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# The Fate if 2, 4-D in Intact Soybean (Glycine Mix)

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# The Fate of 2,4-D in Intact Soybean (*Glycine max*)

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## ABSTRACT

About 479,000 pounds of 2,4-D (2, 4-dichlorophenoxyacetic acid) was applied to South Dakota's crops in 2000. 2,4-D can injure or reduce yield of soybean (*Glycine max*) if deposited on growing plants. This study determined uptake, translocation, and metabolism of ring-labeled-$^{14}$C -2,4-D in soybean at the third trifoliate (V3) stage of growth. Plants were harvested and partitioned into four parts from 1 hr (HAT) to 10 d (DAT) after treatment. Thin layer chromatography techniques were used to determine if $^{14}$C remaining in the tissue was parent chemical or metabolite. 2,4-D uptake ranged from 39% at 1 HAT to 74% 6 DAT. By 10 DAT, $^{14}$C translocated to the youngest tissue (10%) and older tissue (8%). All $^{14}$C recovered from the treated leaf 1 HAT was parent 2,4-D, however, at 24 HAT and later, only about 30% of the $^{14}$C remained as 2,4-D whereas 70% of the $^{14}$C was observed as a more water soluble compound.

## INTRODUCTION

In South Dakota, 100% of corn acres, 98% of soybean acres, 93% of spring wheat acres and 56% of winter wheat acres were treated with herbicide in 2000 (USDA National Agricultural Statistics Service, 2001). 2,4-D (2, 4-dichlorophenoxyacetic acid) is a chlorinated phenoxy compound. 2,4-D is a plant growth regulator used worldwide to control broadleaf weeds. 2,4-D has been available since the mid-1940's and is applied in many situations today. About 479,000 pounds acid equivalent (a.e.) 2,4-D was applied to 56% of SD spring wheat acres, 26% of SD winter wheat acres and 2% of SD corn acres in 2000. Application rates of 2,4-D to corn, spring wheat, and winter wheat averaged about 0.95, 0.37, and 0.50 lb a.e. per acre, respectively (USDA National Agricultural Statistics Service, 2001). According to the 2,4-D label this product cannot be sprayed when the wheat crop is in the boot to dough stage. These figures do not include the additional pounds of 2,4-D used in pastures, rangeland, woodlands, or home usage.

2,4-D may be applied to land prepared for soybean crops, but only as a pre-emerge application for burn down of emerged broadleaf weeds. The rate that is used depends on the interval before soybean planting. A producer can apply up to 0.5 lb a.e. per acre seven days before planting soybeans, or a 1 lb a.e. per acre rate can be used if there is a 30 day interval before planting (2,4-D Label). 2,4-D has very little soil activity; therefore, when applied preplant it should not affect soybeans.

The main cause of the problem with 2,4-D on soybean is drift or volatilization of the chemical from target areas onto emerged soybean plants. The fact that so much of this chemical is used in South Dakota greatly increases the potential for drift to occur. Drift occurs when 2,4-D is applied to adjoining corn or wheat fields and chemical is carried to soybean fields by the wind. The average wind speed in South Dakota during May and June is 5 to 8.5 MPH (SDSU Electrical Engineering, 2001). Volatilization is the movement of chemical due to evaporation and redeposition occurs when 2,4-D is applied at high temperatures around 85° F (2,4-D, Pesticide Fact Sheet, 2005). The average temperature in Brookings, South Dakota in May and June is 70° F with actual day to day temperatures as high as 90° F SDSU(Climate, 2005). In 2002, 16 days in June recorded temperatures of 80° or higher, within the first month after soybean emergence (June 1) (SDSU, Climate, 2005). These temperatures increase the risk of volatilization at a time when soybean is very sensitive to 2,4-D injury.

When 2,4-D comes into contact with soybeans, it can be very detrimental to a soybean crop. Some symptoms of 2,4-D affected plants include stem twisting (epinasty), leaf discoloration (chlorosis), and eventually death (necrosis). Some of these symptoms can be seen in plants just hours after application (Kelley et al., 2002). In addition it takes very little 2,4-D to cause damage to soybeans, only 10% or 0.06 lb a.e. per acre of the 0.61 lb a.e. per acre labeled rate in corn has been shown to cause a 7% yield loss in soybeans (Andersen et al., 2004).

