistic equation parameters and resistance factors for herbicide control at 49 days after treatment (DAT) for Sumatran fleabane biotypes RN (2,4-D rapid necrosis resistant) and S (2,4-D susceptible), after application of 2,4-D, dicamba and triclopyr in three plant growth stages in the application (S1: 5-8 cm and 10-12 leaves; S2: 30-45 cm and 22-25 leaves; S3: 45-60 cm and 30-40 leaves).

Biotype	Herbicide	*b*	*c*	*d*	*e* (ED_{50})	ED_{80}	RF
		%			g ae ha^{-1}		
Stage 1 (5-8 cm and 10-12 leaves)							
S	2,4-D	-6.27^{ns}	0.01^{ns}	100	43.05^{ns}	53.71*	
RN		-0.30*	0.61*	100	341.46*	803.92*	7.93*
S	dicamba	-3.18*	-0.08^{ns}	100	18.93*	29.24*	
RN		-2.62*	-0.08*	100	22.64*	39.05*	1.20^{ns}
S	triclopyr	-0.92*	0.10*	100	11.62*	30.33*	
RN		-4.26*	0.10*	100	41.91*	57.14*	3.61*
Stage 2 (30-45 cm and 22-25 leaves)							
S	2,4-D	-0.88*	0.02^{ns}	100	47.89*	187.45*	
RN		-0.25*	0.04*	100	3950.12*	18601.00	82.48*
S	dicamba	-0.47*	0.12^{ns}	100	68.72*	164.74*	
RN		-0.71*	0.37^{ns}	100	95.37*	351.49*	1.39^{ns}
S	triclopyr	-0.64*	0.23^{ns}	100	65.05*	224.46*	
RN		-1.22*	0.17^{ns}	100	69.48*	314.08*	1.01^{ns}
Stage 3 (45-60 cm and 30-40 leaves)							
S	2,4-D	0.84*	0.42^{ns}	100	195.17*	405.65*	
RN		0.63*	0.12^{ns}	69.46*	4845.96*	33271.00*	24.83*
S	dicamba	-0.90*	-0.22*	100	59.23*	227.11*	
RN		-0.30*	-0.45*	100	139.42*	4572.51*	2.35*
S	triclopyr	-0.84*	0.23^{ns}	100	72.05*	230.96*	
RN		-0.62*	1.48^{ns}	100	397.46*	749.11*	5.52^{ns}

Difference * statistically significant or ^{ns} not statistically significant for parameter b (curve slope) with 0; parameter c (lower limit) with 0; parameter d (upper limit) with 100; parameter e (effective dose for 50 % control) between S and RN biotypes; RF (resistance factor) with 1.


A
Control (%)
Biotype S
Biotype RN
15 30 60 120 240 480 960 1920
Dicamba (g ha-1)
B
Control (%)
Biotype S
Biotype RN
0,2 0,4 0,9 1,8 3,5 7 14 28
Halauxifen-methyl (g ha-1)
C
Control (%)
Biotype S
Biotype RN
23 45 90 180 360 720 1440 2880
Triclopyr (g ha-1)
D
Control (%)
Biotype S
Biotype RN
9 19 37 75 150 300 599 1199
Fluroxypyr (g ha-1)
E
Control (%)
Biotype S
Biotype RN
0,1 0,2 0,5 0,9 1,9 3,8 7,5 15 30
Florpyrauxifen-benzyl (g ha-1)
F
Control (%)
Biotype S
Biotype RN
8 15 30 60 120 240 480 960
Picloram (g ha-1)
G
Control (%)
Biotype S
Biotype RN
25 50 101 201 402 804 1608 3216 6432 12864
2,4-D (g ha-1)
H
Injury (%) at 03 DAT
Biotype S
Biotype RN
25 50 101 201 402 804 1608 3216 6432 12864
2,4-D (g ha-1)

551

552 **Figure 1** Dose-response curves for Sumatran fleabane biotypes RN (2,4-D rapid necrosis
553 resistant) and S (2,4-D susceptible) at 35 days after treatment (DAT) to dicamba (A),
554 halauxifen-methyl (B), triclopyr (C), fluroxypyr (D), florpyrauxifen-benzyl (E), picloram (F)
555 and 2,4-D (G) and at 03 DAT to 2,4-D (H). Vertical bars indicate the confidence interval (α =
556 0.05).

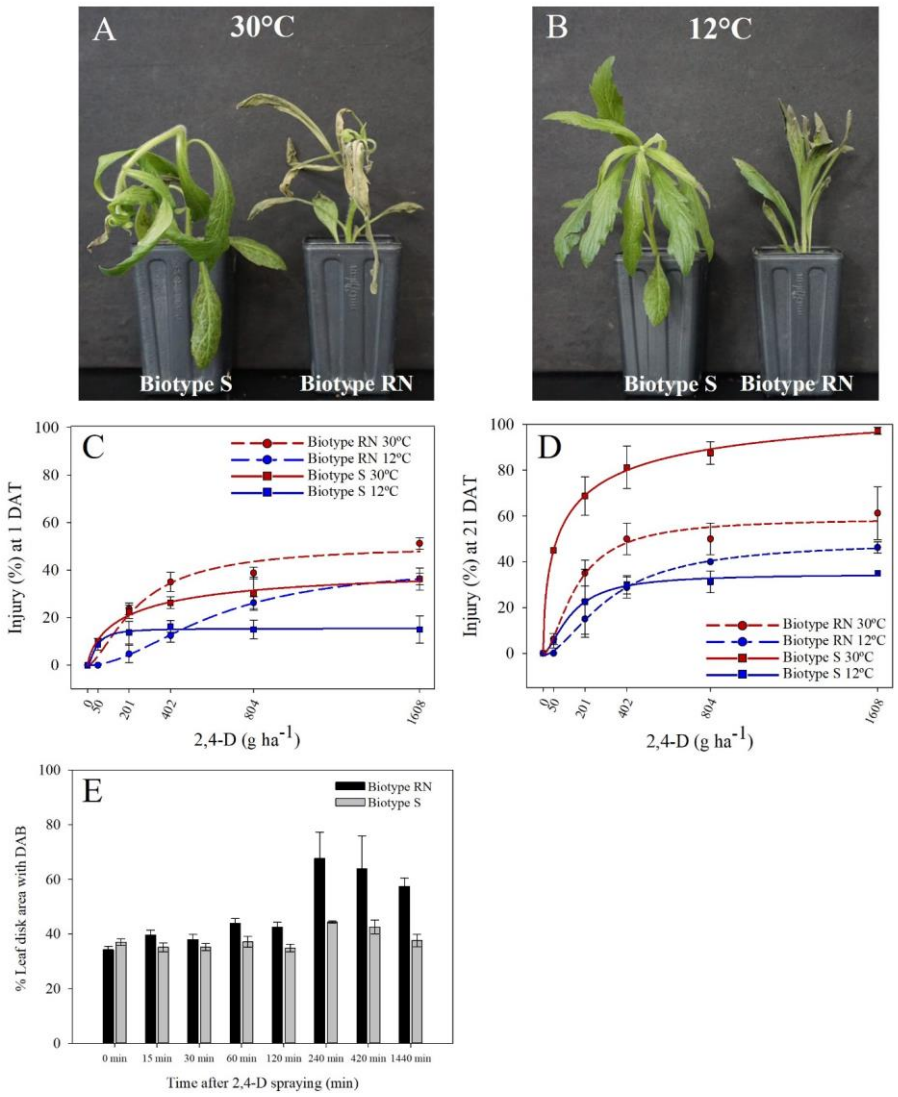


Figure 2 Effect of temperature on 2,4-D symptoms on Sumatran fleabane biotypes RN (2,4-D rapid necrosis resistant) and S (2,4-D susceptible) at one day after treatment (DAT) with 804 g ae ha^{-1} 2,4-D at 30 C (A) and 12 C (B), plant injury (%) after 2,4-D application at 12 and 30 C evaluated at 1(C) and 21 DAT (D) and ROS accumulation (%) at different times after 2,4-D spraying at 12 C (E). Vertical bars indicate the confidence interval ($\alpha = 0.05$).

