4. EFFECT OF TEMPERATURE AND CHEMICAL ADDITIVES ON THE RESPONSE OF LIBERTY-LINK® AND ROUNDUP-READY® SOYBEANS TO GLUFOSINATE AND GLYPHOSATE

4.1 ABSTRACT

The influence of temperature and chemical additives on the response of transgenic crops to herbicide treatments was investigated using soybeans engineered with either a metabolism-based resistance (Liberty-Link®), or an altered target site resistance (Roundup-Ready®). Liberty-Link® soybeans engineered with the *pat* (phosphinothricin acetyl transferase) gene, detoxify glufosinate to the non-toxic metabolite, acetyl-glufosinate, while Roundup-Ready® soybeans, transformed with an altered EPSP synthase enzyme, prevent glyphosate from binding to its target site. For temperature studies, V1 stage soybeans were grown in chambers with constant temperatures of 15°, 25°, or 35° C. Liberty-Link® soybeans were treated with glufosinate rates ranging from 0.25 to 2.0 kg/ha, and Roundup-Ready® soybeans, with glyphosate rates ranging from 0.5 to 4.0 kg/ha. Chlorophyll measurements revealed a rate-dependent loss of chlorophyll in the center leaflet of the first trifoliolate of glufosinate-treated Liberty-Link® soybeans which was greater at 15° C rather than 25° or 35° C. Conversely, the rate of chlorophyll loss in the center leaflet of the terminal trifoliolate of glyphosate-treated Roundup-Ready® soybeans was greater at 35° than at 15° or 25° C. The absorption, translocation, and metabolism of ^{14}C-glufosinate in Liberty-Link® soybeans at 3, 12, 24, and 48 hours after treatment (HAT), and ^{14}C-glyphosate in Roundup-Ready® soybeans, at 1, 3, 5, 7 days after treatment (DAT), were examined under different temperatures in order to explain the observed injury from these herbicides at 15° C and 35° C respectively. Absorption of ^{14}C-glufosinate was significantly greater at 25° C than 15° C at 12, 24, and 48 HAT. There were only slight differences in ^{14}C-glufosinate translocation in Liberty-Link® soybeans. ^{14}C-glufosinate metabolism to the ^{14}C-acetyl-glufosinate metabolite was significantly less at 3 hours in 15° C than 25° C.

After 12 hours, however, metabolism was not different at the two temperatures. Roundup-Ready® soybean ^{14}C-glyphosate absorption was similar at 15° and 35° C. Soybeans grown at 35° C translocated more ^{14}C-glyphosate to shoots and leaves above the treated leaf, while those kept in 15° C translocated more to the shoots and leaves below the treated leaf. These results suggest that glufosinate injury to Liberty-Link® soybeans at 15° C may be due to reduced or slowed ^{14}C-glufosinate metabolism. Glyphosate injury in Roundup-Ready® soybeans at 35° C may be due to increased translocation of ^{14}C-glyphosate to leaves above the treated leaves. V1 stage transgenic soybeans were also treated with the chemical additives ammonium sulfate (AMS) and pelargonic acid (PA) in combination with glufosinate or glyphosate. Both transgenic soybean varieties showed no increase in injury with the addition of 5% (w/v) AMS to the spray mix, however, a herbicide rate-dependent loss of fresh weight was found in both Liberty-Link® and Roundup-Ready® soybeans when the herbicide was applied in combination with 3% (v/v) PA. Absorption and translocation studies on Liberty-Link® soybeans showed increased ^{14}C-glufosinate absorption in plants treated with AMS plus glufosinate at two time periods, while PA plus glufosinate treatments were no different than glufosinate alone treatments. Translocation and metabolism of ^{14}C-glufosinate did not differ between treatments. ^{14}C-glyphosate absorption in Roundup-Ready® soybeans was less in both AMS and PA treatments than glyphosate alone treatments. Translocation of ^{14}C-glyphosate did not vary with treatment. These data suggest that the injury found in PA treatments is due more to the herbicidal properties of PA than increased absorption or translocation of the herbicide.

Nomenclature: Glufosinate, 2-amino-4-(hydroxymethylphosphinyl) butanoic acid; Glyphosate, *N*-(phosphonomethyl) glycine; PA, pelargonic acid (nonanoic acid); AMS, ammonium sulfate; *pat*, phosphinothricin acetyl transferase; EPSPS, 5-enolpyruvylshikimate-3-phosphate synthase, DAT, days after treatment; HAT, hours after treatment.

4.2 INTRODUCTION

Environmental factors such as light intensity, water stress, relative humidity, nutrient levels, atmospheric pollution and temperature stress have all been shown to affect the efficacy of a herbicide, as well as its crop safety (Cole, 1983). Temperature changes can rapidly affect herbicide efficacy by either changing the rate of metabolism, herbicide absorption, or translocation to target sites (Hatzios and Penner, 1982). Even crops that are normally tolerant to a herbicide can show injury symptoms at different temperatures. Thompson et al. (1970) reported that maize, which is normally tolerant to atrazine, was injured at lower temperatures because the glutathione conjugation and hydroxylation reactions that normally detoxify this herbicide were retarded at low temperatures resulting in crop damage. With transgenic herbicide-resistant crops entering the market, it is important to investigate whether environmental factors such as temperature will influence the level of resistance that these crops possess.

Glufosinate is a non-selective post emergence herbicide that has its effect in plants by inhibiting glutamine synthetase (Wendler et al., 1990). Inhibition of this enzyme causes a large buildup of ammonia in plant cells as well as depleting the plant of crucial amino acids. Anderson et al. (1993 a, 1993b) found that the efficacy of glufosinate, on barley and green foxtail was highly dependent on environmental factors such as rainfall, soil moisture, temperature, and relative humidity. Light was also shown to affect the ammonia accumulation and tissue necrosis of treated plants (Kocher, 1983).

Liberty-Link® soybean lines containing the *pat* (phosphinothricin acetyl-transferase) gene conferring glufosinate tolerance by metabolism, are currently under development. The nonselective herbicide glufosinate can be applied to Liberty-Link® soybeans with good weed control, as well as safety to soybeans. Roundup-Ready® soybeans contain a glyphosate insensitive EPSP synthase gene introduced from *Agrobacterium* sp. conferring tolerance to the non-selective herbicide glyphosate (Padgette et al., 1995).

Presently there is little information regarding the absorption, translocation, or metabolism of glyphosate and glufosinate in these herbicide resistant soybean varieties at different temperature regimes. The enzyme encoded by the *pat* gene contained in

Liberty-Link® soybeans has been shown to have very low activity at 10° C and increasing activity to 45° C (Botterman et al., 1991). Night temperatures as low as 10° to 15° C are not uncommon during the soybean-growing season. Therefore, the potential for injury to soybeans transformed with the *pat* gene, due to reduced or slower glufosinate detoxification, is present.

The primary mode of action of glyphosate is inhibition of the enzyme EPSP synthase (Duke, 1985). However, glyphosate has also been shown to inhibit δ-aminolevulinic acid synthesis in barley and corn resulting in a blockage of chlorophyll synthesis, as well as other porphyrin ring compounds (Kitchen et al., 1981).

