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Absorption and translocation tolerance of glyphosate

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The purpose of this experiment was to relate the tolerance of *Commelina benghalensis* to two different kinds of glyphosate formulation, in function of the absorption time and translocation of this molecule in the cells of this plant. The experiment was conducted in a greenhouse. The experimental design is a completely randomized design (CRD), with four treatments follows; glyphosate potassium salt and isopropylamine tested with commercial dosage, haematoxylin dye and a control, with three repetitions. Sample from the leaves, stems and roots were collected and subdivided in twenty-five subsamples. The evaluations were performed by microscopic views every 30 min, until the herbicide was absorbed into the parenchyma of the mesophyll. After 30 min of the application of isopropylamine glyphosate, there was accumulation of this molecule inside leaf apoplast which indicated the process of penetration of isopropylamine glyphosate. For the glyphosate potassium salt the absorption was observed 2 h after application. After 7 h, the presence of a small concentration of the product in the stem was verified. 12 h later, a low concentration was verified in the root. After 36 h the phloem was full of the isopropyl amine salt glyphosate, 86 h later its presence in the xylem and phloem, together with the symptoms of destruction of the parenchyma tissue was visualized. The solution with isopropyl amine salt, after 7 h, was seen in the phloem, with a 12 h concentration of isopropyl amine salt decrease in the stem, which overloaded the phloem of the root. After 86 h, all the parenchyma tissue of the xylem and phloem were destructed. The absorption and translocation of glyphosate depended on its formulation, which represented more or less tolerance of the plant to the herbicide, but tolerance was not the only factor influenced by anatomy, the physiological, biochemical and genetic features were also critical.

Key words: Phloem, morphology, tropical spiderwort.

INTRODUCTION

Glyphosate is the most utilized herbicide in the word. After the technology of Roundup Ready® crops, its utilization has increased. Some weeds present glyphosate tolerance and this has increased the cost of the weed control due to the need to mix different molecules with different modes of action to have a better control of these weeds. Tolerance is a natural process found in some species that may be related to the gene, the anatomy of the plant and the process of absorption and translocation of the herbicide. Deuber (1982)

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reported that absorption is limited by the amount of product passing through the cuticle of the leaves, being influenced by the environ-mental conditions in which the weed is growing.

There are currently a lot of glyphosate trademarks available in the market; however, they all have the same mechanism of action, regardless of the used salts, especially the isopropylamine, ammonium and potassium (Rodrigues and Almeida, 2005). For transgenic soybeans is registered for utilization in Brazil, the Roundup Ready®. Its formulation contains the isopropylamine which also is also present in Roundup Original formulation (Rodrigues and Almeida, 2005). Different formulations can change the absorption and translocation of the herbicide, influencing the control efficiency (Silva et al., 2006) and its behavior in the environment (Werlang et al., 2005; Santos et al., 2007).

Weeds cause greater damage to agriculture than the pests and diseases together, and constitute a major barrier to food production and economic development in many regions of the world. It is estimated that losses caused to crops by weed interference in Brazil are around 20 to 30%.

The Benghal dayflower (also known as tropical spiderwort) (*Commelina benghalensis* L.) is a perennial weed, a herb, erect or semi-prostrate, which reproduces vegetatively or by seed (Lorenzi, 2014). It is quite common in annual crops and shows a clear preference for loamy, moist, shaded soil. That plant is considered a difficult weed to control, especially when in single applications of glyphosate, which can cause problems in the mechanical harvesting and can be a problem for the natural drying of grain crops. According to Monquero et al. (2004), the mechanisms of tolerance of *C. benghalensis* to glyphosate are related to absorption and differential metabolism. Meschede et al. (2008) observed a control of the specie even when it resists small doses of glyphosate.

Glyphosate passes through the cuticle at moderate speed, requiring, on average, six hours without raining, after application to have adequate control of susceptible plants. It is possible that the relatively slow absorption of glyphosate occurs due to the very low value of octanol to water (-4) compared to other herbicides which give low lipophilicity and high solubility in water. Thus, new formulations have surfactants to provide greater apolarity to the solution that facilitates the absorption of the inhibitors (EPSPs) by the leaves (Velini, 2009; Mac Isaac et al., 1991).

In areas where glyphosate has been used frequently, the population of two species of *Commelina genus*, *Commelina diffusa* and *C. benghalensis*, popularly known as tropical spiderwort, has increased due to tolerance to applications of this herbicide. Monquero et al. (2005) found that plants of *C. benghalensis* were tolerant to glyphosate application. Rocha et al. (2007) found that treatment with glyphosate applied in isolated form was not able to completely inhibit the development of plants of *C. benghalensis*, *C. diffusa* and *Commelina erect*.

The objective of the present study is to evaluate the absorption and translocation of glyphosate potassium and isopripilamina salt formulation, by anatomical views of tropical spiderwort (*C. benghalensis*) in different periods, relating the tolerance of this weed to the glyphosate.

MATERIALS AND METHODS

The study was conducted in two stages: in the greenhouse and in the laboratory of physiology and plant anatomy of the Agronomy Faculty of Paraguaçu Paulista - ESAPP, in 2013.