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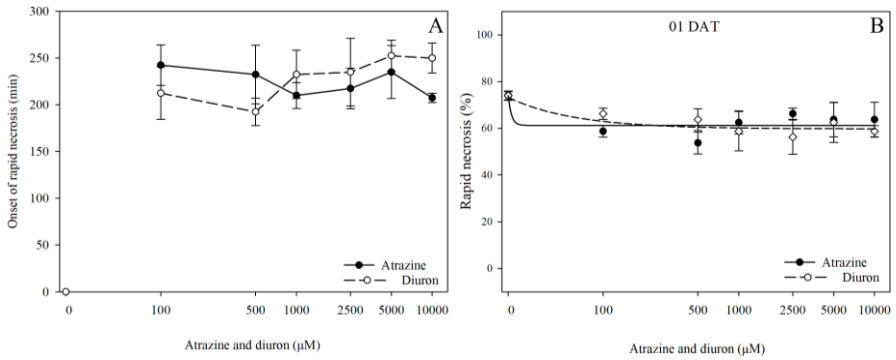
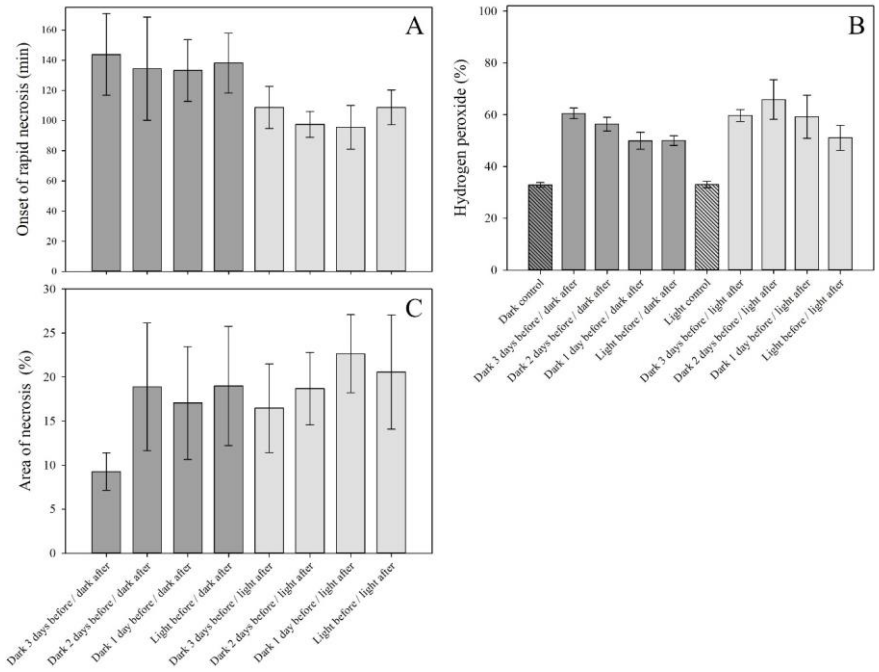


563

564 **Figure 3** Effect of photosystem II inhibitors atrazine and diuron before application of 2,4-D
565 (2000 µM) on the onset of symptoms (A) and injury (%) from rapid necrosis one day after
566 treatment (DAT) (B) on Sumatran fleabane biotype RN (2,4-D rapid necrosis resistant).
567 Vertical bars indicate the confidence interval ($\alpha = 0.05$).

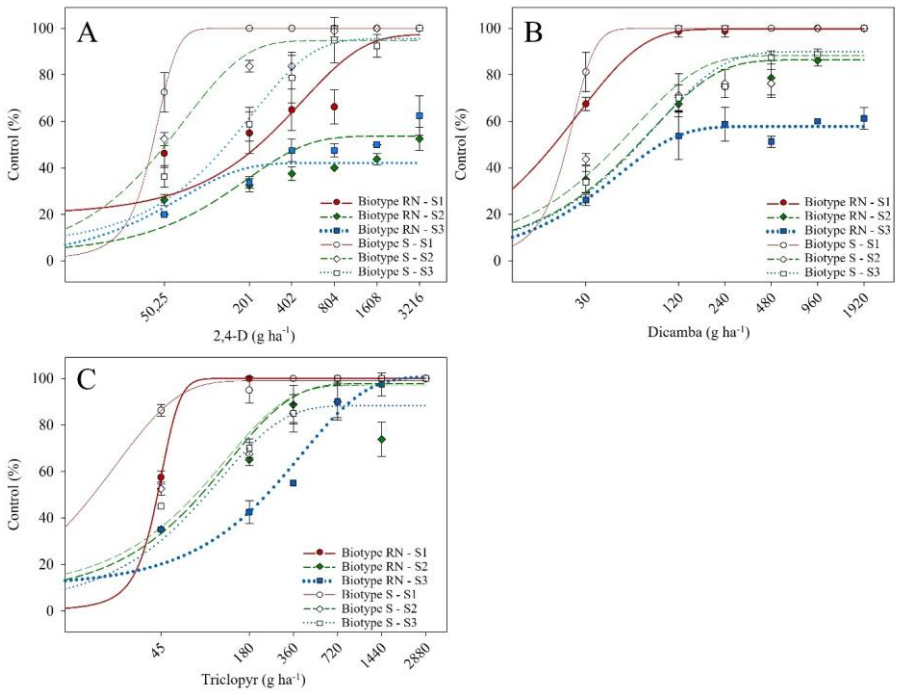
568



569

570 **Figure 4** Onset of symptoms in minutes (min) (A), accumulation of hydrogen peroxide (%) (B)
571 at 90 min after treatment, and area of leaf necrosis (%) (C) at 5 h after 2,4-D application (4.02
572 g ae L^{-1}) in plants kept under different light conditions on Sumatran fleabane biotype RN (2,4-
573 D rapid necrosis resistant). Dark control: untreated plants kept in the absence of light; light
574 (intensity 400 μmol m^{-2} s^{-1}). Vertical bars indicate the confidence interval ($\alpha = 0.05$).

575



576

577 **Figure 5** Efficacy of control (%) at 49 days after treatment (DAT) of Sumatran fleabane
578 biotypes RN (2,4-D rapid necrosis resistant) and S (2,4-D susceptible) at three plant growth
579 stages. Dose-response curves to 2,4-D (A), dicamba (B) and triclopyr (C) (S1: 5-8 cm and 10-
580 12 leaves; S2: 30-45 cm and 22-25 leaves; S3: 45-60 cm and 30-40 leaves). Vertical bars
581 indicate the confidence interval ($\alpha = 0.05$).

Short Title: Rapid necrosis and 2,4-D resistance

Title: Rapid necrosis: Implications of environmental conditions and plant growth stage on 2,4-D resistance and effect of other auxinic herbicides in Sumatran fleabane (*Conyza sumatrensis*)

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Abstract

Resistant plants of Sumatran fleabane with an unusual rapid necrosis symptom after application of 2,4-D were characterized in previous studies. Field observations indicated variability in the occurrence of the rapid necrosis (RN) caused by 2,4-D, but the causes of the variation are unknown. This study aimed to investigate the effect of environmental conditions, plant growth stage, and simultaneous and sequential herbicide mixtures with other auxin mimics on the occurrence of RN caused by 2,4-D. Application at temperature of 12 C delayed the symptoms and decreased the intensity of the RN, but still resulted in plant survival to 2,4-D. The absence of light after herbicide application caused a slight delay in the symptoms, but the production of hydrogen peroxide and the size of necrosed area were not affected by the light treatments before and after 2,4-D application. Changes in plant photosynthesis through inhibiting photosystem II do not prevent the occurrence of the RN symptom. The auxinic herbicides dicamba, triclopyr, and halauxifen-methyl do not cause RN symptoms and are effective at controlling the resistant biotype when applied without 2,4-D, but the effectiveness of these herbicides was reduced when sprayed on the resistant biotype either together, 4 h or 24 h after 2,4-D. The RN phenotype does not occur for dicamba and triclopyr, even in advanced plant growth stages and high doses on the resistant biotype. The herbicides dicamba and triclopyr effectively controlled resistant plants, especially when sprayed at the initial growth stages. The results of this study identified environmental, plant development effects, and herbicide interactions, that interfere with the occurrence of RN symptoms caused by 2,4-D in Sumatran fleabane. These data provide insights about the mechanisms behind the RN symptoms caused by 2,4-D and are important for identifying the causes of variability of the herbicide symptomology and performance under experimental and field conditions.

49 **Nomenclature:** 2,4-D; Sumatran fleabane, *Conyza sumatrensis* (Retz.) E. Walker, ERISU

50

51 **Keywords:** auxinic herbicide resistance; low temperature; light effect; photosynthesis
52 inhibitors; plant growth stage

Introduction

Species of the genus *Conyza* are important weeds due to their high abundance, easy seed dispersion, and occurrence of hybridization. These species are cosmopolitan weeds, that settle mainly in disturbed areas (Tremmel and Peterson 1983). The germination and establishment in the crop fields occur mainly during the late fall to winter, which in Brazil are fallow or cultivated with pastures, cover crops, or winter grain cereals (Vidal et al. 2007). The seeds are positive photoblastic and do not germinate in soil depths greater than 0.5 cm (Nandula et al. 2006). Generally, the *Conyza* seeds germinate between 10 to 25 C, and 20 C is regarded as optimum for germination (Zinzolker et al. 1985). The wide genetic diversity of *Conyza* species also favors the emergence of herbicide-resistant biotypes (Bajwa et al. 2016). Herbicide resistance is one of the largest agricultural problems. In Brazil, herbicide resistance is estimated to occur on 20.1 million ha, resulting in US $1,63 billion yearly losses (Adegas et al. 2017). In this country, the most important herbicide resistant weeds are *Conyza* sp., sourgrass (*Digitaria insularis (L.) Mez ex Ekman*), italian ryegrass (*Lolium perenne* L. ssp. *multiflorum* (Lam.) Husnot), goosegrass (*Eleusine indica* (L.) Gaertner), and *Echinochloa* sp. (Adegas et al. 2022; Heap 2022). Cross-resistance occurs in Sumatran fleabane, and cases of glyphosate (5-enolypyruvyl-shikimate-3-phosphate synthase - EPSPS inhibitor, HRAC code 9) and chlorimuron (acetolactate synthase - ALS inhibitor, 2) double resistance have been in Brazil since 2011, limiting the use of these two mechanisms of action (Santos et al. 2014). Following the appearance of resistance, herbicides with other mechanisms of action were used to control the resistant population, mainly 2,4-D, an auxinic herbicide (4); the photosystem I (PSI, 22) inhibitors paraquat and diquat, ammonium glufosinate, an inhibitor of the enzyme glutamine synthetase (GS, 10), and saflufenacil, an inhibitor of the enzyme protoporphyrinogen oxidase (PPO, 14). However, the intensification of the use of these herbicides has contributed to the

emergence of biotypes resistant to these mechanisms of action. In fact, in Brazil, cross-resistance was identified in Sumatran fleabane to paraquat, chlorimuron, and glyphosate in 2016 (Albrecht et al. 2020), and to 2,4-D, paraquat, diuron, glyphosate and saflufenacil, in 2017 (Pinho et al. 2019).