Herbicide combinations, as well additives, have been shown to affect the safety of glufosinate or glyphosate on transgenic herbicide-tolerant soybeans (Bennett et al., 1998). Pline, (Thesis chapter 2, 1999), showed that the herbicidal additive pelargonic acid caused significant injury to Liberty-Link® and Roundup-Ready® soybeans when applied in combination with glufosinate or glyphosate. It is unclear whether this injury is due to the herbicidal activity of the glufosinate or glyphosate overcoming the resistance mechanism, or to the herbicidal activity of pelargonic acid.

The specific objectives of this study were to first determine whether variable temperature affects the safety of Liberty-Link® and Roundup-Ready® soybeans to applications of glufosinate or glyphosate. Secondly, studies were conducted to determine whether these temperature effects are due to differential absorption, translocation, or metabolism of glufosinate or glyphosate. Finally, studies to determine whether the additives AMS or PA affect the absorption, translocation, or metabolism of glufosinate or glyphosate in transgenic soybeans were conducted.

4.3 MATERIALS AND METHODS

4.3.1 Growth chamber studies

Liberty-Link® (variety 5547 LL) and Roundup-Ready® soybeans (variety Asgrow 4501) were grown in 450-ml Styrofoam cups containing Metro-Mix 360, (Scotts-

Sierra Horticultural Products Co., Marysville, OH). Soybeans were kept in a greenhouse at 25° ± 2° C where natural sunlight was supplemented with mercury-halide lights providing 650 μmol/m^2/sec and 16 hours day length. Three days before soybeans reached the V1 growth stage, plants were transferred to growth chambers set at 15° or 35° C, 75% relative humidity and a 16 hour day length, with the 25° C treatments remaining in the greenhouse. After 3 days in the respective temperature treatments, Liberty-Link® soybeans were treated with 0, 0.25, 0.5, 0.75, 1, or 2 kg/ha glufosinate, and Roundup-Ready® soybeans with 0, 0.5, 1, 2, or 4 kg/ha glyphosate with a track sprayer delivering 237 L/ha using a 8001E nozzle. Immediately following treatment, plants were returned to their respective temperature treatments for 10 days where plants were kept watered as needed.

Visual ratings, fresh weights, and chlorophyll measurements were taken 10 DAT to quantify herbicide injury. For chlorophyll measurements, the first trifoliolate of Liberty-Link® soybeans and terminal trifoliolate of Roundup-Ready® soybeans was weighed at the time of harvest.

The methodology of Hiscox and Israelstam (1979) was used for chlorophyll extraction. Excised trifoliolates were soaked in 10-ml of dimethyl-sulfoxide (DMSO) for 24 hours in the dark to extract chlorophyll. One ml of DMSO/chlorophyll solution was read in a spectrophotometer at 645 and 663 nm wavelength. Chlorophyll content was calculated per gram of leaf tissue using the equation of Arnon (1949): Chlorophyll (g/L) = $(0.0202\ A_{645} + 0.00802\ A_{663})$ x dilution factor using DMSO as the solvent instead of acetone. Once the chlorophyll content of the 10-ml test tube of DMSO was determined, it was divided by the fresh weight of the trifoliolate and expressed as mg chlorophyll/ g leaf tissue.

4.3.2 Temperature and additive effects on herbicide absorption, translocation, and metabolism

Absorption, translocation, and metabolism studies on Liberty-Link® soybeans were conducted using ^{14}C labeled-glufosinate and on Roundup-Ready® soybeans using ^{14}C labeled-glyphosate. Soybeans for temperature studies were grown as in greenhouse studies and transferred to 15° or 25° C growth chamber for Liberty-Link® soybeans and 15° or 35° C for Roundup-Ready® soybeans 48 hours prior to treatment at the V1 growth stage, while soybeans for additive studies remained in greenhouse conditions. For temperature studies, Liberty-Link® soybeans were treated with 0, 1.0, or 2.0 kg/ha formulated glufosinate and Roundup-Ready® soybeans with 0, 2.0, or 4.0 kg/ha formulated glyphosate. For additive studies, Liberty-Link® soybeans were then treated with 1 kg/ha formulated glufosinate, and Roundup-Ready® soybeans with 2 kg/ha formulated glyphosate, alone or in the presence of 5% (w/v) AMS or 3% (v/v) PA applied at 237 L/ha output on a track sprayer.

The center leaflet of the first trifoliolate was then immediately treated with 5-µl of solution containing 0.01% Silwet L-77 and 1.25 kBq of ^{14}C-glufosinate [specific activity of (3,4-^{14}C)-glufosinate of 1917 kBq/mg] for Liberty-Link® soybeans, and 0.83 kBq [specific activity of 2.04 GBq/mmol] of ^{14}C-glyphosate for Roundup-Ready® soybeans. Soybeans used for temperature studies were returned immediately to their respective temperature treatments. Harvests were made at 3, 12, 24, 48 HAT for Liberty-Link® soybeans, 1, 3, 5, 7 DAT for Roundup-Ready® soybeans. At harvest, the treated leaflet was washed with 10-ml of a solution of 0.05% Silwet L-77 (OSI, Danbury, CT). A one-ml sample of the wash was mixed with 10-ml scintillation cocktail (ScintiVerse®, Fisher, Pittsburgh, PA) and the ^{14}C was quantified using liquid scintillation spectrometry (Beckman LS 5000TA Model, Beckman Instruments, Fullerton, CA). Plants were separated into treated leaf, shoots and leaves above the treated leaf, shoots and leaves below the treated leaf, and roots. Parts were then oxidized (Model B307, Packard Instrument Company, Downers Grove, IL) to measure absorption and translocation of labeled herbicides, and replicate plants were used for metabolism studies in Liberty-Link® soybeans.

Metabolism studies were conducted on Liberty-Link® soybeans at each temperature by glufosinate rate by time treatment for temperature studies, and additive by time treatment for additive studies. Plants were stored at –20° C until metabolism studies were conducted. The treated trifoliolate of each soybean plant was pulverized in liquid nitrogen. The ground material was transferred to 10-ml test tubes containing 3-ml of water to extract ^{14}C-glufosinate and ^{14}C-glufosinate metabolites, then 0.5-ml Hajra's solution (0.2 M H_3PO_4 / 1 M KCl) (Hajra, 1974) and 3-ml chloroform was added to each tube to dissolve lipids and precipitate proteins. Tubes were vortexed and centrifuged at 15,000 rpm for 5 minutes. Water extract was then transferred to 5-ml test tubes and dried down. Plant extract was then re-suspended with 1-ml 80% methanol, centrifuged for 1 minute, and eluate transferred to a 1.5-ml microfuge tube. Eluate was again dried and resuspended with 0.2 ml 80% methanol. Thin layer chromatography plates (Silica Gel 60 F_{254} TLC Plates, EM Science, Gibbstown, NJ) were prepared by baking for 1 hour at 110° C. Standard ^{14}C-glufosinate and ^{14}C-glufosinate metabolites, and 50 μl of each plant extract were spotted onto separate 1.5 cm wide lanes on TLC plates and were developed in an isopropyl alcohol: glacial acetic acid: water (2:1:1) solvent system. After plates were dry, they were exposed to X-ray film for three weeks. Parent and metabolite bands were scraped from plates and quantified using liquid scintillation spectrometry. The percent metabolite was determined by its proportion of the total radioactivity recovered from TLC plates.