2,4-D is a post-emergence translocated herbicide. It enters the plant through the leaf, and stem tissue and is then translocated through the plant via the phloem. 2,4-D has been used for more than four decades, yet research detailing the metabolism of the herbicide in intact soybean is difficult to find. Soybean was the focus of this study due to the 4.2 million acres of soybean planted per year in South Dakota and their susceptibility to 2,4-D damage. Injury complaints on soybean and other broadleaf plants due to growth regulator compounds, such as 2,4-D and dicamba, comprised about 66% of the injury complaints seen by the pesticide testing facility at SDSU in 2004 (D. Matthees, personnel communication). In most cases, however, the samples sent in near the end of the season contain little if any 2,4-D, although injury symptoms were often rated as severe. The objectives of this study were twofold. The first was to determine how much chemical entered the plant in a given time period and translocation patterns within a plant that has been affected by a low rate of 2,4-D. The second objective was to determine if soybeans metabolized 2,4-D and if so, the timeframe of when this occurred and what metabolites were formed.

## MATERIALS & METHODS

Soybean plants (variety Surge) were grown in the greenhouse until they reached the third trifoliate (V3) growth stage. These plants were treated with the methyl-ester formulations of 2,4-D at an equivalent rate of 0.112 kg ae/ha (0.1 lb ae/a). 2,4-D was mixed with water (1:748) and applied to the plants using a garden spray bottle with the nozzle set to a fine mist. Aluminum foil was cut to fit around the stem of the plant and

covered the soil. The plant was misted twice with the 2,4-D solution to cover the leaf surfaces. The aluminum foil was removed and the amount of herbicide solution per plant was determined.

Once the soybean plants had been treated, the leaves were allowed to air-dry. The middle leaflet of the second trifoliate leaf was marked for identification. The marked leaf was then treated with 10 ul of uniformly ring labeled $^{14}$C-2,4-D with a radioactivity level of 2.2 X $10^6$ DPM. The plants were harvested at 1 hour after treatment (HAT), 6 HAT, 48 HAT, 6 days after treatment (DAT) and 10 DAT. Each harvest time was replicated three times, and the experiment was replicated in time.

At harvest, the leaflet treated with $^{14}$C-2,4-D was removed from the plant, and the surface was rinsed with 3 ml of methanol. The rinsate was collected and 100-ul aliquot was transferred into a scintillation vial. Two ml of scintillation cocktail (Ultima Gold, Packard Bioscience BV, Meriden CT) was added to the rinsate aliquot and the vial was shaken and placed in a scintillation counter (Packard Bioscience Company, Meriden CT) to determine the level of radioactivity on the leaflet surface. The maximum counting time was 10 min or until 2 sigma reached 0.05. The remaining rinsate was labeled and stored.

Once the treated leaflet had been rinsed, the leaflet was freeze dried in liquid nitrogen and weighed. The leaflet was then placed into a bag and stored at -18 C until further processing. The remainder of the plant was partitioned into 1) the remaining 2 leaflets of the trifoliate, 2) leaves and stem above the treated leaf, and 3) leaves and stem below the treated leaf and stored as previously described.

One plant from each treatment period was selected for further processing. Each plant part was freeze dried using liquid nitrogen and weighed. The leaves were placed into a mortar, more liquid nitrogen was added and the leaves were ground up using a pestle. Five ml of methanol was added to the finely ground leaf tissue to extract the remaining herbicide in the remaining 2 leaflets of the trifoliate and leaves and stems above the treated leaf. Ten ml of methanol was added to the leaves and stems below the treated leaf and ground. The liquids from the ground materials were decanted into vials. A 100-ul aliquot was placed in 2 ml of scintillation cocktail and radioactivity determined as described above.

A 50-ul aliquot of extract (in two 25-ul increments) from the ground plant tissue of the treated leaflet and remaining 2 leaflets of the third trifoliate were spotted onto thin layer chromatography (TLC) plates (Whatman Inc. Clinton NJ). The first 25- ul aliquot was pipetted onto the plate, allowed to dry for several hours and then the second 25-ul aliquot was applied. One ul of $^{14}$C- 2,4-D standard, containing about 24,000 DPM, was placed on 1 or 2 lanes on the thin layer chromatography plates.