A unique case of resistance to the herbicide 2,4 D with an unusual resistance mechanism was identified in a biotype of Sumatran fleabane from the state of Paraná, Brazil in 2015. Rapid necrosis (RN) symptoms begin about 2 h after herbicide spraying and later the plants regrow from the axillary buds, resulting in a resistance factor of 18.6 compared with a susceptible biotype (Queiroz et al. 2020). Recently, a second study on this case of resistance identified that the RN mechanism may be related to changes in auxin transport or in the Transport Inhibitor Response 1 (TIR1) receptor, and it is not related to the 2,4-D detoxification by glutathione-S-transferase or cytochrome P450 monooxygenase enzymes (Queiroz et al. 2022). Furthermore, the oxidative stress related to RN was responsive to temperature and was not light-dependent in Sumatran fleabane resistant plants that also showed rapid photosynthetic damage (Leal et al. 2022). There is no report of other species showing similar resistance to auxinic herbicides in the literature (Figueiredo et al. 2022; Peterson et al. 2016). However, a similar phenotype has been reported in giant ragweed (*Ambrosia trifida* L.) resistant to glyphosate in the USA (Brabham et al. 2011). This mechanism has been proposed to increase the production of hydrogen peroxide and it is influenced by temperature and light (Harre et al. 2018a; Moretti et al. 2017). In the resistant biotype of giant ragweed, the RN limited the action of other herbicides and caused antagonism between glyphosate and the herbicides atrazine, cloransulam, dicamba, lactofen, and topramezone (Harre et al. 2018b). Despite their similarity, the 2,4-D RN-resistant biotype of Sumatran fleabane does not develop the RN symptoms in response to glyphosate (Queiroz et al. 2020).

A previous study identified that the RN caused by 2,4-D in Sumatran fleabane was influenced by temperature, indicating the possible involvement of metabolic and/or transporter proteins (Leal et al. 2022). There are only a few studies about the influence of the temperature on the 2,4-D efficacy in plants of the genus *Conyza* even in susceptible biotypes (Montgomery et al. 2017; Silva et al. 2021). A study in horseweed [*Conyza canadensis* (L.) Cronq.] identified higher control efficiency of 2,4-D at noon (11 to 13:30 h, 16-26 C) than in the early morning (6 to 6:30, 6-13°C) (Montgomery et al. 2017). In general, low temperatures reduce the efficacy of auxinic herbicides due to a reduction in herbicide uptake and translocation (Richardson 1977).

The occurrence of rapid necrosis has been reported as a variable in field conditions. Anecdotal evidence related to temperature and light has been associated with the low effect of the herbicide 2,4-D and with the intensity of the rapid necrosis. A previous study indicated that under low light (29 μmol m^{-2} s^{-1}) the H_2O_2 production was reduced in Sumatran fleabane, and the onset of RN symptoms was delayed in comparison to high light conditions (848 μmol m^{-2} s^{-1}) (Queiroz et al. 2020). A similar response was observed in another 2,4-D resistant biotype of Sumatran fleabane, which showed similar levels of H_2O_2 under dark and under light (520 μmol m^{-2} s^{-1}) conditions, and it was higher in the resistant biotype than in the susceptible biotype (Leal et al. 2022). Another factor affecting the onset of rapid necrosis is the plant growth stage in the timing of herbicide spraying which is variable in field conditions. Due to the increasing occurrence of plants with rapid necrosis caused by 2,4-D, there is a necessity for more information on the effect of mixtures of 2,4-D and other auxinic herbicides to control resistant biotypes. In addition, alternative herbicides can also be applied after the visualization of the rapid necrosis, and the efficacy of such applications is also unknown. The aim of this study was to investigate the effect of environmental conditions, plant growth stage, and simultaneous and sequential herbicide mixtures on the occurrence of rapid necrosis caused by 2,4-D in Sumatran fleabane.

Material and Methods

Plant Material and data analysis

The resistant biotype MARPR9-RN (biotype RN) was collected in the city of Maripá, Paraná, Brazil (24.55°S, 53.72°W) and the susceptible biotype LONDS4-S (biotype S) was collected in Londrina, Paraná, Brazil (23.33°S, 51.21°W). Both biotypes were described in Queiroz et al. (2020). Resistant plants were bagged and selfed for two generations after selection with 804 g ae ha^{-1} 2,4-D (DMA® 806 BR SL, DMA® 806 BR SL, Corteva Agrisciences, São Paulo, SP, Brazil; labeled use rate of 1005 g ae ha^{-1} for Sumatran fleabane control) in a greenhouse to produce the seeds (Queiroz et al. 2020). Sowing was carried out in plastic trays measuring 15 cm by 10 cm, filled with substrate. The trays were maintained in a greenhouse at 28 ± 5°C and daily irrigated to promote seed germination. One seedling at the stage of four immature leaves was transplanted into individual 200 mL plastic pots previously filled with substrate, maintained in a greenhouse, and irrigated daily. All the studies were conducted twice in a completely randomized design with four replicates. The statistical software R v.4.2.1 was used for data analysis (R Core Team 2022). Data were submitted to the non-parametric tests of Shapiro-Wilk and histogram to verify the normal distribution and transformed as necessary. After that, data were submitted to ANOVA, and when significant ($p \leq 0.05$) the means were compared by Tukey´s HSD test ($p \leq 0.05$) using the *Expdes.pt* package (Ferreira et al. 2021). Herbicide dose-response curves were adjusted using the three-parameter nonlinear log-logistic model using the *drc* package (Ritz et al. 2015). Data from two replicates of each experiment were submitted to Bartlett's test for homogeneity of variance using the car

package, and when considered homogeneous, the data were analyzed together. All the repeated experiments were similar, and the replications of each experiment were analyzed together.

Dose-response evaluation of seven auxinic herbicides

The study evaluated the occurrence of rapid necrosis and plant in response to increasing doses of auxinic herbicides. Resistant and susceptible plants at 10-15 cm of height (8-10 leaves) were sprayed with the herbicides dicamba (Clarity® SL, BASF, Durham, NC, USA) at 15, 30, 60, 120, 240, 480, 960 and 1920 g ae ha^{-1}; halauxifen-methyl (Arylex™ SC, Dow AgroSciences Industrial) at 0.2, 0.4, 0.9, 1.8, 3.5, 7, 14 and 28 g ae ha^{-1}; triclopyr (Garlon 480 BR® EC, Dow AgroSciences Industrial) at 23, 45, 90, 180, 360, 720, 1440 and 2880 g ae ha^{-1}; fluroxypyr (Starane® EC, Dow AgroSciences Industrial) at 9, 19, 37, 75, 150, 300, 599 and 1199 g ae ha^{-1}; florpyrauxifen-benzyl (Loyant™ SL, Dow AgroSciences, Indianapolis, IN, USA) at 0.2, 0.5, 0.9, 1.9, 3.8, 7.5, 15 and 30 g ae ha^{-1}; picloram (Padron® SL, Dow AgroSciences Industrial) at 8, 15, 30, 60, 120, 240, 480 and 960 g ae ha^{-1}. For the herbicide 2,4-D the rates for susceptible biotype were 25, 50, 101, 201, 402, 804, 1608, and 3216 g ae ha^{-1} and for the resistant biotype were 101, 201, 402, 804, 1608, 3216, 6432 and 12864 g ae ha^{-1}. The considered labeled rate for Sumatran fleabane control was 560 g ae ha^{-1} of dicamba, 7 g ae ha^{-1} of halauxifen-methyl, and 1005 g ae ha^{-1} of 2,4-D. The dose for the other herbicides was selected based on the recommendation for similar species because there is no label recommendation for *Conyza* species. The label rates considered were 720 g ae ha^{-1} of triclopyr, 300 g ae ha^{-1} of fluroxypyr, 7.5 g ae ha^{-1} of florpyrauxifen-benzyl, and 360 g ae ha^{-1} for picloram. Plants were sprayed in a spray chamber (Generation III Research Sprayer, DeVries Manufacturing, Hollandale, MN) calibrated at 262 kPa delivered by a TJ8002E nozzle, resulting in an output volume equivalent to 200 L ha^{-1}. Plant injury was evaluated by a visual percentage scale rating the RN in the

biotype RN and the occurrence of epinasty in the susceptible biotype at 35 d after treatment (DAT), where 0% corresponded to the absence of symptoms and 100% to total plant control.