4.3.3 Experimental design and statistical analysis

Growth chamber and temperature studies on absorption, translocation, and metabolism were designed as split plot designs with a main plot of temperature and subplots of herbicide rate in growth chamber studies, and time after treatment in ^{14}C studies, with 4 replications. Experiments were repeated. Analysis of variance was run using SAS (SAS version 6.12, SAS Institute, inc.) and data from repeated experiments were combined due to no significant interactions. Linear regression was conducted on growth chamber study data using Excel (Excel version 8.0, Microsoft Corp.). Temperature studies on absorption, translocation, and metabolism revealed that absorption, translocation, and metabolism were independent of herbicide rate, so only main plot effect of temperature and subplot of time after treatment were compared. Means were separated using SAS Fisher's Protected LSD method at α=0.05 or 0.1.

4.4 RESULTS AND DISCUSSION

4.4.1 Temperature sensitivity of transgenic soybeans to herbicide applications

Chlorophyll measurements showed a glufosinate rate dependent reduction in chlorophyll that was greater at 15°C than at 25° or 35°C (Figure 4.1 A). The slopes of the lines at each temperature treatment over the range of glufosinate rates were used as a measure of relative herbicide sensitivity. The chlorophyll content of Liberty-Link® soybean leaves was greatest at 35°C followed by 25°C, and 15°C. The rate of chlorophyll loss over increasing glufosinate rates was equal at 25 and 35° C (-0.16 and –0.17 mg chlorophyll/g leaf tissue per kg/ha glufosinate respectively). However, at 15° C the rate of loss was 2 times greater, (-0.37 mg chlorophyll/g leaf tissue per kg/ha glufosinate), indicating an increase in glufosinate sensitivity at 15° C. Plants grown at 15° C and treated with rates of 1 kg/ha and higher glufosinate had visual injury such as chlorosis and necrosis of the treated tissue (Figure 5A, 6A), which somewhat resembled glufosinate injury to plants which are not resistant to glufosinate (Bellinder, 1987).

In Roundup-Ready® soybeans, the rate of chlorophyll loss over increasing rates of glyphosate in the terminal trifoliolate was greater at 35° C than at 15° and 25° C (Figure 4.1 B). The chlorophyll content in non-treated terminal trifoliolates of Roundup-Ready® soybeans was greatest at 35° C, followed by 15° and 25° C. The rate of chlorophyll loss over increasing glyphosate rates was lowest at 15° C (-0.05 mg chlorophyll/g leaf tissue per kg/ha glyphosate), followed by 25° C (-0.16 mg chlorophyll/g leaf tissue per kg/ha glyphosate). At the 35° C the rate of loss was 11 times greater than at the 15° C treatment, (-0.55 mg chlorophyll/g leaf tissue per kg/ha glyphosate), indicating a sharp increase in glyphosate sensitivity at 35° C. In plants grown at 35° C, chlorophyll loss was evident at 0.5 kg/ha glyphosate and increased as rate increased. Plants grown at 35° C and treated with high rates of glyphosate showed symptoms of severe chlorosis, which increased in severity in every newly developing trifoliolate. The newest trifoliolates at the 4.0 kg/ha rate appeared completely chlorotic (Figure 4.5 B, 4.6 B).

4.4.2 Temperature effects on absorption, translocation, and metabolism of ^{14}C-glufosinate

Absorption of ^{14}C-glufosinate by Liberty-Link® soybeans was temperature dependent (Figure 4.2 A). Absorption at 25° C was significantly greater at 12 and 48 HAT, and than at 15° C. Absorption appeared to slow at 12 HAT. At 48 HAT absorption at 25° C reached 48% and 34% at 15° C indicating that Liberty-Link® soybeans at 25° C generally absorb glufosinate more rapidly and a greater amount over time than soybeans at 15° C. Anderson et al. (1993), found that lower temperatures delayed the activity of glufosinate in barley and green foxtail, but only small differences in glufosinate activity remained at 288 HAT. This reported delay in glufosinate activity could be due to slower initial glufosinate absorption, as the present data demonstrate. However, differences in ^{14}C-glufosinate absorption in Liberty-Link® soybeans over temperature, do not seem to explain the increased sensitivity seen at 15° C over 25° C.

Translocation of ^{14}C-glufosinate by Liberty-Link® soybeans did not vary with temperature until 48 HAT (Table 4.1 A). The majority of glufosinate (80-84%) at 12 HAT remained in the treated leaf with 3-5% being translocated to shoots above the treated leaf, 7-10% to leaves and shoots below the treated leaf, and 6% translocated to the roots. The proportion of ^{14}C-glufosinate moving out of the treated leaf increased with time. At 48 HAT, significantly more ^{14}C-glufosinate remained in the treated leaf at 15° C than at 25° C. Translocation to the roots was also significantly higher at 25° C than at 15° C (14% vs. 4% respectively). With slightly more ^{14}C-glufosinate remaining in the treated leaf at 15° C versus 25° C, it is possible that if this glufosinate is non-metabolized, it could be responsible for the greater injury observed in the treated trifoliolate at 15° C versus 25° C.

Metabolism of ^{14}C-glufosinate by Liberty-Link® soybeans was temperature dependent at 3 HAT (Figure 4.3). In soybeans kept at 15° C, metabolism of ^{14}C-glufosinate to the nontoxic ^{14}C-acetyl-glufosinate metabolite was significantly lower than at 25° C (39% versus 49% respectively). After 12 hours, metabolism of ^{14}C-glufosinate to the ^{14}C-acetyl-glufosinate metabolite was not significantly different at 15° or 25° C. Approximately 50% of the ^{14}C-glufosinate was found as parent ^{14}C-glufosinate, and 50% as ^{14}C-acetyl-glufosinate. Dröge et al. (1992) reported that the *PAT* enzyme contained in transgenic, herbicide resistant plants is specific to the L-enantiomer of glufosinate, therefore only 50% of the racemic mixture of DL-^{14}C-glufosinate is *N*-acetylated. Our results, indicating approximately 50% of the ^{14}C-glufosinate metabolized after 3 hours, agree with those of Dröge et al. (1992).

Differences in absorption and translocation of ^{14}C-glufosinate at 15° C versus 25° C fail to adequately explain the increased glufosinate sensitivity at 15° C. However, lower metabolism of ^{14}C-glufosinate to the non-toxic *N*-acetyl-glufosinate metabolite could affect sensitivity by leaving more active L-glufosinate available in the plant to bind to glutamine synthetase causing ammonia buildup and injury in tissue. These results, showing a significant difference in metabolism at 3 HAT in 15° C versus 25° C, but a subsequent recovery of metabolism after 12 hours, suggest that metabolism is simply slower at 15° C versus 25° C. The enzyme kinetics of the *PAT* enzyme over temperature and pH showed very low *PAT* activity at 10° C, with increasing activity up to 40° C (Botterman et al., 1992). Temperature sensitivity to glufosinate application at 15° C in Liberty-Link® soybeans could therefore be due to slow *PAT* activity in the initial hours after treatment. Another possibility for increased glufosinate sensitivity at 15° C could be possible lower *pat* promoter activity and a subsequently lower amount of active *PAT*. Many metabolic processes in plants are affected by temperature, and it is possible that a combination of these processes could account for the increased sensitivity observed in these studies.