Greenhouse experiment

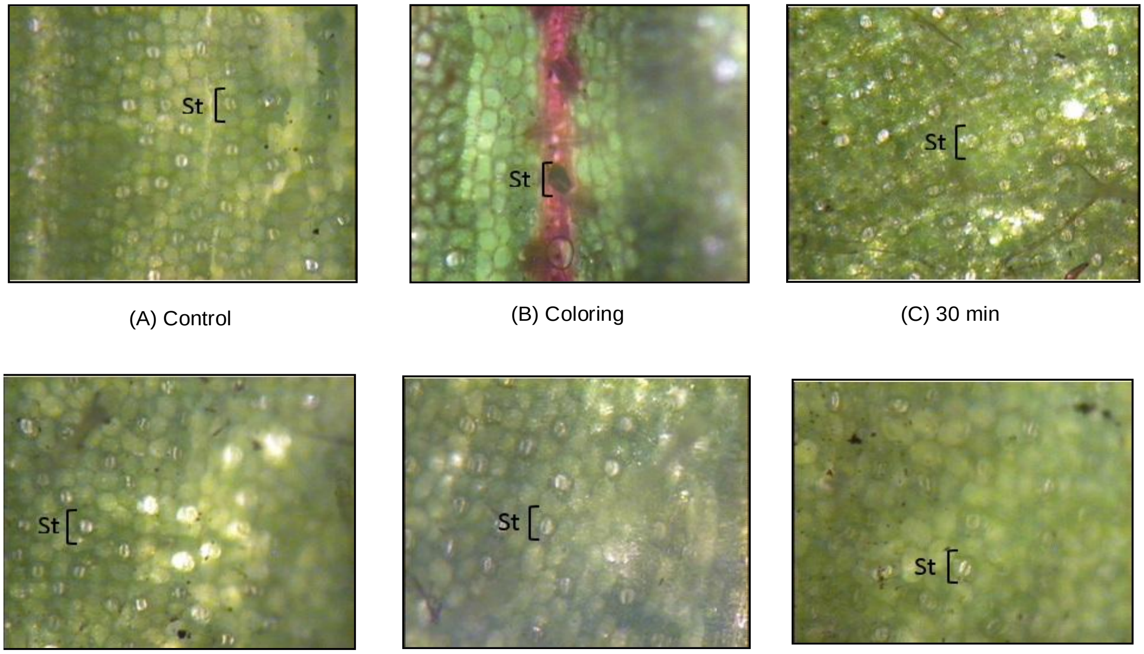
The vegetative parts of tropical spiderwort were selected at the field and transplanted in pots with a capacity of 5 L of soil by placing 2 rods in each pot, with a total of 12 samples that recived daily irrigation. For the fixation and standardization of seedlings, the same size and numbers of leaves were retained in the greenhouse for 20 days after transplanting, until uniformity in order to receive product application. The treatments consisting 2 commercial formulations of glyphosate, Roundup Ready® and original Roundup, formulation with potassic and isopropylamine were applied. T1: glyphosate 360 g e.a (potassic salt formulation) + dye; T2: glyphosate 720 g e.a (isopropylamine formulation) + dye; T3: dye; T4: water (control). The experimental design was completely randomized with 3 replications.

The solution was prepared by adding 100 ml of glyphosate (potassium salt formulation) and 50 ml (formulation isopropylamine) with 0.1 ml of previously prepared hematoxylin dye. The herbicides were applied with a common brush n^{o}. 12 in all surface of the newest fully extended leaf, thus avoiding contact of the product with other plant parts and providing a perfect distribution of the herbicide. The control followed the same procedure of application of water and application of hematoxylin dye. This facilitated the checking of the occupancy of the different tissues by the herbicide.

Anatomical studies

Fully expanded leaves were selected, of which the most important were fragments of the base, middle third (midrib, intercostal region and board) and summit. The microchemical tests were performed on sections obtained hands-free, with the help of razor blade in unfixed material. Stomatal count was made in the intercostal region of the leaf (base, middle and upper) in dissociated fragments of leaves, according to the method of Jeffrey (Johansen, 1940), by counting 15 fields for each species in each of the mentioned regions. The comparison of stomatal density data was performed by Student Newman Keuls method with 0.05% significance level. The patterns of stomatal and epicuticular wax were described according to the parameters proposed by Wilkinson (1979). In order to examine the vascular bundles, was accompanied the way of the leaf sheath to the apical region of the leaf to dissociate elements of the cell, we used the Jeffrey method (Johansen, 1940).

The accumulation of glyphosate in the different parts of the plant was evaluated by staining with hematoxylin, according to the method proposed by Polle et al. (1978), which was modified only to the concentration of the dye. The solution was prepared by dissolving 1 g of hematoxylin, 0.1 g $NaIO_3$ and one drop of 0.1 mol L^{-1} NaOH. Hematoxylin is a basic purple dye that carries a



(A) Control (B) Coloring (C) 30 min

(D) 1 h (E) 1 h 30 min (F) 2 h

Figure 1. View of the cross sections of the adaxial face of *C. bengalensis* leaves, subject to application of glyphosate potassium salt formulation, after periods of 30 min, 1 h, 1 h 30 min and 2 h.

positive charge, capable of giving color to the tissue or the negative charge componds, which includes phosphate groups (glyphosate), nucleic acids, glycosaminoglycans sulfates and carboxyl proteins. The evaluations were made of temporal and spatial form, where for every part of the plant (leaf, stem and root), the tissue was viewed every 30 min, until it attains the stem phloem, later evaluations began to be hourly until the product reached the root. Later, the views occurred 12 to 12 h, until the full discharge of glyphosate in all spiderwort plant tissues and the redirection of translocation upwards, through the xylem. The data were subjected to analysis of variance. Pearson correlation tests were performed ($p \leq 0.05$) between the measured parameters and the averages were grouped according to the criteria of Scott-Knott ($p \leq 0.05$). As the data were normally distributed, Shapiro-Wilk was used in the transformation before analysis of variance. Sisvar software was used for statistical analysis (Ferreira, 2004).