The entire extract from the 48 HAT, 6 DAT and 10 DAT ground plant tissues above the treated leaf was reduced to near dryness using a stream of warm air. An aliquot of methanol was added back to the near dry materials. A 25-ul aliquot of this concentrated extract was counted by scintillation counting techniques previously described. Then 50-ul aliquots of these extracts were spotted onto TLC places as previously described.

The chromatography plate was developed using a (42:4:8) benzene/acetic acid/methanol solution (Eastin, 1986). The solution traveled to a height of 15 cm. The plate was allowed to air dry in the fume hood for 20 minutes and was then placed into a 100°C oven for 4 minutes. The plates were placed against a phosphorescent screen for

1 to 4 days. The energy of $^{14}C$ decay resulted in dark spots on the screen. The screen was then placed in a counter that detected the darkened areas and was reported as a function of pixel density.

Pixel density of the $^{14}C$-2,4-D standard was used to determine the Rf value of 2,4-D in this developing solution. The Rf value of the standard was determined using the equation: Rf = height of 2,4-D movement/height of solvent movement (Weete, 1986).

The Rf values of high pixel density areas that were found outside the standard 2,4-D spot were calculated and were considered to be 2,4-D metabolites (or breakdown products), since they were still radioactive but did not have the same characteristic movement of 2,4-D.

## RESULTS AND DISCUSSION

### *Plant Injury, 2,4-D Uptake and Translocation*

There was a large amount of variation in the amount of 2,4-D applied to each plant as seen in Table 1. The variation in the amount of 2,4-D applied to each treatment ranged from 1X (0.78 mg/plant) to 4X (3.22 mg/plant). Although the chemical amount applied was the least for the 6 and 10 day harvest times, the injury symptoms were more severe than plants treated and harvested at the earlier times (Table 2 and Figure 1). Symptoms of 2,4-D injury included stem curling, epinasty, upper leaf curling, leaf cupping, chlorosis, and, finally, death. This increase in symptom severity was also observed in a field study done by Andersen et al. (2004).

The amount of 2,4-D taken up over the 10-day experiment varied with the amount of time that the 2,4-D was left on the plant (Table 3). Total uptake ranged from 40% 1 HAT to 74% 10 DAT. The percent of radioactivity that entered the plant at 6, 24, and 48 HAT was about 55% of the total added. The percent of uptake at 6 and 10 DAT was about 70% of the amount added. The amount of $^{14}C$ that remained in the treated leaflet (TL) ranged from 98% 1 HAT to 76% 10 DAT.

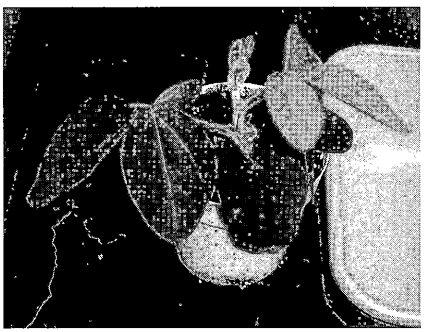
| Harvest Time after application | Amount of 2,4-D applied (mg/plant) |
|---|---|
| 1 hr | 2.24 |
| 6 hrs | 1.6 |
| 24 hrs | 1.9 |
| 48 hrs | 3.22 |
| 6 days | 0.78 |
| 10 days | 0.86 |

**Table 1.** Amount of 2,4-D applied to V3 soybean plants.

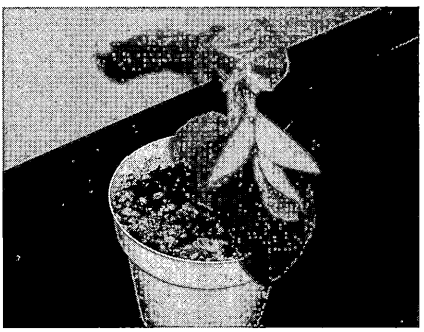
| Time After Treatment | Severity of Symptoms[1] |
|---|---|
| 1 hr | 2 |
| 6 hr | 2 |
| 24 hr | 3 |
| 48 hr | 3 |
| 6 days | 6 |
| 10 days | 8 |

[1]Scale of 1-10 with 10 being dead plants and 1 showing no injury.