4.4.3 Temperature effects on absorption, translocation, and metabolism of ^{14}C-glyphosate

Absorption of ^{14}C-glyphosate by Roundup-Ready® soybeans was temperature independent with soybeans kept at 15° C and 35° C absorbing similar amounts of ^{14}C-glyphosate at all time periods investigated (Figure 4.2 B). Absorption after 7 days reached a high of 62% at 35° C and 56% at 15° C. The rate of ^{14}C-glyphosate absorption was very high in the first 24 HAT, and increased slowly to 7 DAT.

The pattern of translocation of ^{14}C-glyphosate in Roundup-Ready® soybeans was dependent on temperature (Table 4.1 B). The amount of ^{14}C herbicide remaining in the treated leaves did not vary with temperature, but did gradually decrease at every harvest period. The amount of ^{14}C-glyphosate translocated to the shoots and leaves above the treated leaf was highly dependent on temperature treatment. Soybeans kept at 35° C translocated a greater proportion of ^{14}C-herbicide to the above treated leaf portion than those kept at 15° C at 3, 5, and 7 DAT. Translocation to shoots and leaves above the treated leaf reached a maximum of 25% of absorbed ^{14}C-glyphosate 7 DAT at 35° C. In contrast, Roundup-Ready® soybeans kept at 15° C translocated a greater proportion of ^{14}C-glyphosate to shoots and leaves below the treated leaf than did those kept at 35° C, reaching a maximum of 17% of absorbed ^{14}C-glyphosate at 3 DAT at 15° C. Translocation of ^{14}C-glyphosate to roots varied with temperature at 1 and 3 DAT, but was similar at 5 and 7 days. Soybeans kept at 35° C translocated a greater proportion of ^{14}C-glyphosate to roots than those kept at 15° C up to 3 days. This was transitory however, as root translocation at 5 and 7 days was not different. It appears that translocation to the roots at 15° C is slower than at 35° C, with translocation to roots of plants grown at 35° C reaching 11% 1 DAT, and roots of plants grown at 15° C not reaching this level until 3 DAT.

Transport of sugars in phloem tissue has been shown to be lower as temperature decreases. Since glyphosate is a phloem translocated compound, it is possible that translocation to metabolic sinks such as roots, and developing leaves and shoots above the treated leaf, is slower at 15° C than at 35° C. This could account for the lower proportion of radioactivity present in these tissues at 15° C than 35° C.

It is clear from this study that a greater proportion of ^{14}C-glyphosate reaches the newly developing trifoliolates at 35° C than at 15° C. This does not however, explain the chlorosis observed in these trifoliolates. The transgenic EPSP synthase enzyme in Roundup-Ready® soybeans is insensitive to glyphosate therefore, injury due to EPSP synthase inhibition should not be observed. Glyphosate has been proposed to have secondary modes of action in addition to inhibition of EPSP synthase. One of these is the inhibition of chlorophyll biosynthesis. Inhibition by glyphosate of δ-aminolevulinic acid synthesis resulting in inhibition of chlorophyll and other porphyrin ring compound synthesis has been reported (Kitchen et al., 1981). Glyphosate has also been shown to form metal complexes while in spray solution (Nalewaja and Matysiak, 1991). If these chelating effects were to carry over in-vitro, inhibition of chlorophyll and other metal ion containing molecules is possible. Although Roundup-Ready® soybeans contain a glyphosate insensitive EPSP synthase enzyme, it is possible that glyphosate accumulating in developing tissue such as soybean trifoliolates could inhibit chlorophyll biosynthesis causing a chlorotic appearance, as was observed in this study at 35° C at high glyphosate rates. It seems unlikely that the injury is caused by the transgenic EPSP synthase enzyme losing glyphosate insensitivity, because one would expect chlorosis and tissue death on all tissue, not simply the newly developing trifoliolates. It is also possible that the transgenic EPSP synthase gene is not expressed as efficiently in developing tissue or at higher temperatures, or that the native EPSP synthase is simply saturated causing a lack in chorismate production.

4.4.4 Additive effects on absorption, translocation, and metabolism of herbicide in soybeans

Absorption of ^{14}C-glufosinate by Liberty-Link® soybeans was significantly higher in the presence of AMS than in glufosinate alone at 12 and 24 HAT (Figure 4.3 A). PA treatments showed ^{14}C-glufosinate absorption similar to that of glufosinate alone at each time period. Absorption increased rapidly in the first 3 HAT, but did not significantly change after 3 hours in any of the additive treatments.

Differences in translocation were only present at 12 HAT (Table 4.2 A). ^{14}C-glufosinate remaining in the treated leaf was significantly higher in the presence of AMS than glufosinate alone in Liberty-Link® soybeans. The amount of ^{14}C-glufosinate translocated to the shoots and leaves above the treated leaf was significantly lower in the presence of PA than glufosinate alone at 12 HAT. Metabolism of ^{14}C-glufosinate by Liberty-Link® soybeans did not differ over time or by additive treatments.

Roundup-Ready® soybean absorption of ^{14}C-glyphosate alone and in the presence of AMS or PA increased very rapidly in the first 24 HAT and more slowly after 24 hours (Figure 4.3 B). Soybeans treated with AMS and PA had decreased absorption of ^{14}C-glyphosate than those treated with glyphosate alone. Translocation of ^{14}C-glyphosate by Roundup-Ready® soybeans out of the treated leaf and to the above and below treated leaf portions and roots was the similar in treatments with AMS or PA as in those with glyphosate alone over all time periods investigated (Table 4.2 B).

These data suggest that the phytotoxicity observed in glufosinate and glyphosate treatments in combinations with PA on Liberty-Link® and Roundup-Ready® soybeans (Pline, Thesis chapter 2, 1999), is not due to increased absorption, translocation, or metabolism of glufosinate or glyphosate by these soybeans. It is possible that the increasing phytotoxicity as glufosinate or glyphosate rate increases could be due to a corresponding increase in the concentration of adjuvants in the spray solution. These adjuvants could act to increase the phytotoxicity of PA over those treatments with PA applied alone.

In summary, the results of these studies show that temperature sensitivity in transgenic herbicide tolerant soybeans is likely due to decreased herbicide metabolism in Liberty-Link® soybeans at low temperatures, and to increased translocation to sensitive tissue in Roundup-Ready® soybeans at high temperatures. The glufosinate or glyphosate rate-dependent increase in injury to Liberty-Link® and Roundup-Ready® soybeans observed in treatments containing 3% (v/v) PA is not due to increased absorption and translocation or decreased metabolism of glufosinate or glyphosate by transgenic soybeans.