RESULTS AND DISCUSSION

Figure 1 shows the views from absorption of glyphosate potassium salt formulation at different times. At 30 min after application, the accumulation of hematoxylin dye was observed in the apoplast and symplast leaves (Figure 1B). It is remarkable that the accumulation of the product in the apoplast and retention of open stomatal in the treatment (T1) only indicated the penetration of the solution (Figure 1C). At period of 1 h after application (Figure 1D) initiated the accumulation of the product in nearby cells while 1:30 h initiated the closure of most stomata. 2 h after application, the product inside the cell was noted, thereby initiating the process of absorption which was already without filling the phloem leaves (Figure 1F). This showd that glyphosate in salt formulation potassium takes an average of 2 h to begin absorption and reaches the tissues of the leaf symplast with all closed stomatall. The absorption of glyphosate potassium salt formulation is slow and if there is rainfall, it may be washed and this reduces the control of % of these species. The obtained results by several researchers have shown that the absorbtion time of glyphosate in the potassium salt formulation ranges from 4 to 8 h (Bastiani et al., 2000; Jakelaitis et al., 2001; Martini et al., 2003; Werlang et al., 2003; Martins et al., 2009).

During evaluation of the size of the stomata, a correlation was verified with the microscopic analysis discussed before (Figure 2) and it was observed that with the passage of time, there was a reduction in the width while the length did not experience any substantial change. Every half hour, there was closing of the stomatal almost linearly; however, from two hours after application the width did not change, showing that after this period the stomatal continued to be closed. This behavior interferes directly with the water plant process, with changes in transpirational flow and consequently reducing the translocation of the product in the plant. Galon et al. (2010a) observed that the studied herbicides directly affect stomatal conductance on sugar cane.

Shaner (2000) and Yanniccari et al. (2012) reported that

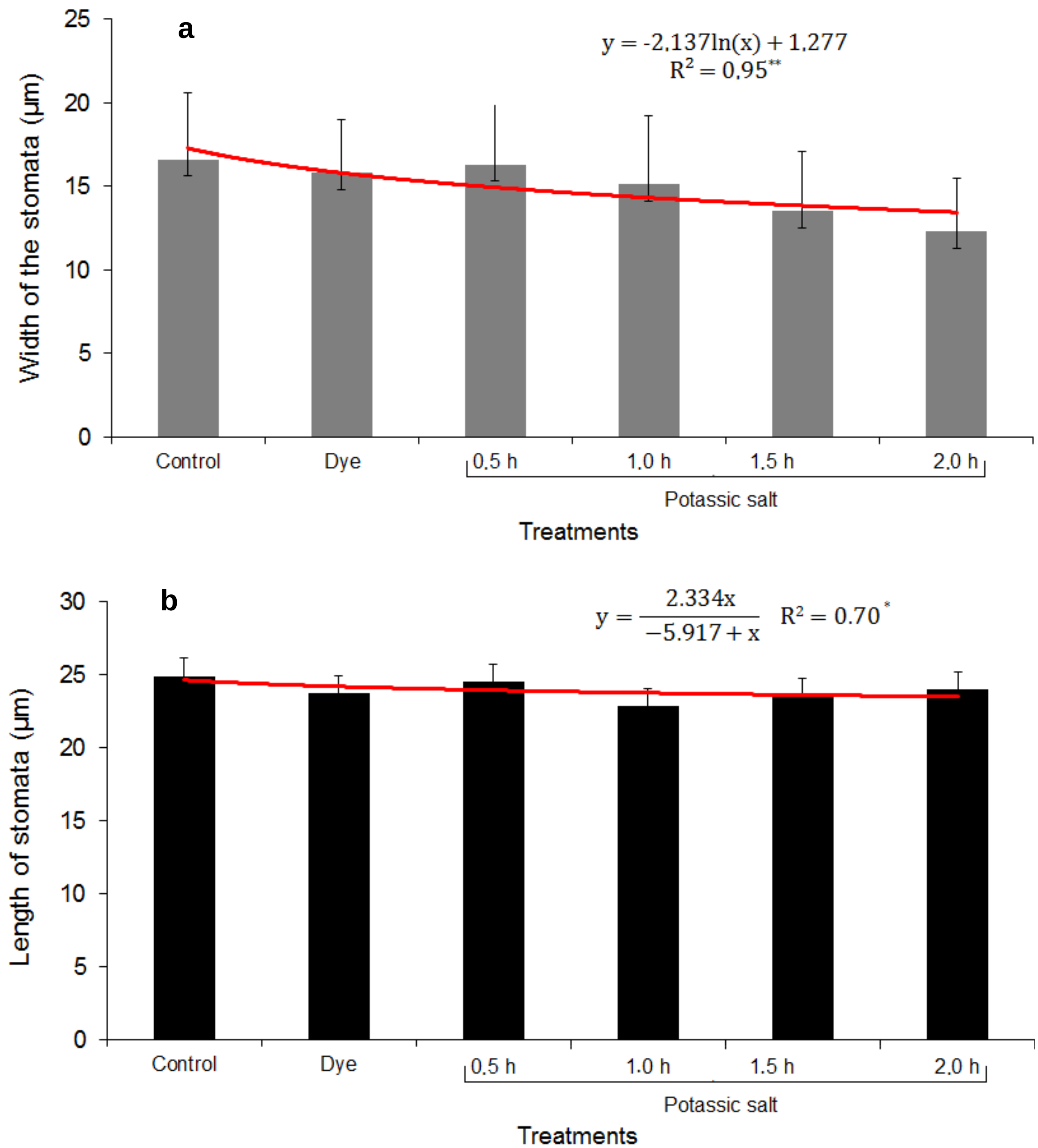


Figure 2. Width (A) and length (B) of stomatal of adaxial surface of leaves of *C. bengalensis*, subject to the application of glyphosate potassium salt formulation, after periods of 30 min, 1 h, 1 h 30 min and 2 h.

glyphosate can directly affect the guard cells and directly interfer in stomatal closure. After 7 h of application, it was found that the same had reached the phloem of the leaf; however, the herbicide had not arrive in the stem yet. At 8 h after application, the presence of glyphosate was visualized in small concentrations in the stem (Figures 3C and D). At 12 h (Figures 3E and F) and after the phloem parenchyma cells of the stem were filled with the herbicide, this indicated an average time of 12 h for the glyphosate to translocate to the parenchyma leaf and stem tissues (Figure 2). The approximate time of 12 h for the translocation of glyphosate isopropylamine formulation, until it reaches the stem should be related to lower translocation of the product due to the reduction of transpiracional flow promoted by stomatal closure.