Effect of the rapid necrosis on the effect of other auxinic herbicides

Plants of the biotypes RN and S at 10-15 cm of height (8-10 leaves) were sprayed with the herbicides 2,4-D at 670 g ae ha^{-1} alone and in a simultaneous mixture with dicamba at 480 g ae ha^{-1}, halauxifen-methyl at 7 g ae ha^{-1}, or triclopyr at 720 g ae ha^{-1}. These herbicides were also applied 4 and 24 h after 2,4-D spraying. The occurrence of RN was evaluated at 3 DAT and plant injury at 35 DAT as described above. Data were submitted to ANOVA, $p \leq 0.05$, and means were compared by Tukey´s test ($p \leq 0.05$). Analysis of the effect of interactions between herbicides was performed using the Colby method (Colby 1967), which compares the effect of control of herbicides in mixture with the effect of the herbicides used alone, and reveals additive, synergistic or antagonistic responses. Synergism occurs when the observed effect is higher than the expected effect of the mixture, antagonism occurs when the observed effect is less than expected, and the additive response occurs when the observed effect is equal to the expected. Expected and observed values were compared using the t-test ($p<0.05$).

Effect of temperature on the occurrence of rapid necrosis

The first experiment evaluated the time course of the rapid necrosis symptom at low temperature. Initially, plants of the resistant, and the susceptible biotypes were grown in a greenhouse at a temperature of 25 ± 5°C. Four days before spraying the plants were transferred to a growth chamber (Percival, Boone, IA) at 12 C and 13 h of photoperiod (300 μmol m^{-2} s^{-1}). Plants at 10-15 cm of height (8-10 leaves) were sprayed with 804 g ae ha^{-1} of 2,4-D. Four

12mm-diameter leaf discs were collected from the fifth leaf of four plants at different times after 2,4-D spraying and kept at 10 C. A hydrogen peroxide assay was performed using the 3,3'-diaminobenzidine (DAB) staining method (Thordal-Christensen et al. 1997). The presence of H_2O_2 was visualized by color change (brown) where DAB polymerized with this compound. The staining associated with H_2O_2 was determined in the Image J program (National Institutes of Health, Bethesda, MD).

The second experiment evaluated the effect of 2,4-D doses and temperatures on the occurrence of RN symptoms. Factor A was the biotypes S and RN. Factor B comprised the temperatures of 12 and 30°C, and factor C was the 2,4-D doses of 50.25; 201; 402; 804, and 1608 g ae ha^{-1}. After spraying half of the plants were kept in a growth chamber (Percival) at 12°C and 13 h photoperiod and the other half was kept in a growth chamber (ATC40, Conviron) at 30°C and 13 h of photoperiod, both with light intensity of 300 μmol m^{-2} s^{-1}. Plant visual injury on a percentage scale was evaluated for RN in the resistant biotype and epinasty in the susceptible biotype at 1 and 21 DAT.

Effect of changes in photosynthesis on the occurrence of rapid necrosis

Plants of the biotype RN at 5 cm in height (4-5 leaves) were grown in nutrient solution. Treatments consisted of the herbicide 2,4-D singly or preceded by the application of the photosystem II inhibitor herbicides atrazine (Aclamado BR® SC, Ouro Fino Química S.A, Uberaba, MG, Brazil) and diuron (Diox® SC, Ouro Fino Química S.A) at 100, 500, 1000, 2500, 5000, and 10000 μM and maintained for nine hours. Then, the nutrient solution was renewed, and the herbicide 2,4-D was applied to the nutrient solution at the concentration of 2000 μM and maintained for six hours. After this period, the solution was renewed again. The evaluation of symptoms was performed at 1 DAT using a percentage visual scale, in which 0 %

corresponds to the absence of injury and 100% to plant death. The resistant plants were evaluated for rapid necrosis and the susceptible plants for the epinasty symptoms. The time for onset of rapid necrosis symptoms after herbicide application was also evaluated at intervals of 15 minutes until 5 h after herbicide spraying.

A second study evaluated the biotype RN submitted to different periods of light. Plants were initially grown in a greenhouse at 25 ± 5°C. When the plants were at 10-15 cm of height (8-10 leaves), they were transferred to a growth chamber with a temperature of 25 C and absence of light for zero, one, two, and three days before the herbicide treatment. After that, four drops of 2,4-D herbicide were applied with a micropipette at a concentration of 4.02 g ae L^{-1} per leaf sampled. Half of the plants remained in the absence of light and the other half were transferred to a growing chamber with a temperature of 25°C and 400 μmol m^{-2} s^{-1} of light after herbicide application. The evaluation was performed on 11 mm diameter leaf discs collected from the herbicide application site 90 min after application. For each treatment, four leaf discs were collected, and each disc consisted of a repetition. The leaf disc was incubated in a solution with DAB (1 mg mL^{-1}, pH 3.8) at room temperature for eight hours. The staining associated with H_2O_2 was determined for each disc in the Image J program as described earlier. In addition, the plants were photographed at the onset of the symptoms and at 5 h later. The necrotic area of four leaves per treatment was measured using the Image J program. Each experimental unit consisted of a leaf disc obtained from an individual leaf where the herbicide was applied. The time for onset of rapid necrosis symptoms after herbicide application was also recorded for each leaf collected for the necrosis area measurement. An auxiliary green light was used to evaluate the onset of symptoms in plants kept in the dark.

Effect of plant growth stage on the occurrence of rapid necrosis

Factor A was the biotypes S and RN. Factor B corresponded to the plant growth stage 1, corresponding to 5-8 cm of height and 10-12 leaves plants (S1), stage 2 for 30-45 cm plants with 22-25 leaves (S2), and stage 3 for plants with 45-60 cm and 30-40 leaves (S3). The factor C was herbicides doses of 2,4-D at 50.25; 201; 402; 804; 1608 and 3216 g ae ha^{-1}; dicamba at 30; 120; 240; 480; 960 e 1920 g ae ha^{-1} and triclopyr at 45; 180; 360; 720; 1440 and 2880 g ae ha^{-1}. Plant visual injury on a percentage scale was evaluated at 49 DAT.

Results and Discussion

Dose-response evaluation of auxinic herbicides

In a previous study, the resistant biotype showed RN symptoms to 2,4-D and MCPA herbicides, both classified as phenoxy herbicides, and only showed epinasty symptoms to other auxinic herbicides applied at labeled use rates (Queiroz et al. 2020). However, some field observations have identified the occurrence of RN in overlapping herbicide applications in some populations. In the present study, the effect of several auxinic herbicides was evaluated using dose-response curves. The symptoms of RN were observed only in the biotype RN in response to the 2,4-D herbicide. The other auxinic herbicides dicamba, halauxifen-methyl, triclopyr, fluroxypyr, florpyrauxifen-benzyl, and picloram, even applied at high rates in the dose-response assay promoted only the typical symptom of epinasty and controlled both RN and susceptible biotypes (Figures 1A to 1F). The 2,4-D herbicide controlled susceptible plants with the dose of 804 g ae ha^{-1}, but the resistant biotype showed only 40 % control at that dose (Figure 1G). The resistance factor (RF) for 2,4-D at 3 DAT was 0.66, because the susceptible plants were evaluated for epinasty and the resistant plants for the rapid necrosis symptoms, which were equivalent in some doses. At 35 DAT the RF was 7.39 for 2,4-D (Table 1).

Auxinic herbicides are an important group of selective herbicides used to control dicot weeds (Peterson et al. 2016). Resistance to these herbicides limits the options for controlling *Conyza* species, in which herbicide resistance has already been reported to other five mechanisms of action (inhibitors of photosystems I and II, EPSPS, ALS, and PPO inhibitors) (Santos et al. 2014; Pinho et al. 2019). The results obtained in this study are important to confirm the efficacy of other six auxinic herbicides in the control of the biotype RN.

Rapid necrosis caused by 2,4-D results in antagonism to other auxinic herbicides

The herbicides dicamba, halauxifen-methyl, and triclopyr were applied singly, mixed with, or 4 h after the application of 2,4-D to evaluate the effect of RN on the efficacy of these alternative herbicides. The absence of antagonism was observed in the evaluation at 3 DAT of the simultaneous or sequential application of 2,4-D and either dicamba, halauxifen-methyl, or triclopyr in the biotype RN (Table 2). However, after plant regrowth, the herbicide injury at 35 DAT indicated an antagonism between 2,4-D and these three herbicides for controlling biotype RN when these herbicides were used either in association with or 4 h after 2,4-D (Table 2). After 2,4-D spraying, the R-RN plants developed the symptoms of RN, with partial leaf wilt and necrotic spots that expand over time (Queiroz et al. 2020). It is possible that the leaf necrosis could have reduced the herbicide absorption and mobility from the leaf to the meristems when used in a simultaneous or sequential application. Similar antagonism was found in giant ragweed resistant to glyphosate by RN for five herbicides with different mechanisms of action (Harre et al. 2018b). The prevention of herbicide resistance is dependent on rotation and mixtures of different herbicide mechanisms of action, especially for the increasing problem of resistance in *Conyza* species (Cantu et al. 2021), but the occurrence of antagonism in the RN plants jeopardizes this strategy and increases the herbicide resistance problem. In addition,

herbicide resistance management also requires other nonchemical weed control and agronomic measures that contribute to resistance prevention.