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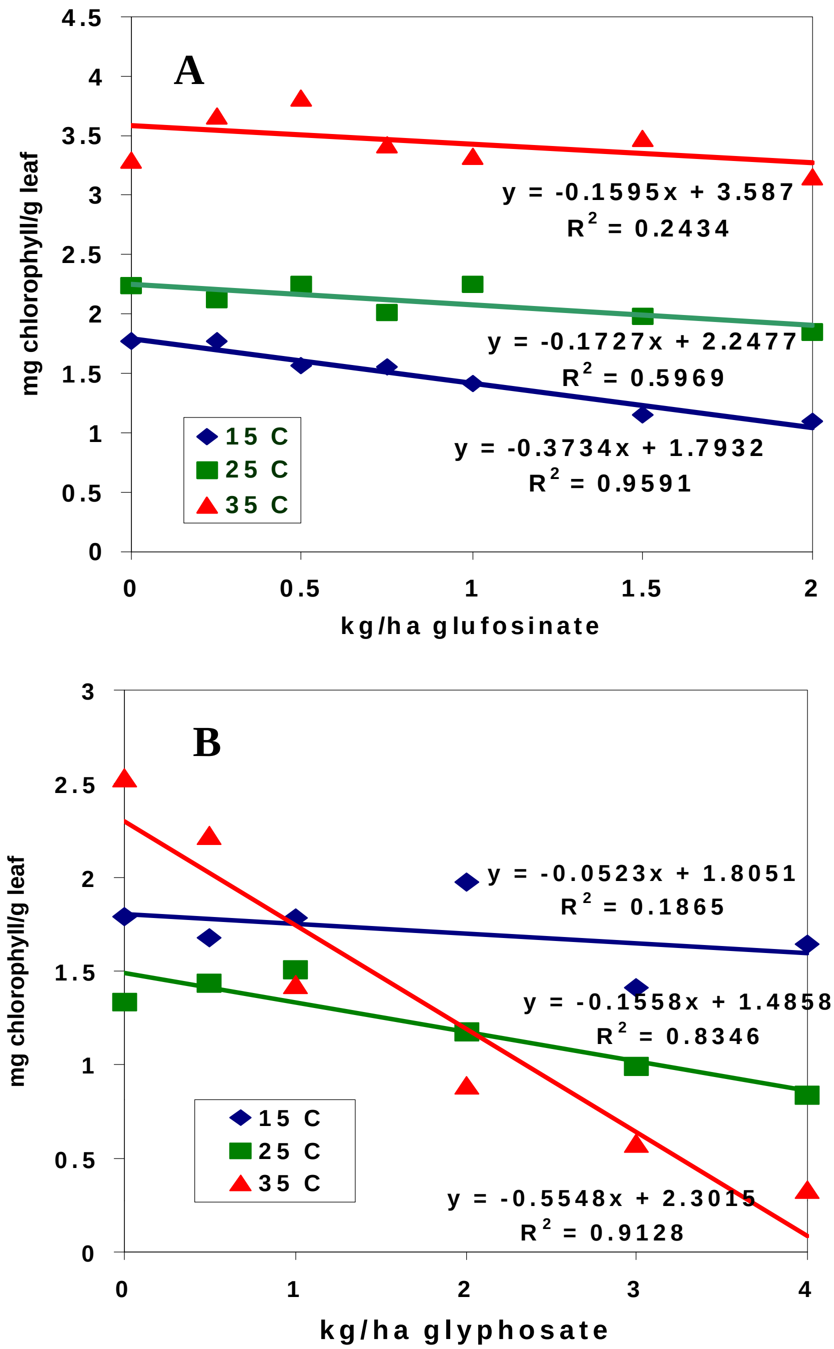

Figure 4.1. Effect of glufosinate on chlorophyll content of Liberty-Link® (A.) and glyphosate on chlorophyll content of Roundup-Ready® (B.) soybeans grown at 15°, 25°, or 35° C, 10 DAT.

Linear regression analysis was used to measure the rate of herbicide rate dependent chlorophyll loss of Liberty-Link soybeans treated with glufosinate and Roundup-Ready soybeans treated with glyphosate at variable temperatures.

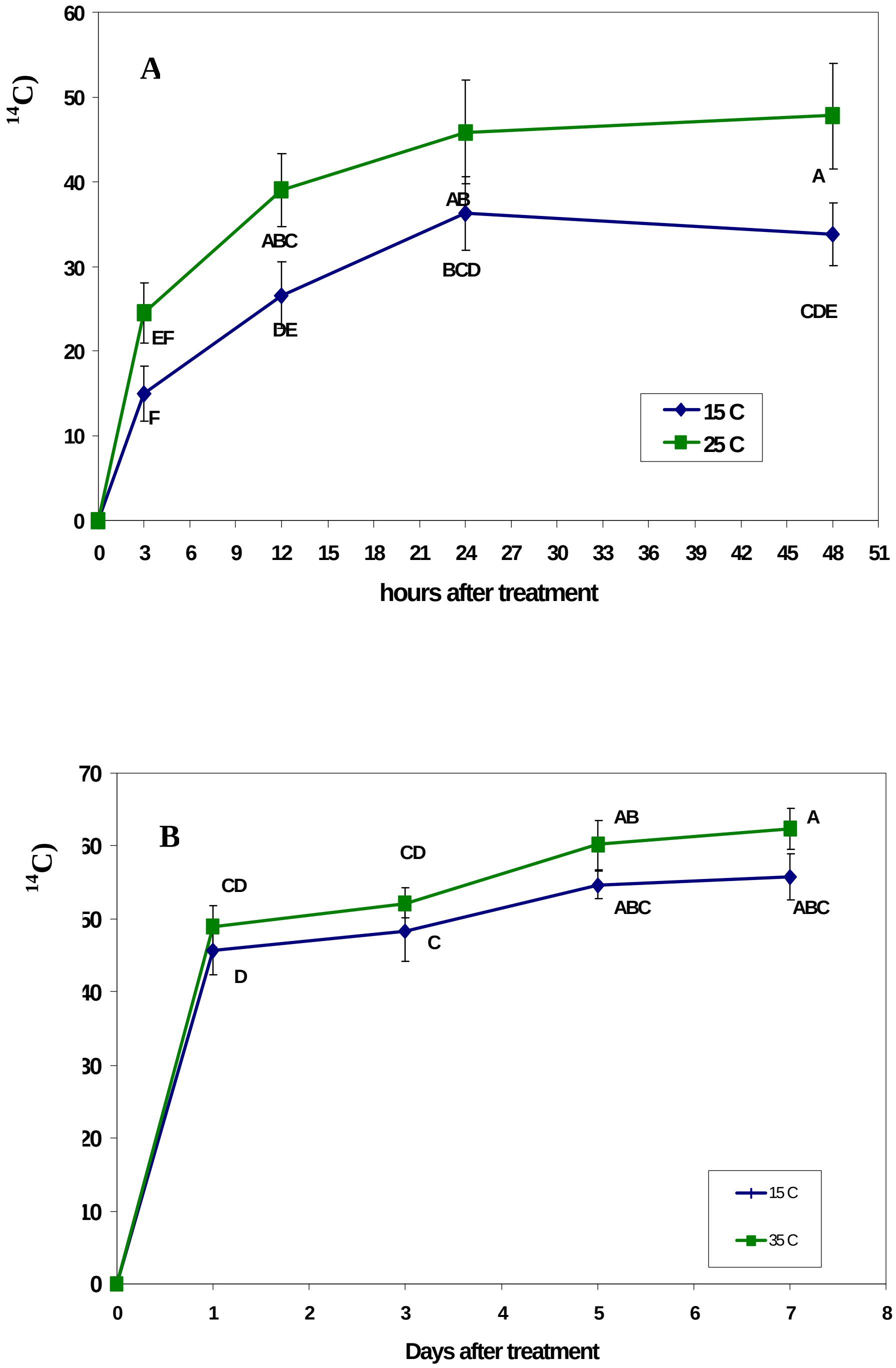

Figure 4.2. Absorption of ^{14}C-glufosinate by Liberty-Link soybeans grown at 15° and 25° C (A) and ^{14}C-glyphosate by Roundup-Ready soybeans grown at 15° and 35° C (B).