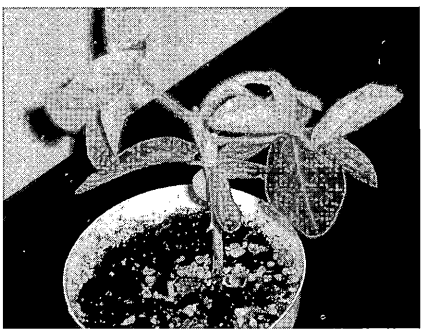
**Table 2.** Severity of soybean rating of injury symptoms that were observed in V3 soybean plants at corresponding harvest times.



1 HAT - injury severity 2



48 HAT – injury severity 3



10 DAT – injury severity 8

**Figure 1.** Severity of 2,4-D injury symptoms to a soybean plant at the V-3 stage, 1 hour after treatment (HAT), 48 HAT, and 10 days after treatment (DAT).

As time to harvest increased more of the 2,4-D was translocated to other parts of the plant. The areas of next highest concentration of $^{14}C$ was plant material above the treated leaf, the amount of $^{14}C$ that was in the area above the treaded leaf ranged from 0.8% 1 HAT to 10% 10 DAT. The remaining plant parts had very little $^{14}C$ in them, even 6 and 10 DAT.

## METABOLISM AND CONJUGATION OF 2,4-D

The use of $^{14}C$ as a tracer for a nonlabelled chemical is an indication of the uptake and translocation patterns for the chemical. The question that needs to be answered is "Is the $^{14}C$ in the original herbicide form or has the herbicide been changed into a different chemical?" The process of changing the original chemical into a metabolite (breakdown of a parent compound to a smaller product) or a conjugate (adding a plant product to the original parent compound resulting in a larger product) can be accomplished several ways. Plants have been shown to join 2,4-D with a sugar, tripeptides, or single amino acids to form conjugates (Feung et al. 1972). In most cases, the joining of these compounds with 2,4-D turns the original chemical into an inactive compound. 2,4-D has also been shown to be metabolized in plants by undergoing B-oxidation (removal of the 2C chain of the carboxyl group) which also leads to a nonphytoxic product (Loos 1975). In the injury data, the plants that were harvested 10 DAT showed more severe symptoms than the plants harvested 48 HAT. This leads one to believe that after 10 d the chemical is still able to cause harm to the plant, therefore metabolism and/or conjugation are not likely to be occurring.

Thin Layer Chromatography (TLC) was used to evaluate the remaining $^{14}C$ in the plant and determine if the $^{14}C$ recovered was 2,4-D, or some other form. The amount of radioactivity in the aliquots of extract was too low to be detected by this method, except in the treated leaflet. Therefore, only results from the treated leaflet are presented.

| | Total Applied | | Partitioning of $^{14}C$ in Plant | | | |
|---|---|---|---|---|---|---|
| Time after Application | Leaf Surface | Uptake | Treated Leaflet | Remaining Trifoliate | Above Treated Leaf | Below Treated Leaf |
| | % of $^{14}C$ applied | | % of $^{14}C$ taken up | | | |
| 1 hr | 57.5 | 42.5 | 96.5 | 0.6 | 2.5 | 0.5 |
| 6 hr | 45.2 | 54.8 | 96.8 | 0.9 | 0.4 | 1.9 |
| 24 hr | 35.4 | 64.6 | 86.2 | 3.2 | 2.7 | 7.9 |
| 48 hr | 34.4 | 65.6 | 81.8 | 1.3 | 2.5 | 1.4 |
| 6 day | 26 | 74 | 92.6 | 2.4 | 2.5 | 2.6 |
| 10 day | 35.3 | 64.7 | 75.9 | 5.3 | 10.7 | 8.2 |

**Table 3.** Partitioning of $^{14}C$ in a V3 soybean plant from 1 hour to 10 days after application of $^{14}C$-2,4-D to the middle leaflet of the 2nd trifoliate leaf.

A reproduction of the TLC plate is shown in Figure 2. The first lane is an aliquot of $^{14}$C-2,4-D. The other lanes were spotted with treated leaf extract from each harvest time. The light spots in the lanes represent where the radioactive compounds are located. In the control lane only 2,4-D parent material is present. It moved the most in the developing solution, indicating that it was the most benzene soluble; the Rf value was calculated to be 0.55. This Rf value correlated with the Rf value reported by Feung et al. (1972). They used a TLC developing solution that contained 1-butanol-95%, ethanol-3 N ammonium hydroxide (4:1:5), and the Rf value found for 2,4-D was 0.42. In this study any light spots that had an Rf value of 0.55 would be assumed to be 2,4-D parent material.