Low temperature delays the occurrence of RN

At a temperature of 25 C or higher, necrosis symptoms were detected in the biotype RN about 2 h after spraying, and leaf desiccation after 1 DAT (Queiroz et al. 2020). Considering the field observation of variable occurrence of RN, we postulated that low temperature might modulate the effect of 2,4-D. Therefore, we evaluated the effect of 2,4-D at the temperature of 12°C. In this situation, the RN symptoms were detected only after 1 DAT and were less intense in comparison with the application at 30 C (Figures 2A, B, and C). The typical 2,4-D symptoms of epinasty were also less intense in the susceptible biotype at 12 C in comparison with 30 C (Figures 2A and B). At 21 DAT, the injury caused by 2,4-D was higher in the susceptible biotype in comparison with biotype RN, characterizing the occurrence of resistance to 2,4-D (Figure 2D). In this evaluation, the application of 2,4-D at the temperature of 12 C for biotype RN resulted in lower plant injury in comparison to 30°C (Figure 2D). The obtained results are in agreement with the standard Q10 (temperature quotient) principle in biology, which indicates that for most biochemical reactions the rate of reaction changes by a factor of 2 for every 10 degree C change in temperature.

Not only was the necrosis delayed at low temperature but ROS accumulation was also delayed. Previous results indicated that the onset of ROS accumulation was about 15 min after 2,4-D spraying at the temperature of 25°C and high light (848 μmol m^{-2} s^{-1}) and results in about 62 % of the leaf area stained with DAB (Queiroz et al. 2020). In the present study carried out at 12°C, the onset was 60 to 120 min after spraying and ROS formation was maximum at 240 min covering 63% of leaf area (Figure 2E). A similar change has been reported in giant ragweed

with the resistance to glyphosate by RN, in which the amount of H_2O_2 at 30 C was more than twice that obtained at 10°C, after 2.5 h of spraying (Harre et al. 2018a). Also, in that study, the temperature affected the occurrence of antagonism with other systemic herbicides. The control of giant ragweed resistant to glyphosate with the association of the herbicides atrazine, cloransulam, dicamba, and topramazone was 12 to 21% less effective than expected at 30 C and 11 to 16% less efficient at 10 C. Overall, the antagonism was up to 10% greater at 30 C than at 10 C (Harre et al. 2018a).

Effect of light and photosynthesis inhibitors on the occurrence of rapid necrosis

The effect of light and photosynthesis inhibitors could provide insights about the mechanisms behind the symptoms of the RN caused by 2,4-D and also regarding the variability of the RN intensity under field conditions. The application of photosystem II inhibitors, atrazine, and diuron in nutrient solution did not delay the onset (Figure 3A) or decrease the intensity (Figure 3B) of RN at any of the concentrations evaluated in relation to the application of the herbicide 2,4-D alone. These results indicate that alterations in plant photosynthesis through photosystem II inhibition do not prevent the occurrence of symptoms related to the resistance in the biotype RN.

In the second study related to the effect on photosynthesis, treatments were evaluated in the presence and absence of light. Plants maintained in the absence of light after the application of the 2,4-D herbicide started the RN symptoms from 134 to 144 min after treatment, and in plants exposed to 400 μmol m^{-2} s^{-1} light after application, the RN symptoms began earlier at 95 to 109 min after treatment (Figure 4A). The periods of one to three days of dark treatment before herbicide spraying resulted in similar results for RN.

Regarding the effect on oxidative stress, no difference was observed in the production of H_2O_2 in leaf discs evaluated at 90 min after herbicide application, independent of the light regime (Figure 4B). Similar results were observed for the area of RN at 5 h after treatment when the RN symptom was consolidated (Figure 4C). These results indicate that the absence of light after herbicide application causes a slight delay in the RN symptoms, but the production of hydrogen peroxide and the size of necrosed area were not affected by the light treatments neither before nor after 2,4-D application.

Similar results were observed in previous studies, wherein low light (29 μmol m^{-2} s^{-1}) delayed the onset of symptoms of RN compared with higher light (848 μmol m^{-2} s^{-1}) (Queiroz et al. 2020). However, the production of H_2O_2 was not dependent on the presence of light in this species and the symptoms also occurred in the dark condition. Another study evaluated the consequences on photosynthesis after 2,4-D application on an RN biotype of Sumatran fleabane (Leal et al. 2022). The photosynthetic performance was reduced by 20 % in 1 h after the application of the 2,4-D herbicide, showing lower performance of the electron transport chain. After 4 h of treatment, these metabolic alterations were also observed in the susceptible biotype. Photosynthetic damage was rapidly observed in the resistant compared with the susceptible biotype due to the differential physiological response to 2,4-D (Leal et al. 2022). These symptoms are probably related to the increase of ROS and the effect of the necrosis of the leaf tissue on photosynthesis.

The resistance mechanism of RN is affected by light intensity and temperature. In low light there is a delay of 3 h for the symptom onset and the amount of H_2O_2 accumulated is also reduced in comparison to high light (Queiroz et al. 2020). Here, we report that low temperature (12°C) causes a stronger effect; the symptoms were only manifested 1 DAT and were subtler, H_2O_2 began to accumulate between 60 and 120 min. As discussed before, these interactions between environmental conditions and the RN mechanism have been reported also for the

resistance to glyphosate found in giant ragweed (Moretti et al. 2017; Harre et al. 2018a). These findings are important because, in the field, where the environment is more variable than in greenhouse or growth chamber experiments, these interactions may confound the diagnostic of RN and influence the efficacy of other herbicides when applied in association with 2,4-D.

Effect of plant growth stage

Plant regrowth at 49 DAT characterizes plant survival after the occurrence of RN in the biotype RN as determined in previous studies (Queiroz et al. 2020). Resistant plants were effectively controlled only with 2,4-D doses higher than 1340 g ae ha^{-1} applied in plants at stage 1 (5-8 cm and 10-12 leaves). At this stage, the susceptible plants were controlled with the dose of 50.25 g ae ha^{-1} (Figure 5A). The RF for biotype RN in comparison with biotype S was 7.9, 82.5, and 24.8 for applications to stages 1, 2, and 3, respectively (Table 3). Despite the lower RF in stage 1 in comparison with stages 2 and 3, the occurrence of plant regrowth at 49 DAT was observed in treatments with the recommended dose of 804 g ae ha^{-1}.

The RN symptoms were not observed for dicamba and triclopyr treatments in resistant plants, regardless of the dose used and growth stage at application. These plants showed similar symptoms of epinasty to the biotype S for both herbicides, after one day of application (data not shown). The efficacy of dicamba and triclopyr between the biotypes RN and S was similar for application at stages 1 and 2 (Figures 5B and 5C). However, at stage 3, control of the biotype RN was inferior to the biotype S at doses higher than 120 g ae ha^{-1} of dicamba and for the doses of 45 to 360 g ae ha^{-1} of triclopyr (Figures 5B and 5C). The recommended stage of application is stage 1 (5-8 cm and 10-12 leaves). The other two evaluated stages (30-45 cm and 22-25 leaves; 45-60 cm and 30-40 leaves) represent situations where late burndown applications are necessary, which frequently occurs in farm situations. Although the auxinic herbicides are not

recommended for application on plant stages 2 and 3 this is evidence of decreased efficacy of dicamba and triclopyr for the 2,4-D resistant plants caused by RN.

Several studies indicate satisfactory control of *Conyza* plants with the herbicide dicamba, even in advanced stages of growth (Kruger et al. 2010). For 2,4-D, however, the plant stage is important for *Conyza* control (Oliveira Neto et al. 2010; Walker et al. 2012). When evaluating hairy fleabane [*Conyza bonariensis* (L.) Cronq] plants at rosettes of 5 cm and 10-15 cm in diameter to the effect of 2,4-D at 940 and 1250 g ae ha^{-1}, Walker et al. (2012) found 36% lower control effectiveness for taller plants compared to the 5 cm diameter stage, despite the dose increase in late application. However, in a study with glyphosate-resistant horseweed, there was no growth stage effect with 2,4-D when 560 g ae ha^{-1} of the herbicide was applied to plants with heights of 0-7;7-15; 15-30, and 30-45 cm (Kruger et al. 2010). The environmental conditions may complicate further the effect of the plant growth stage on the control of *Conyza* species by auxinic herbicides. The current study indicates that RN-resistant plants treated at early growth stage, such as 1 (5-8 cm and 10-12 leaves) are more affected by 2,4-D than at later herbicide applications. The herbicides dicamba and triclopyr are less effective on plants of the biotype RN treated at growth stage 3 (45-60 cm and 30-40 leaves).

In conclusion, the auxinic herbicides dicamba, triclopyr, and halauxifen-methyl do not cause RN symptoms and are effective at controlling the RN 2,4-D resistant biotype when applied without 2,4-D use. However, the effectiveness of these herbicides was reduced when sprayed on the resistant biotype either together, 4 h or 24 h after 2,4-D herbicide. The temperature at spraying time modulates the occurrence of RN. Application at 12 C delays the symptoms and decreases its intensity, but still results in plant survival after 2,4-D application. The absence of light after herbicide application causes a slight delay in the RN symptoms, but the production of hydrogen peroxide and the size of necrotic area are not affected by the light

425 treatments either before or after 2,4-D application. The RN phenotypic expression does not
426 occur for the herbicides dicamba and triclopyr, even in advanced plant growth stages of
427 application and high doses. The RN-resistant plants treated at the early plant stages of 5-8 cm
428 and 10-12 leaves are more affected by 2,4-D than at later herbicide applications. The herbicides
429 dicamba and triclopyr are less effective on older plants of the biotype RN (45-60 cm and 30-40
430 leaves). This study identified that environmental, plant growth stage effects, and herbicide
431 interactions can interfere with the occurrence of RN caused by 2,4-D in Sumatran fleabane and
432 are important for identifying the causes of variability in herbicide symptomology and
433 performance under experimental and field conditions. Future research through transcriptome
434 and genetic mapping will be important to characterize the mechanism of resistance related to
435 rapid necrosis caused by 2,4-D in Sumatran fleabane.
436

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441

442 **Conflicts of Interest**

443 No conflicts of interest have been declared.

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532 flowering of *Conyza albida, C. bonariensis* and *C. canadensis*. Phytoparasitica 13:229–
533 230
534

535 **Table 1** Log-logistic equation parameters and resistance factors for herbicide control at 35 days
536 after treatment (DAT) for Sumatran fleabane biotypes RN (2,4-D rapid necrosis resistant) and
537 S (2,4-D susceptible), for seven auxinic herbicides and at 03 DAT after application of 2,4-D.