Percent absorption is the percentage of the applied ^{14}C herbicide that was absorbed at each time period. Vertical bars represent standard errors of means. Means were separated using Fishers' Protected LSD test at α=0.05. Means followed by the same letter are not significantly different.

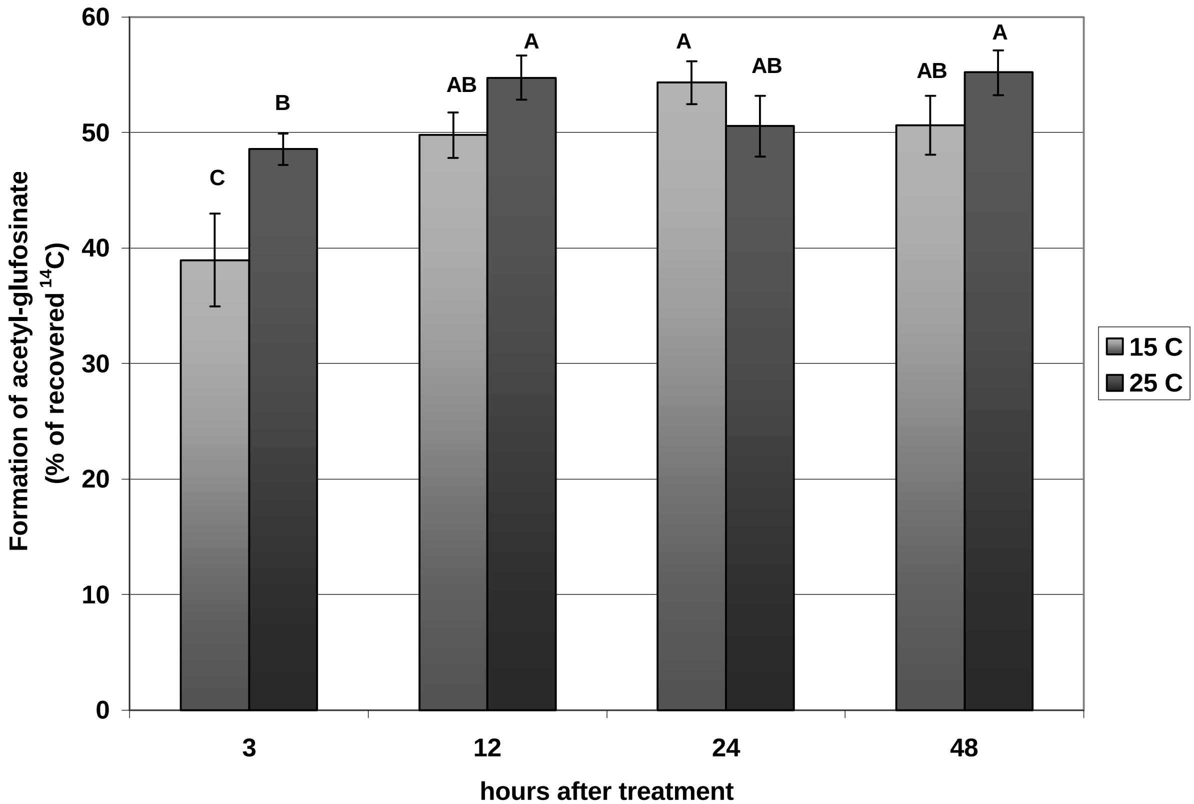

Figure 4.3. Metabolism of ^{14}C-glufosinate by Liberty-Link soybeans grown at 15° C and 25° C.

Means are the % of total ^{14}C extracted in the form of ^{14}C-*N*-acetyl-glufosinate at each time period. Means were separated using Fishers' Protected LSD $_{0.10}$ test. Means with the same letter are not significantly different. Vertical bars represent standard errors of means.

Table 4.1 A. Translocation of ^{14}C-glufosinate by Liberty-Link® soybeans grown at 15° and 25° C.

Hours after treatment	temperature	treated leaf	above treated leaf	below treated leaf	roots
		^{14}C (% of absorbed)			
3	15° C	91 a	1 d	6 c	2 c
	25° C	86 ab	2 cd	8 c	4 bc
12	15° C	84 abc	3 cd	7 bc	6 bc
	25° C	80 c	5 ab	10 abc	6 b
24	15° C	80 bc	5 ab	12 ab	5 bc
	25° C	81 bc	4 abc	8 bc	6 b
48	15° C	78 bc	6 a	12 a	4 bc
	25° C	71 d	6 a	10 abc	14 a
	LSD (0.05)	7	2	4	4

Table 4.1 B. Translocation of ^{14}C-glyphosate by Roundup-Ready® soybeans grown at 15° and 35° C.

Days after treatment	temperature	treated leaf	above treated leaf	below treated leaf	roots
		^{14}C (% of absorbed)			
1	15° C	75 a	7 d	14 b	5 e
	35° C	71 a	10 cd	8 c	11 cd
3	15° C	61 b	12 cd	17 a	5 d
	35° C	59 bc	22 ab	5 d	15 abc
5	15° C	61 b	12 c	14 b	13 bcd
	35° C	55 bc	23 a	5 d	16 ab
7	15° C	52 c	18 b	14 b	17 ab
	35° C	50 c	25 a	6 cd	19 a
	LSD (0.05)	9	5	3	4