Figure 4 shows the translocation of glyphosate in *C. bengalensis* in root cuttings in periods of 24, 48, 72 and 96 h after product application. Observing the product at the root was from 24 h after application; the time of arrival of the product at the root occured after the tissues of the stems were already filled with the product which indicated that the time between the application of product in the leaves and its translocation to the root is approximately24 h in a very low concentration (Figure 4C). Hourly, there

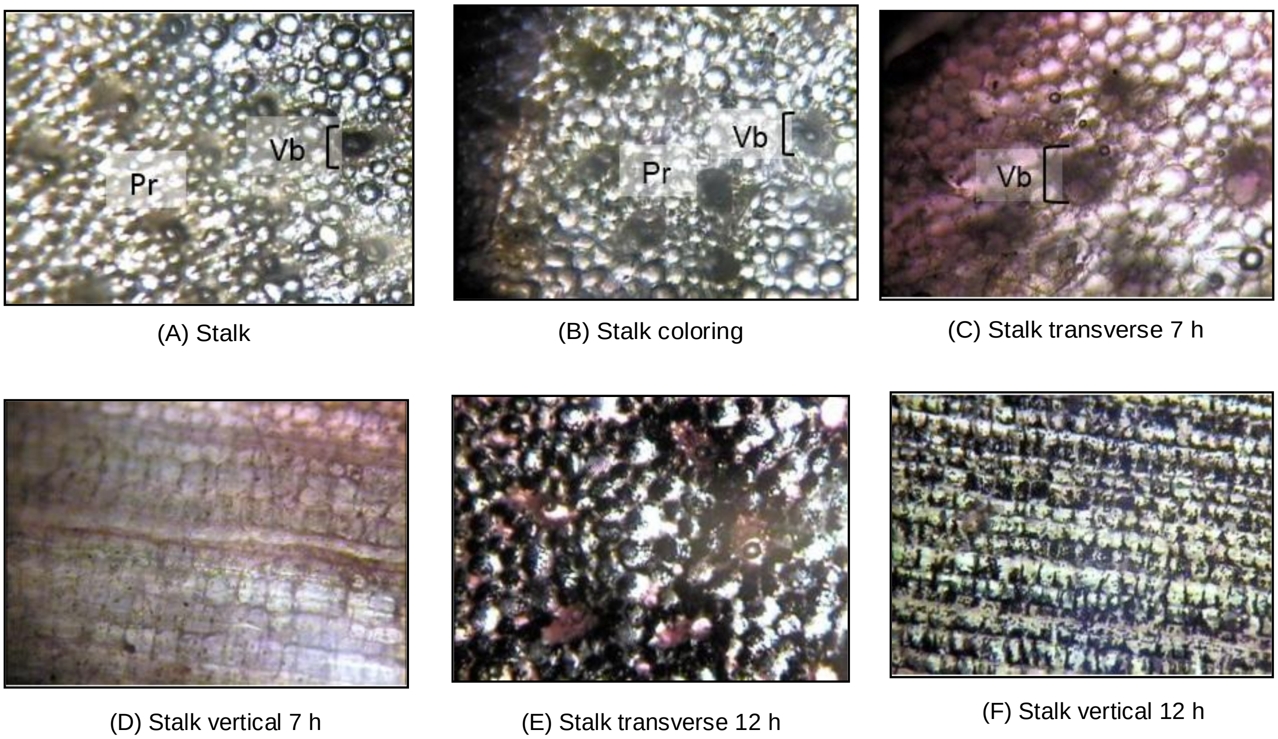


Figure 3. Longitudinal sections from different regions of the stem of *C. bengalensis*, 7 and 12 h after application of glyphosate potassium salt in the formulation. Pr: parenchyma; VB: vascular bundle; Xl: Xylem; Fl: phloem.