Herbicide	Biotype	Evaluation	*b*	*c*	*d*	*e* (ED_{50})	ED_{80}	RF
				%		g ae ha^{-1}		
dicamba	S	35 DAT	-1.20*	3.10^{ns}	100	34.46*	109.25*	
	RN		-0.91*	3.37^{ns}	100	42.05*	194.06*	1.22^{ns}
halauxifen-methyl	S	35 DAT	-0.78*	-0.49^{ns}	100	0.15*	0.87*	
	RN		-0.42*	0.85^{ns}	100	0.61*	13.89*	4.13^{ns}
triclopyr	S	35 DAT	-0.97*	0.53^{ns}	100	25.03*	104.87*	
	RN		-0.99*	1.16^{ns}	100	36.37*	147.29*	1.45^{ns}
fluroxypyr	S	35 DAT	-1.42*	4.48^{ns}	100	41.15*	109.49*	
	RN		-1.37*	1.52^{ns}	100	37.16*	102.38*	0.90^{ns}
florpyrauxifen-benzyl	S	35 DAT	-0.93*	3.08^{ns}	100	1.57*	7.01*	
	RN		-0.98*	0.04^{ns}	100	1.45*	5.97*	0.92^{ns}
picloram	S	35 DAT	-1.36*	0.10^{ns}	100	7.87*	21.80*	
	RN		-1.07*	0.10^{ns}	100	8.94*	32.74*	1.14^{ns}
2,4-D	S	35 DAT	-2.52*	3.98^{ns}	100	108.41*	188.05*	
	RN		-4.11*	28.24*	100	801.00*	3483.60*	7.39*
2,4-D	S	03 DAT	-0.38*	-1.93^{ns}	100	1214.51*	46593.1*	
	RN		-0.46*	0.77^{ns}	100	799.63*	11652.0*	0.66^{ns}

538

539 Difference * statistically significant or ^{ns} not statistically significant for parameter b (curve
540 slope) with 0; parameter c (lower limit) with 0; parameter d (upper limit) with 100; parameter
541 e (effective dose for 50 % control) between S and RN biotypes; RF (resistance factor) with 1.

542

Table 2 Rapid necrosis (%) at 3 days after treatment (DAT), injury (%) at 35 DAT, expected effect (Exp.) of the association, and the result of the interaction (Int.) of the 2,4-D herbicide mixed with dicamba, halauxifen-methyl or triclopyr, according to the method proposed by Colby (1967) on the Sumatran fleabane biotype RN (2,4-D rapid necrosis resistant).

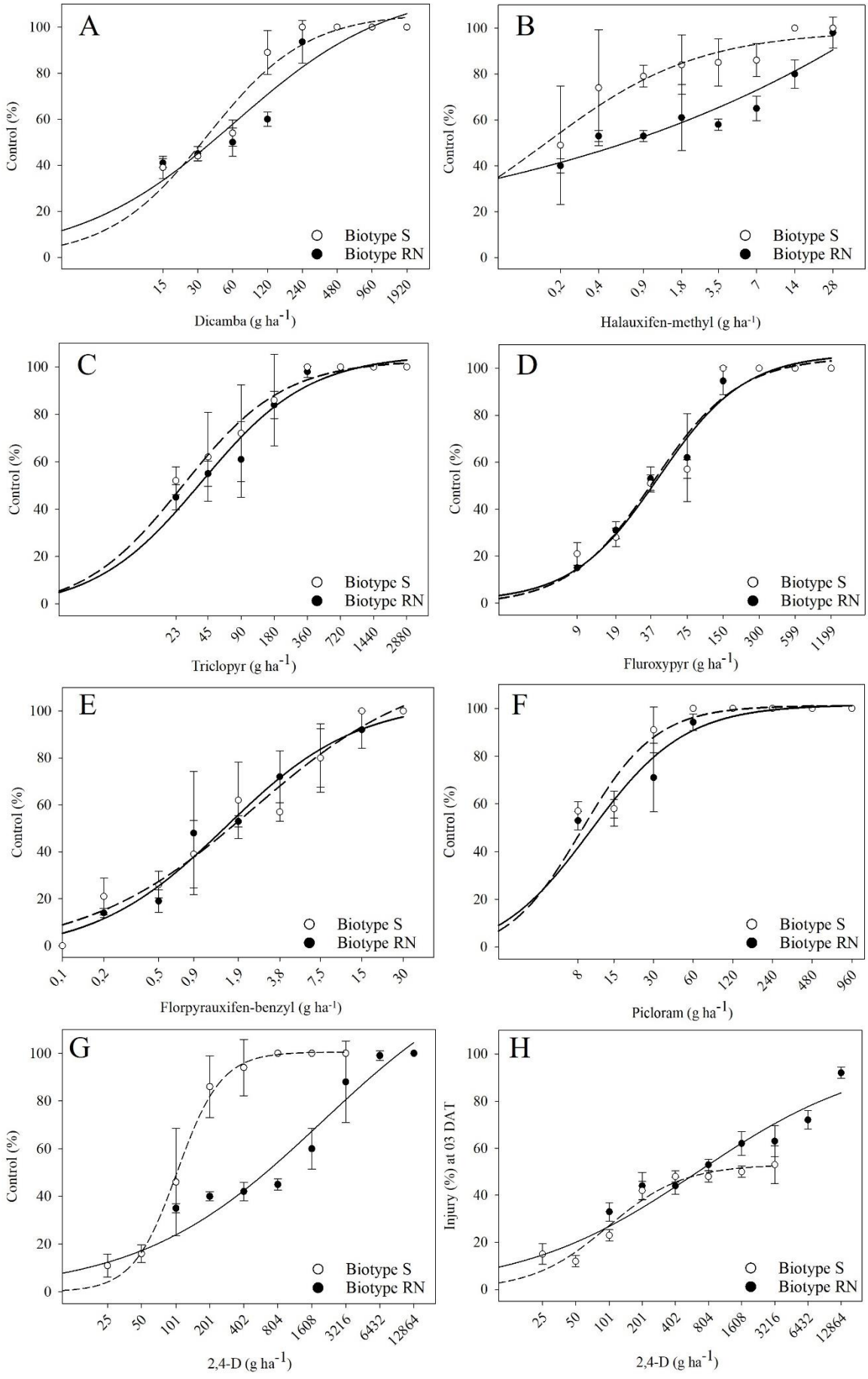
Treatment	*Rapid necrosis - 03DAT*		Colby		*Injury - 35 DAT*		Colby	
			Exp.	Int.			Exp.	Int.
	%				*%*			
Untreated control	0.0	d	-		0.0	g	-	
2,4-D	38,1	c	-		46.2	ef	-	
Dicamba	0.0	d	-		76.9	bcd	-	
Halauxifen-methyl	0.0	d	-		47.5	ef	-	
Triclopyr	3.6	d	-		99.4	a	-	
2,4-D + dicamba	40.0	c	38.1^{ns}	additive	76.3	bcd	88.6^{ns}	additive
2,4-D + halauxifen-methyl	41.9	c	38.1^{ns}	additive	57.5	def	71.5*	antagonism
2,4-D + triclopyr	52.5	a	40.3*	synergism	86.3	abc	99.6*	antagonism
Dicamba 4 h after 2,4-D	40.6	c	38.1^{ns}	additive	67.5	cd	88.6*	antagonism
Halauxifen-methyl 4 h after 2,4-D	42.5	c	38.1^{ns}	additive	57.5	def	71.5*	antagonism
Triclopyr 4 h after 2,4-D	49.4	ab	40.3*	synergism	91.3	ab	99.6^{ns}	additive
Dicamba 24 h after 2,4-D	38.1	c	38.1^{ns}	additive	70.0	cd	88.6*	antagonism
Halauxifen-methyl 24 h after 2,4-D	43.1	bc	38.1^{ns}	additive	66.3	cd	71.5^{ns}	additive
Triclopyr 24 h after 2,4-D	40.6	c	40.3^{ns}	additive	86.3	abc	99.6*	antagonism

Lower letters compare means among treatments inside each column by Tukey´s HSD test ($p<0.05$). *Significant difference between the observed and expected values by t-test ($p<0.05$); ^{ns} Non-significant difference between the observed and expected values by t-test ($p<0.05$).