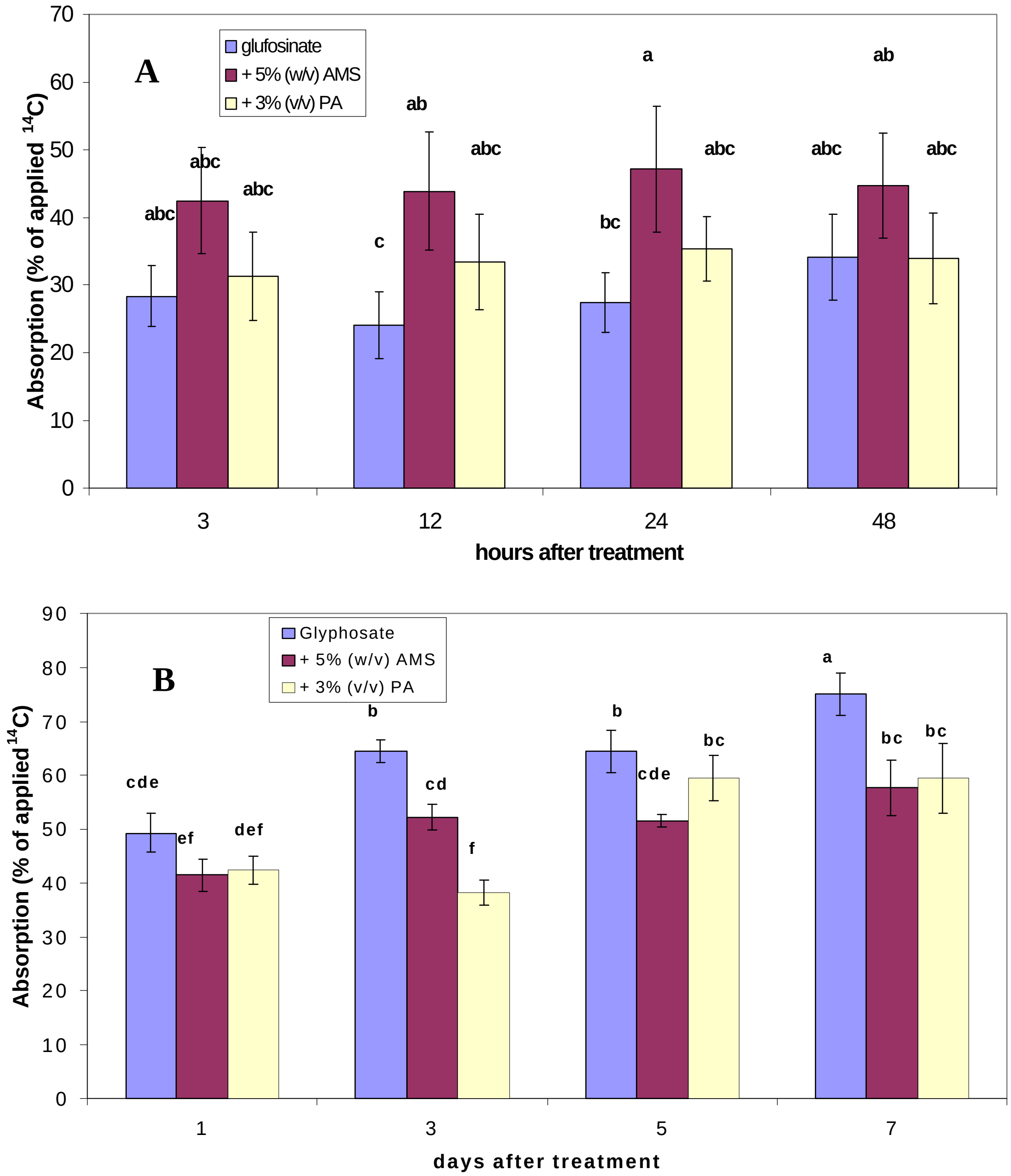

Figure 4.4. Absorption of ^{14}C-glufosinate by Liberty-Link soybeans in the presence of formulated glufosinate, glufosinate + 5% (w/v) AMS, or glufosinate + 3% (v/v) PA (A) and ^{14}C-glyphosate by Roundup-Ready soybeans in the presence of formulated glyphosate, glyphosate + 5% (w/v) AMS, or glyphosate + 3% (v/v) PA (B).

Percent absorption is the percentage of the applied ^{14}C herbicide that was absorbed at each time period. Vertical bars represent standard errors of means. Means were separated using Fishers' Protected LSD test at $\alpha=0.05$. Means followed by the same letter are not significantly different.

Table 4.2 A. Translocation of ^{14}C-glufosinate by Liberty-Link® soybeans as affected by the AMS or PA.

Hours after treatment	treatment	treated leaf	above treated leaf	below treated leaf	roots
	---------------- ^{14}C (%of absorbed) ------------------------				
3	Glufosinate	89 ab	2 bc	7 a	2 a
	+ 5% AMS	91 ab	2 abc	4 a	3 a
	+ 3% PA	88 ab	4 abc	2 a	6 a
12	Glufosinate	82 b	6 ab	7 a	5 a
	+ 5% AMS	93 a	2 abc	2 a	2 a
	+ 3% PA	91 ab	2 c	4 a	3 a
24	Glufosinate	88 ab	4 abc	2 a	6 a
	+ 5% AMS	87 ab	4 abc	5 a	4 a
	+ 3% PA	88 ab	2 abc	6 a	4 a
48	Glufosinate	87 ab	6 a	4 a	3 a
	+ 5% AMS	83 b	5 abc	6 a	6 a
	+ 3% PA	88 ab	4 abc	3 a	5 a
	LSD (0.05)	10.0	3.8	7.1	3.8

Table 4.2 B. Translocation of ^{14}C-glyphosate by Roundup-Ready® soybeans as affected by the AMS or PA.

Days after treatment	treatment	treated leaf	above treated leaf	below treated leaf	roots
	---------------- ^{14}C (%of absorbed) ------------------------				
1	Glyphosate	90 ab	3 d	3 bcd	4 c
	+ 5% AMS	86 abc	5 cd	5 abc	5 abc
	+ 3% PA	92 a	2 d	2 d	4 bc
3	Glyphosate	81 cd	10 bc	5 ab	3 c
	+ 5% AMS	77 cd	10 bc	6 a	7 abc
	+ 3% PA	75 d	12 ab	5 abc	8 ab
5	Glyphosate	79 cd	11 ab	4 abcd	6 abc
	+ 5% AMS	73 d	13 ab	5 abc	9 a
	+ 3% PA	78 cd	11 b	4 abcd	7 abc
7	Glyphosate	80 cd	12 ab	3 cd	5 abc
	+ 5% AMS	72 d	16 a	3 abcd	9 a
	+ 3% PA	81 bcd	11 ab	2 d	5 abc
	LSD (0.05)	9.8	5.5	2.2	4.2