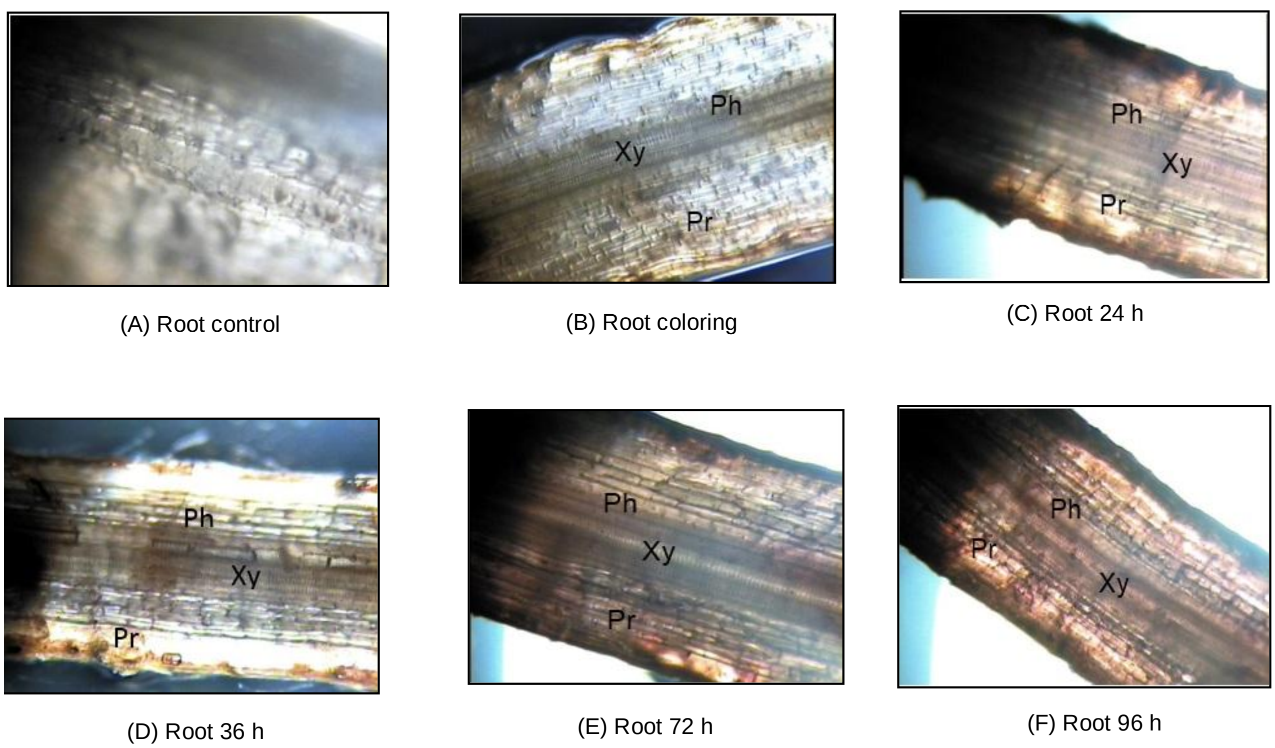


Figure 4. Cross sections from different regions of the *C. bengalensis* root, submitted to the application of glyphosate potassium salt formulation for product detection in conducting vessels. The views were performed 24, 36, 72 and 96 h after glyphosate application on this Roundup formulation. Xy: Xylem; Ph: phloem; Pr: parenchyma.

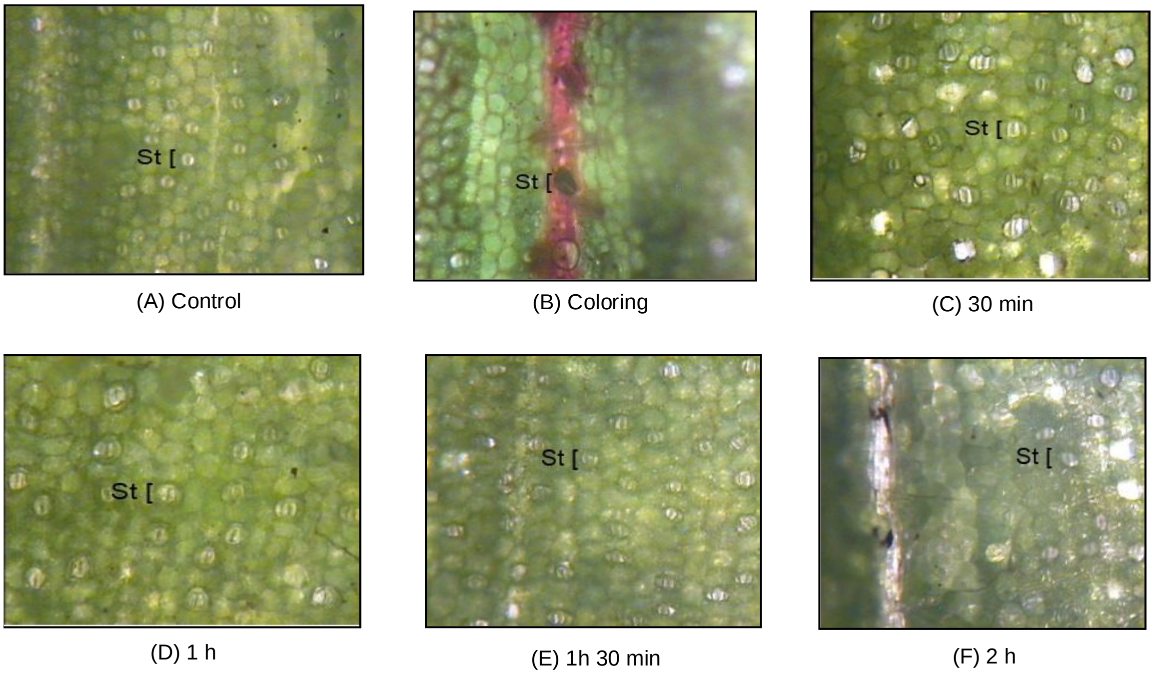


Figure 5. Cross-sections on the adaxial leaf *C. bengalensis*, subjected to glyphosate isopropylamine formulation, and evaluated from time to time: 30 min, 1 h, 2 h and 1 hr 30 min after glyphosate application.

was an increase in concentration of the product in the conducting vessels, and after 48 h the total filling of the product in the phloem was observed (Figure 4D). At 72 h after application, this was observed in phloem tissues and parenchyma (Figure 4E), and after 96 h it was observed within the xylem (Figure 4F), indicating that glyphosate began to be translocated upward, leading the product from root to shoot. The time required for the product to reach the root and be transported to the shoot was at least 96 h or more than three days. This information is very interesting because the application of another product, especially contact action, together with glyphosate or earlier than 3 days after application prevents the discharge of the product at the root and thus the reduction in the percentage dayflower control.