Table 3 Log-logistic equation parameters and resistance factors for herbicide control at 49 days after treatment (DAT) for Sumatran fleabane biotypes RN (2,4-D rapid necrosis resistant) and S (2,4-D susceptible), after application of 2,4-D, dicamba and triclopyr in three plant growth stages in the application (S1: 5-8 cm and 10-12 leaves; S2: 30-45 cm and 22-25 leaves; S3: 45-60 cm and 30-40 leaves).

Biotype	Herbicide	*b*	*c*	*d*	*e* (ED_{50})	ED_{80}	RF
		%			g ae ha^{-1}		
Stage 1 (5-8 cm and 10-12 leaves)							
S	2,4-D	-6.27^{ns}	0.01^{ns}	100	43.05^{ns}	53.71*	
RN		-0.30*	0.61*	100	341.46*	803.92*	7.93*
S	dicamba	-3.18*	-0.08^{ns}	100	18.93*	29.24*	
RN		-2.62*	-0.08*	100	22.64*	39.05*	1.20^{ns}
S	triclopyr	-0.92*	0.10*	100	11.62*	30.33*	
RN		-4.26*	0.10*	100	41.91*	57.14*	3.61*
Stage 2 (30-45 cm and 22-25 leaves)							
S	2,4-D	-0.88*	0.02^{ns}	100	47.89*	187.45*	
RN		-0.25*	0.04*	100	3950.12*	18601.00	82.48*
S	dicamba	-0.47*	0.12^{ns}	100	68.72*	164.74*	
RN		-0.71*	0.37^{ns}	100	95.37*	351.49*	1.39^{ns}
S	triclopyr	-0.64*	0.23^{ns}	100	65.05*	224.46*	
RN		-1.22*	0.17^{ns}	100	69.48*	314.08*	1.01^{ns}
Stage 3 (45-60 cm and 30-40 leaves)							
S	2,4-D	0.84*	0.42^{ns}	100	195.17*	405.65*	
RN		0.63*	0.12^{ns}	69.46*	4845.96*	33271.00*	24.83*
S	dicamba	-0.90*	-0.22*	100	59.23*	227.11*	
RN		-0.30*	-0.45*	100	139.42*	4572.51*	2.35*
S	triclopyr	-0.84*	0.23^{ns}	100	72.05*	230.96*	
RN		-0.62*	1.48^{ns}	100	397.46*	749.11*	5.52^{ns}

Difference * statistically significant or ^{ns} not statistically significant for parameter b (curve slope) with 0; parameter c (lower limit) with 0; parameter d (upper limit) with 100; parameter e (effective dose for 50 % control) between S and RN biotypes; RF (resistance factor) with 1.


A
Control (%)
Biotype S
Biotype RN
15 30 60 120 240 480 960 1920
Dicamba (g ha-1)
B
Control (%)
Biotype S
Biotype RN
0,2 0,4 0,9 1,8 3,5 7 14 28
Halauxifen-methyl (g ha-1)
C
Control (%)
Biotype S
Biotype RN
23 45 90 180 360 720 1440 2880
Triclopyr (g ha-1)
D
Control (%)
Biotype S
Biotype RN
9 19 37 75 150 300 599 1199
Fluroxypyr (g ha-1)
E
Control (%)
Biotype S
Biotype RN
0,1 0,2 0,5 0,9 1,9 3,8 7,5 15 30
Florpyrauxifen-benzyl (g ha-1)
F
Control (%)
Biotype S
Biotype RN
8 15 30 60 120 240 480 960
Picloram (g ha-1)
G
Control (%)
Biotype S
Biotype RN
25 50 101 201 402 804 1608 3216 6432 12864
2,4-D (g ha-1)
H
Injury (%) at 03 DAT
Biotype S
Biotype RN
25 50 101 201 402 804 1608 3216 6432 12864
2,4-D (g ha-1)

561

562 **Figure 1** Dose-response curves for Sumatran fleabane biotypes RN (2,4-D rapid necrosis
563 resistant) and S (2,4-D susceptible) at 35 days after treatment (DAT) to dicamba (A),
564 halauxifen-methyl (B), triclopyr (C), fluroxypyr (D), florpyrauxifen-benzyl (E), picloram (F)
565 and 2,4-D (G) and at 03 DAT to 2,4-D (H). Vertical bars indicate the confidence interval (α =
566 0.05).

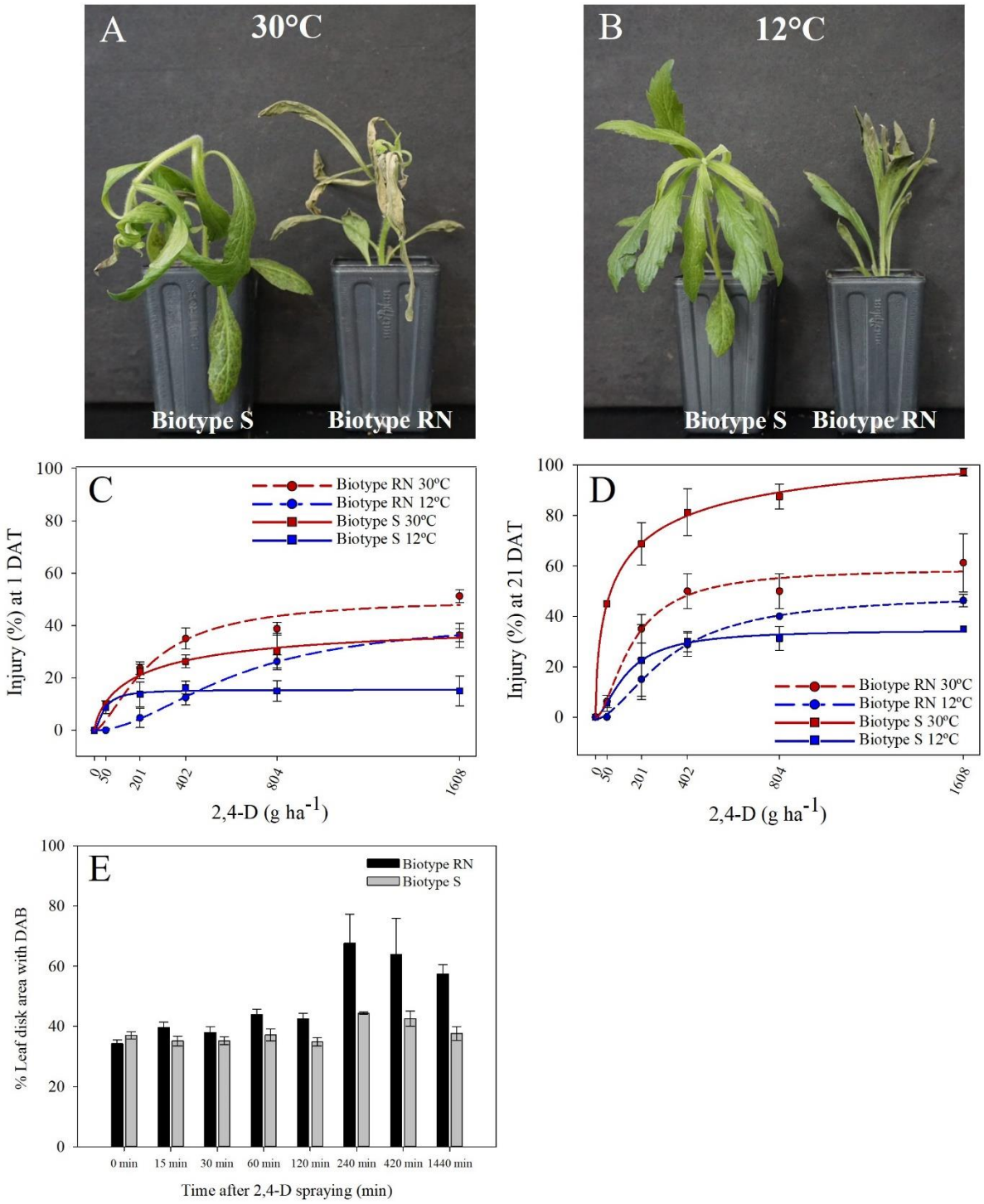
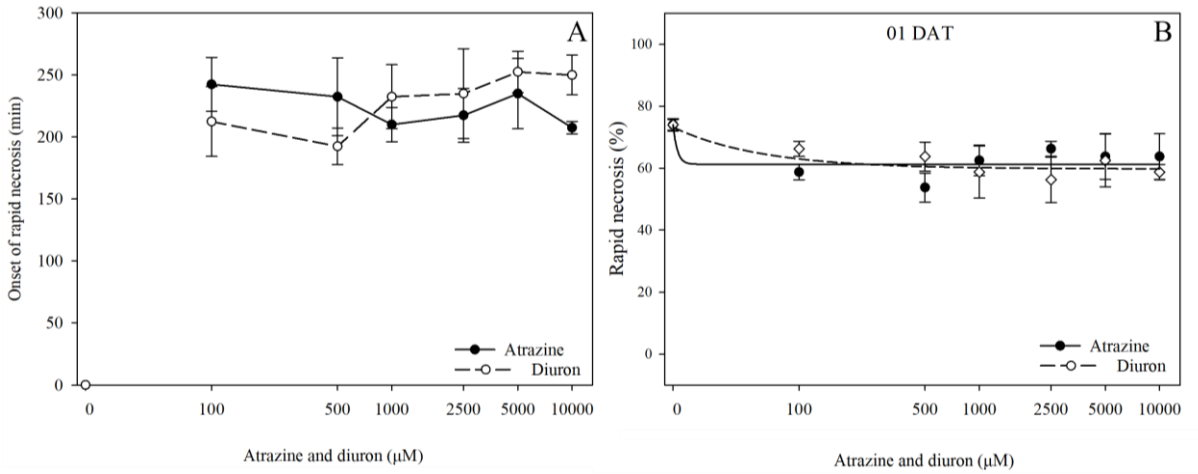