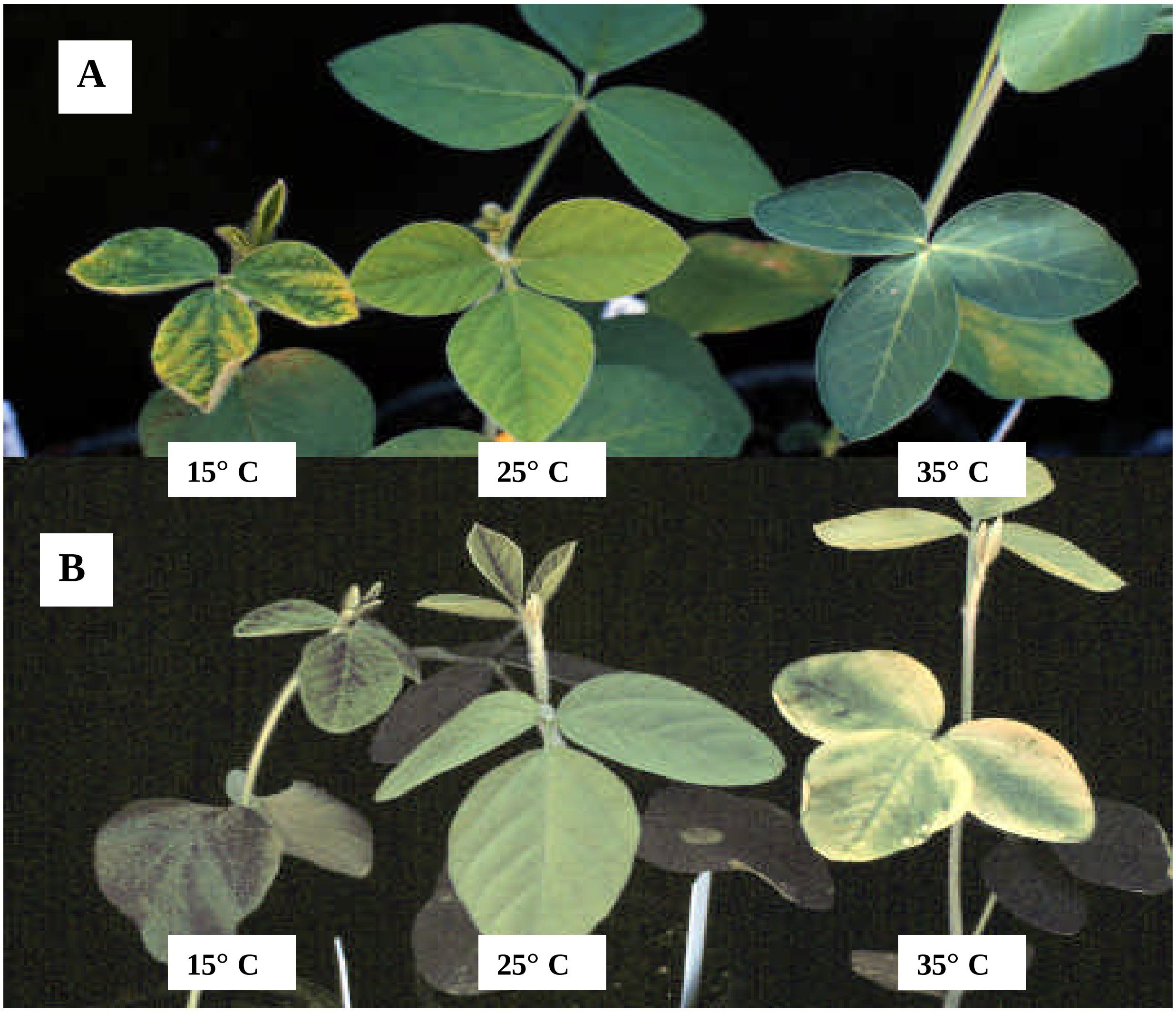

Figure 4.5. Pictorial demonstration of the response of Liberty-Link soybeans to 2 kg/ha glufosinate (A) and of Roundup-Ready soybeans to 4 kg/ha glyphosate (B), 10 DAT, at three temperature regimes.

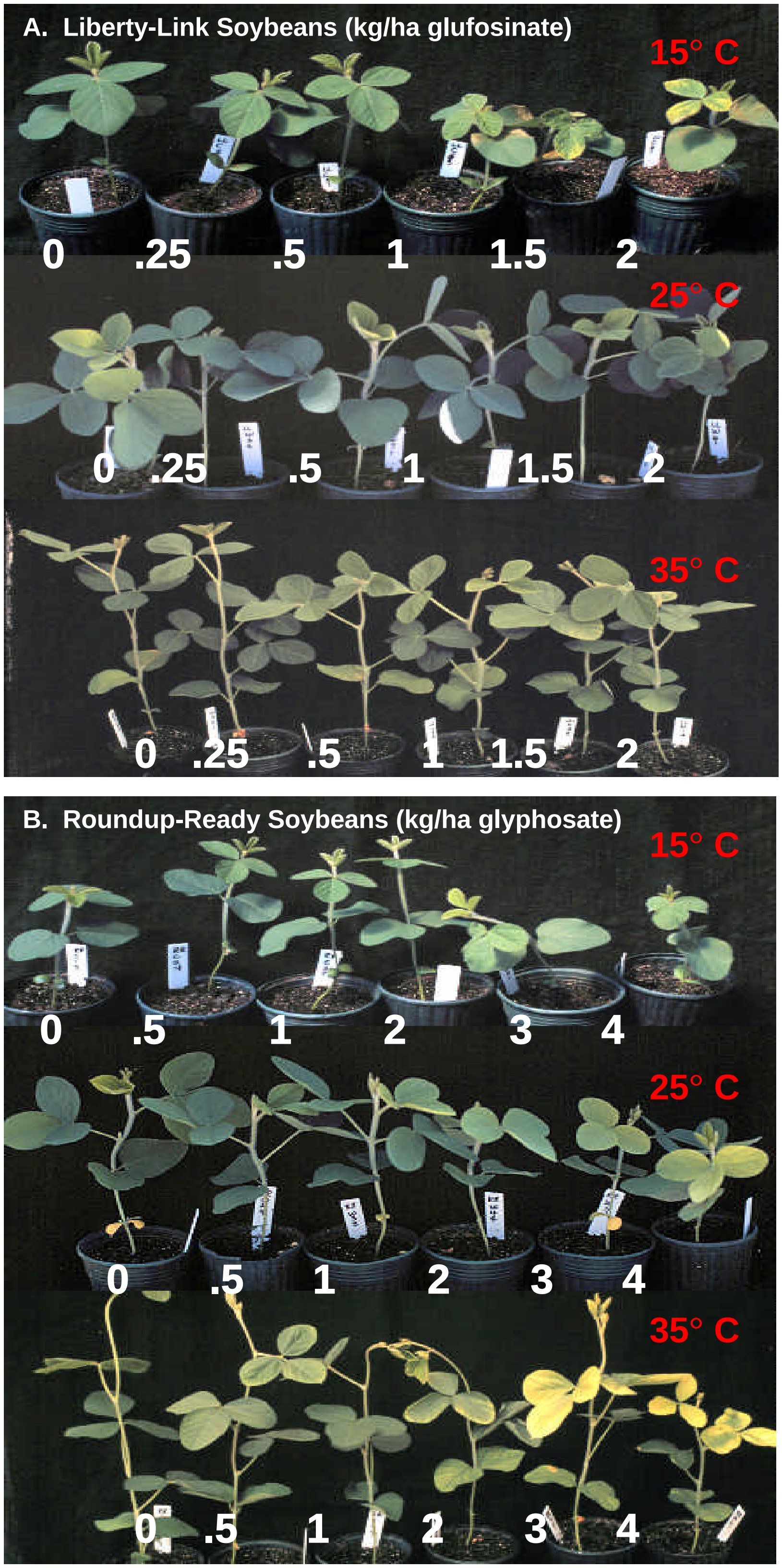

Figure 4.6. Photographs depicting injury to Liberty-Link Soybeans (A.) and Roundup-Ready (B.) Soybeans 10 DAT with 0-2 kg/ha glufosinate (A.) or 0-4 kg ai/ha glyphosate (B.).

Injury is present in Liberty-Link soybeans at 15° C and in Roundup-Ready soybeans at 35° C.