C. benghalensis presents amphistomatic leaves, which is often not a common feature in species of Commelinaceae family. The complex stomatal complex, as seen in Figure 5A was referred to as "six celled stomatal" by Tomlinson (1969). It consists of 2 guard cells and subsidiary cells; with six or four of them arranged parallel and perpendicular to the 2 guard cells. The guard cells and the subsidiaries are kidney-shaped and are about the same height. In the application of glyphosate isopropylamine formulation, 30 min after application of the dye (Figure 5B) the accumulation of glyphosate isopropylamine formulation in symplast can be seen (Figure 5C), indicating the absorption process in the cells of the epidermis and subsidiaries. One hour after application (Figure 5D), the accumulation of the product in all cells was constant, and after two hours, the presence of the product was seen in all mesophyll cell and totally filled the phloem in leaves. This gives a real estimate time for absorption of foliar applied product, which is already being absorbed 30 min after application, and the translocation via apoplast is very intense because it took additional two hours to reach the phloem.

Table 1 shows the significance of data between opening and closure of the stomatal in the leaves by applying the glyphosate potassium salt formulations and isopropylamine in different periods. This significant interaction revealed that the stomatal closed at different times after application of glyphosate in the two formulations. Figure 6 shows the length and width of the stomatal, which when correlated to the visual analysis, infer that from half an hour after glyphosate application, isopropiamina formulation begins its accumulation in the subsidiary cells, which promotes the beginning of the closing of the stomatal. The increased closing takes place quickly and after 2 h of application, since they are completely closed. This result shows the quick uptake of this formulation and accumulation in subsidiaries and guard cells. According to Hamylin (1998), the opening and closing of stomatal depends on the plant species and the treatment in which it is subjected, and translocation related transpiracional stream may represent only 20% cumulative CO_2 via stomatal.

From 7 h after application, both in the cross section (Figure 7C) and longitudinal (Figure 7D), the presence of glyphosate in all vascular stem tissues, except in the

Table 1. Average anatomical features *C. benghalensis* leaves.

Parameter	No. of stomata/mm^2	Length (μm)	Width (μm)
Control	155±4.0	24.9±0.3	16.6±0.3
Dye	148±3.2	23.7±0.2	15.8±0.6
Potassic salt 0.5 h	140±3.6	24.5±0.3	16.3±0.4
Potassic salt 1.0 h	123±4.1	22.9±0.2	15.1±0.2
Potassic salt 1.5 h	130±3.6	23.6±0.2	13.5±0.3
Potassic salt 2.0 h	118±3.2	24.1±0.3	12.3±0.2
Control	147±3.4	25.4±0.2	15.5±0.3
Dye	146±1.1	24.7±0.2	15.3±1.2
Isopropylamine salt 0.5 h	150±2.5	24.5±0.4	13.2±0.2
Isopropylamine salt 1.0 h	152±1.8	23.9±0.2	12.6±0.3
Isopropylamine salt 1.5 h	150±0.4	23.2±0.2	12.3±0.4
Isopropylamine salt 2.0 h	150±2.3	24.8±0.5	11.6±0.2

mean ± standard error.

xylem was observed. With 12 h after application, there was the greatest concentration of product in the conducting vessels and tissues overloading parenchyma in all cells of the marrow parenchyma (Figure 7). Probably, the stomatal closing affected photosynthesis and consequently, the resperation, because they rely on steady stream of CO_2 and O_2 in and out of the cell; this free flow is a function of the concentration of two gases in the intercellular spaces, which in turn depend on the stomatal opening, with the majority controlling the flow of CO_2 and O_2 (Taylor Jr and Gunderson, 1986; Messinger et al., 2006). The stomatal opening, in turn, is largely controlled by the swelling of both guard cells (which control the opening of the stomatal), as the epidermal cells of stomatal that are influenced by hydroelectric potential dependent on the solute potential. A low water potential induces stomatal closure, reduces leaf conductance and minimizes flow transpiration (Attridge, 1990; Hamlyn, 1998). Thus, there is a direct relationship between tolerance and stomatal opening. Therefore, the rapid closure of the stomatal can influence the translocation of glyphosate to other parts of the plant, can promote further degradation of the product in cells, and consequently increase the tolerance of this kind.

Figure 8 shows the translocation of glyphosate in *C. bengalensis* root 24, 36, 48 and 96 h after application. The earliest observation of the product in the roots was from 24 h after application; this explains that the time between the application of the product in leaf and its translocation to the root is approximately 24 h that is, glyphosate in the formulation potassium salt takes about one day to reach the root of spiderwort plants. With the slow passage of time, there was an increased concentration of product in the conductive vessels, and at 36 h after application, visualizing of the product in the phloem and some parenchymal cells can be detected, and only 96 h after application, the product was detected inside the xylem, indicating that glyphosate was discharged into the phloem and passed to the xylem and adjacent cells, including lesions in parenchyma cells. Pline et al. (1999) evaluated the ready roundap absorption rate in soybean plants with addition of ammonium sulfate or pelargonic acid as surfactants. In both cases, more than 40% of glyphosate had penetrated after 24 h of application. Santos et al. (2001) observed that the herbicide was absorbed into roundap ready more than 60% within the first 40 h.

Taking into account the time of absorption and translocation of this herbicide in the plant, it is possible to see the different behavior between the formulations. Considering only the leaf that received application of the product, a higher % in the formulation of isopropylamine was absorbed. Half an hour after application, it had already been absorbed by the cells and promoted stomatall closure, while the formulation with potassium salt took 2 h. However, the translocation of herbicide to stems and roots was similar, and it took an average of 7 h after the application of glyphosate to reach the stem, 12 h to reach the root of the potassium salt formulation, 24 h in isopropylamine, and 96 h for fall in xylem and was brought back to other parts of the plant. Santos et al. (2007) noted that uptake and translocation of glyphosate in different glyphosate formulations took approximately 40 h to overload the root.