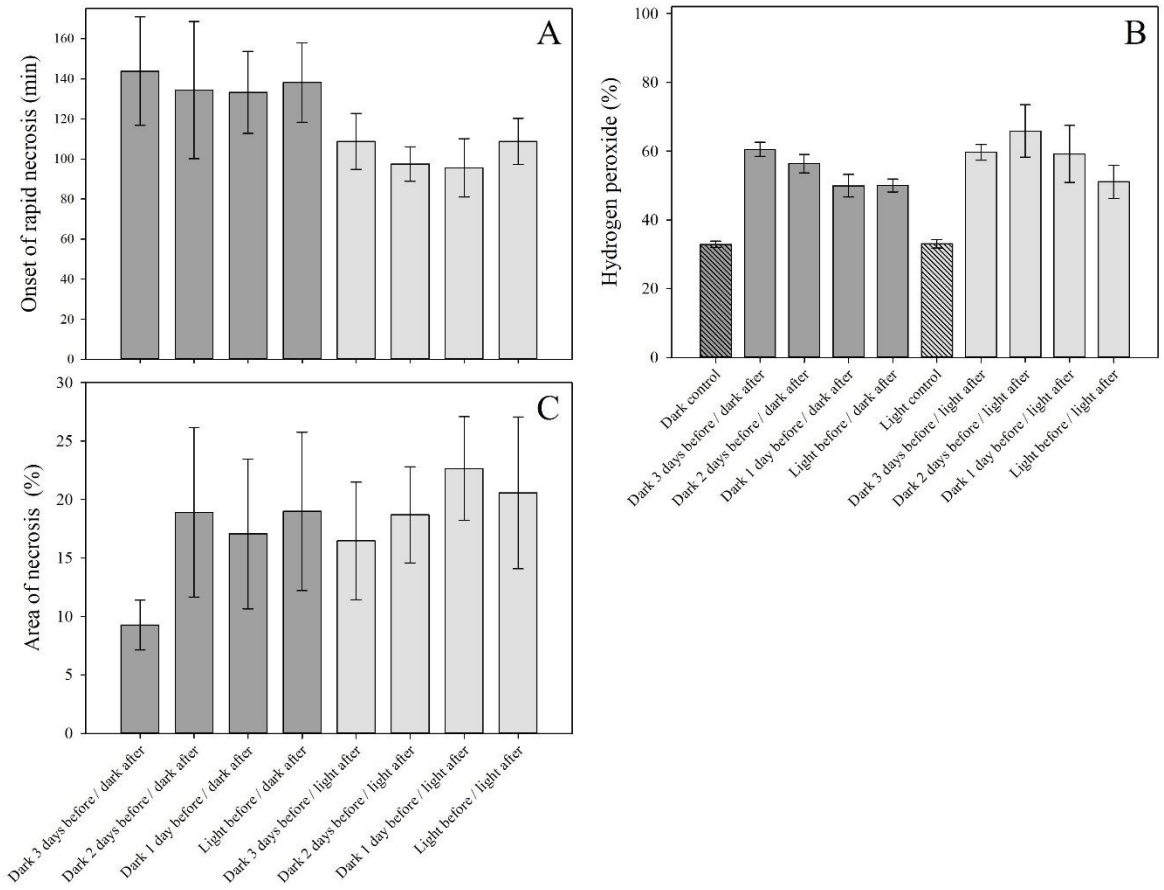
Figure 2 Effect of temperature on 2,4-D symptoms on Sumatran fleabane biotypes RN (2,4-D rapid necrosis resistant) and S (2,4-D susceptible) at one day after treatment (DAT) with 804 g ae ha^{-1} 2,4-D at 30°C (A) and 12°C (B), plant injury (%) after 2,4-D application at 12 and 30°C evaluated at 1(C) and 21 DAT (D) and ROS accumulation (%) at different times after 2,4-D spraying at 12°C (E). Vertical bars indicate the confidence interval ($\alpha = 0.05$).



573

574 **Figure 3** Effect of photosystem II inhibitors atrazine and diuron before application of 2,4-D
575 (2000 μM) on the onset of symptoms (A) and injury (%) from rapid necrosis one day after
576 treatment (DAT) (B) on Sumatran fleabane biotype RN (2,4-D rapid necrosis resistant).
577 Vertical bars indicate the confidence interval ($\alpha = 0.05$).

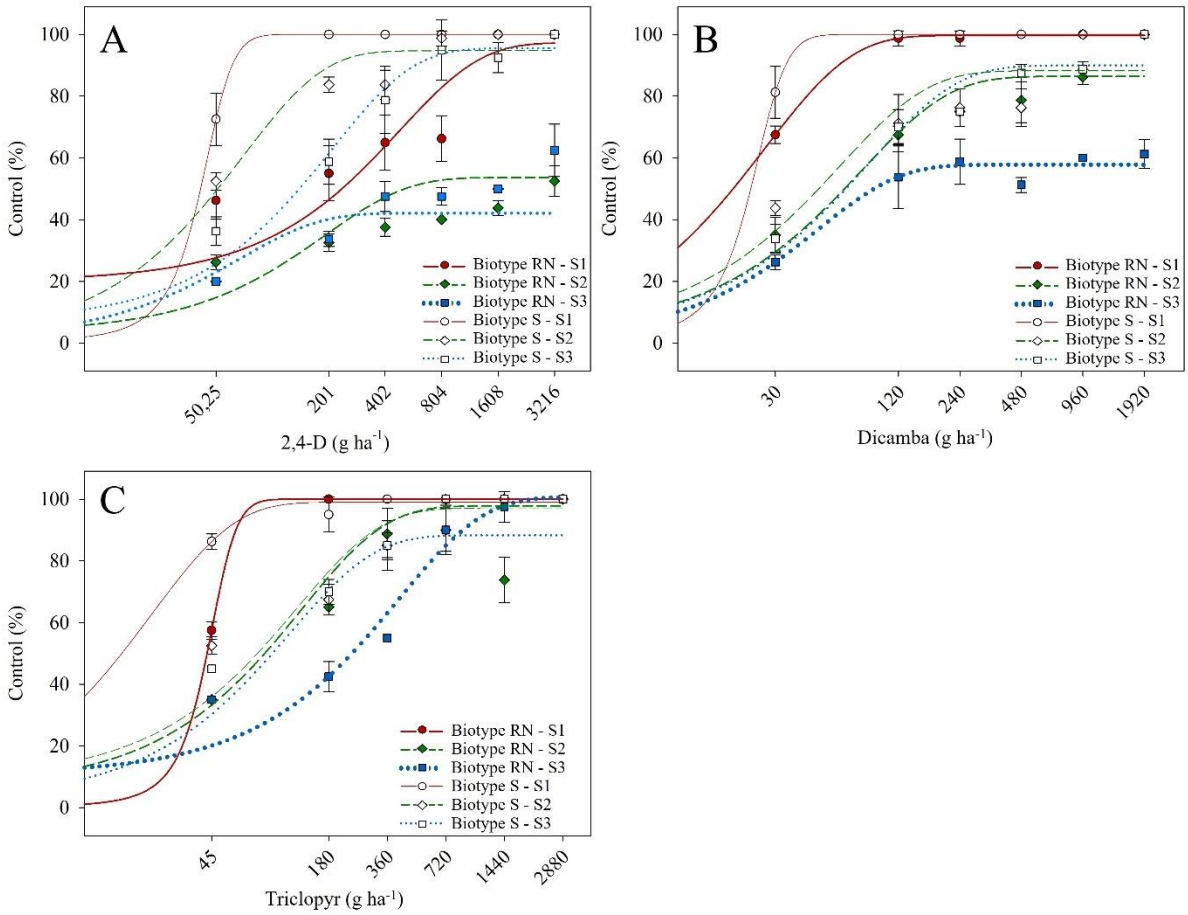
578



579

580 **Figure 4** Onset of symptoms in minutes (min) (A), accumulation of hydrogen peroxide (%) (B)
581 at 90 min after treatment, and area of leaf necrosis (%) (C) at 5 h after 2,4-D application (4.02
582 g ae L^{-1}) in plants kept under different light conditions on Sumatran fleabane biotype RN (2,4-
583 D rapid necrosis resistant). Dark control: untreated plants kept in the absence of light; light
584 (intensity 400 μmol m^{-2} s^{-1}). Vertical bars indicate the confidence interval ($\alpha = 0.05$).

585



586

587 **Figure 5** Efficacy of control (%) at 49 days after treatment (DAT) of Sumatran fleabane
588 biotypes RN (2,4-D rapid necrosis resistant) and S (2,4-D susceptible) at three plant growth
589 stages. Dose-response curves to 2,4-D (A), dicamba (B) and triclopyr (C) (S1: 5-8 cm and 10-
590 12 leaves; S2: 30-45 cm and 22-25 leaves; S3: 45-60 cm and 30-40 leaves). Vertical bars
591 indicate the confidence interval ($\alpha = 0.05$).