Isopropylamine (the glyphosate formulation) was the most efficient in terms of absorption and translocation relative to the potassium salt which took about 2 h to be absorbed, in comparison to isopropylamine formulation which was filled with 2 h in phloem leaf. However, one cannot claim that the action of glyphosate in these plants depends on structural and anatomical features, resulting in the possibility of enzymatic degradation of the herbicide. However, that information is added to the list of information necessary for full understanding of the

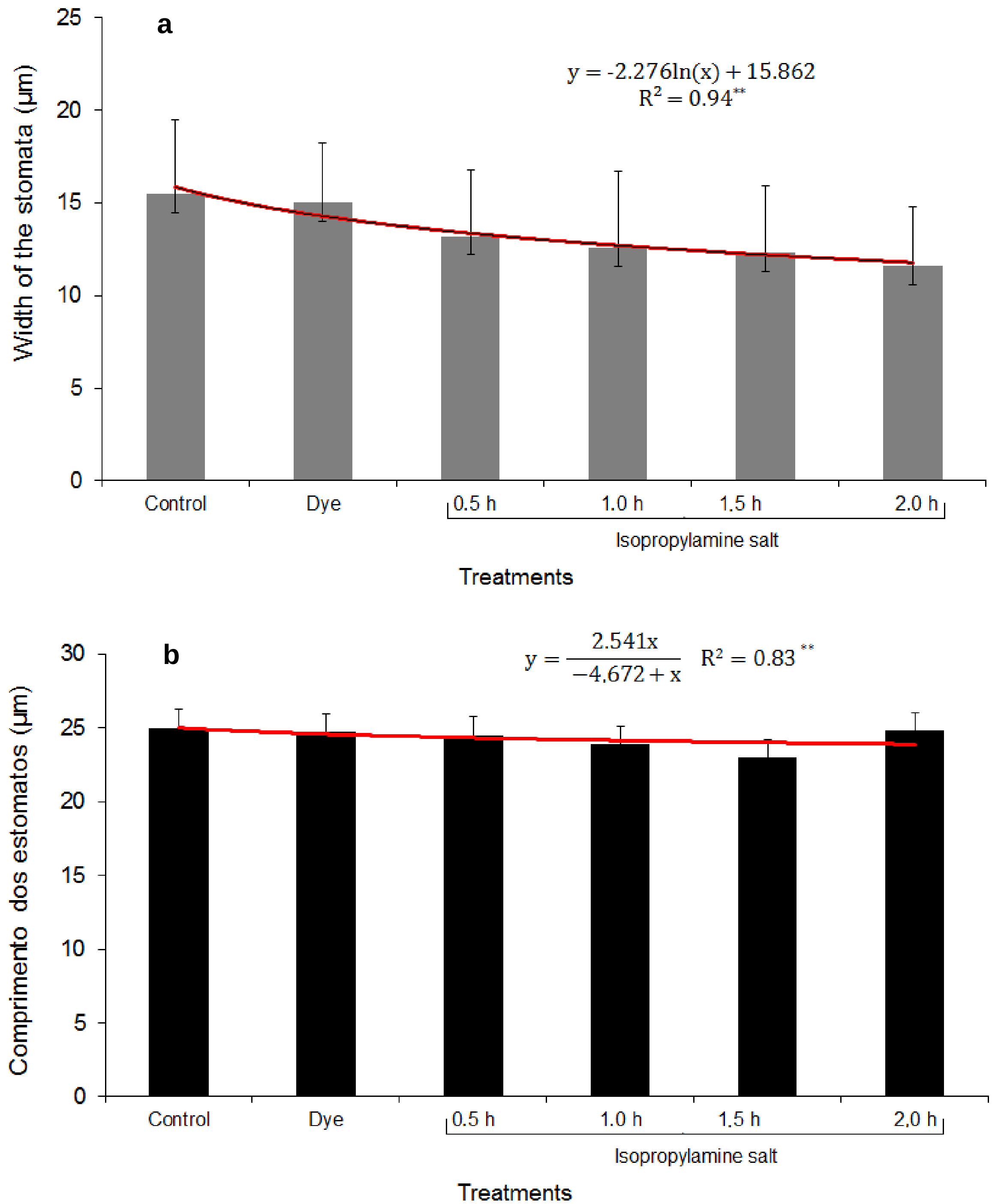
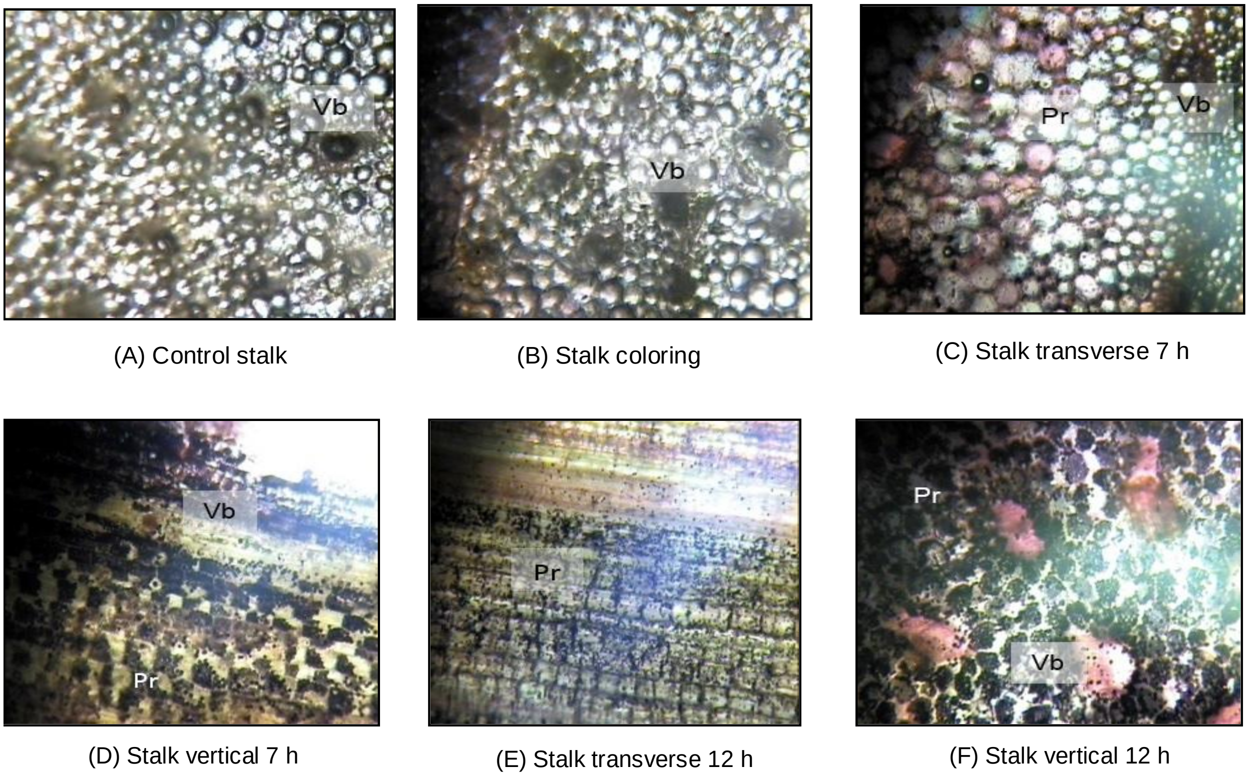


Figure 6. Width and length of stomatal of adaxial surface of leaves of *C. bengalensis*, subject to the application of glyphosate isopropyl amine formulation, evaluated after 30 min, 1 h, 1 h 30 min and 2 h.

phenomenon of glyphosate resistance in *C. benghalensis.*

Conclusion

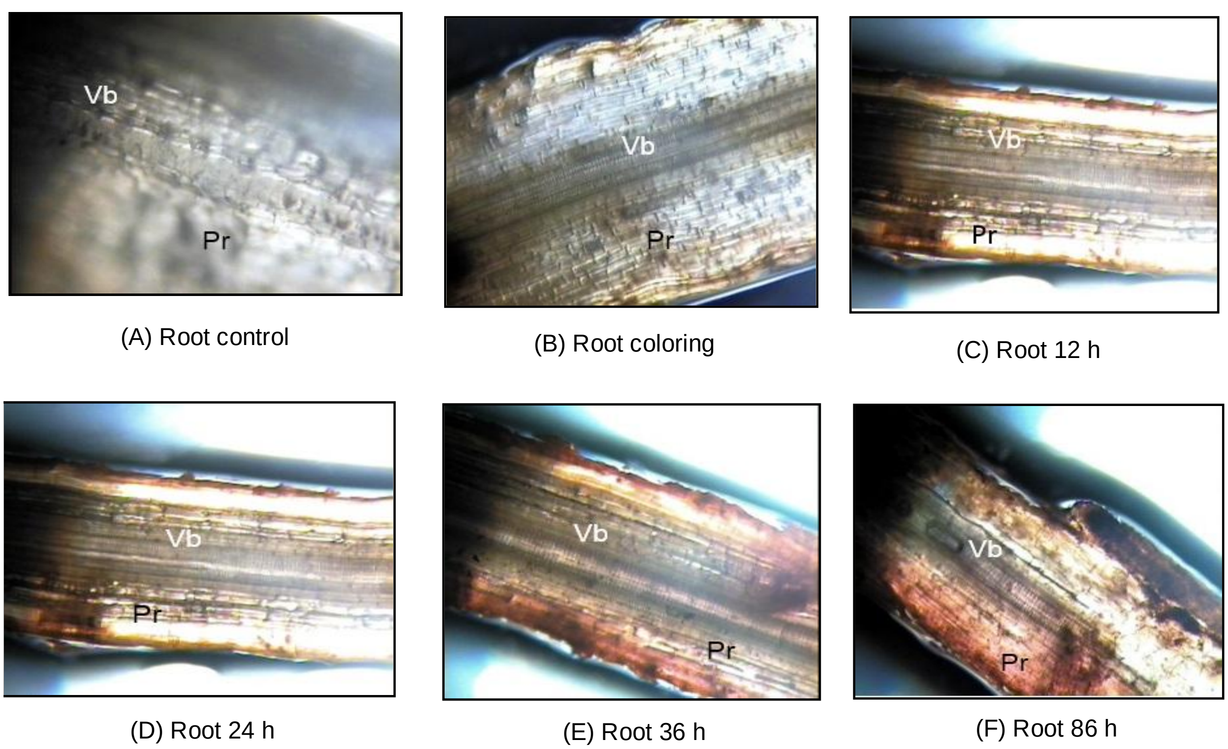
Tolerance of spiderwort to glyphosate cannot be attributed only to the absorption and translocation process, but of course, this aspect is an important factor in this process. This slow absorption by leaves and translocation to the root, regardless of glyphosate formulation, allows the plant to have more time to detoxify through chemical reactions that degrade the herbicide molecules, allowing a greater degree of tolerance. The ratio of absorption and



(A) Control stalk (B) Stalk coloring (C) Stalk transverse 7 h

(D) Stalk vertical 7 h (E) Stalk transverse 12 h (F) Stalk vertical 12 h

Figure 7. Longitudinal sections from different regions of the stem of *C. bengalensis*, 7 and 12 h after application of the glyphosate isopropylamine formulation. Fv: vascular bundle; Pm: pith.



(A) Root control (B) Root coloring (C) Root 12 h

(D) Root 24 h (E) Root 36 h (F) Root 86 h

Figure 8. Longitudinal section of root *C. bengalensis*, 12, 24, 36 and 86 h after application of glyphosate at R. ready formulation.

translocation with openness and stomatal closure still requires more careful studies to define its role due to the treatments which the weed is subjected to.

Conflict of Interests

The authors have not declared any conflict of interests.

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