The plants that were harvested 1 HAT and 6 HAT had almost 100% of the $^{14}$C remaining as parent compound (Table 4). As the time the 2,4-D was in the leaf increased, the amount of chemical that changed into another form increased. Plants that were harvested 24 HAT had only 30% of the $^{14}$C labeled 2,4-D remaining as parent material. The remaining $^{14}$C was found at an Rf value of 0.32. The lower Rf value for this compound indicates that this form was more soluble in methanol than parent 2,4-D since methanol moved up the plates more slowly than benzene (Eastin 1986). The amount of metabolite remained about the same from 24 HAT to 6 DAT with an increase at 10 DAT. Others have reported water soluble forms of 2,4-D-based chemicals in soybean leaf, root, and cotyledon callus tissue (Feung et al. 1972; Davidonis et al. 1980), although these studies were not done on intact plants. Feung et al. (1972) reported both a glucose conjugate and an amino acid conjugate formed in cotyledon callus tissue 24 HAT.

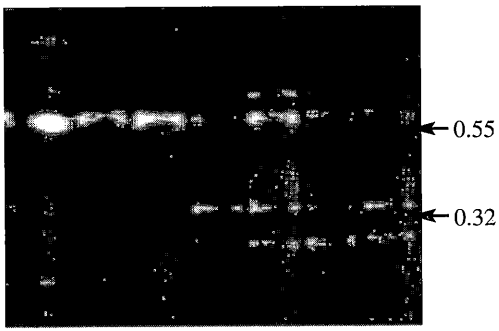



**Figure 3.** Thin layer chromatography of extract from the treated soybean leaflet. Bright spots indicate the presence of $^{14}$C. Each lane is labeled with the time after application, Std is Standard 2,4-D, HAT is hours after treatment and DAT is days after treatment. The Rf values are 0.55 for the 2,4-D standard and 0.32 for the metabolite.

Davidonis et al. (1980) reported that 48 hrs after incubation root callus tissue did not metabolize or conjugate 2,4-D.

Cotyledon callus tissue formed 33% amino acid conjugates with the remainder staying as 2,4-D parent compound; and in the leaf callus tissue, amino acid conjugates were minor metabolites with glucose conjugates being the major metabolite. Davidonis et al. (1980) also showed that the age and type of callus tissue played an important role in the type and speed of alternative 2,4-D products formed, with older soybean callus tissue having a faster conjugation rate.

| Time After Treatment | % Remaining 2,4-D parent compound | % Changed to metabolite |
|---|---|---|
| | % of total $^{14}C$ applied per lane | |
| 1 hr | 100 | 0 |
| 6 hr | 98 | 2 |
| 24 hr | 30 | 70 |
| 48 hr | 36 | 64 |
| 6 days | 38 | 62 |
| 10 days | 24 | 76 |

**Table 4.** The portion of $^{14}C$ that remained as parent compound 2,4-D (Rf value = 0.55) and the amount that was metabolized or conjugated into a more water soluble compound (Rf value = 0.32).

Although extracts of other tissues were used in TLC analysis, the amounts of radioactivity were not detected using the plate imager. A few ways to solve this problem would be to put on more radioactivity per lane by 1) adding more aliquots of the extract or 2) further concentrating the extract. Another method may be to allow more time for development in the plate analyzer. Since the analyzer is a shared piece of equipment, we tried to maximize the time for development without compromising other researchers' needs. Without the $^{14}C$ TLC data, it is not possible to know if the 2,4-D in the other tissue was parent material or the metabolite. This study also did not determine if the plants metabolize the 2,4-D before or after it is translocated.

## CONCLUSIONS

This study showed that 2,4-D uptake by soybean ranged from 39% to 64% in a 10 day period. Most of the $^{14}C$ that was taken up by the plants stayed in the treated leaf with about 10% moving to the youngest plant tissue and 8% moving to older tissue. The

radioactivity did not remain as parent 2,4-D but was fairly rapidly changed to a more water soluble metabolite with only 30 to 40% remaining as 2,4-D after 24 HAT.

Andersen et al. (2004) reported that 6 d after application of 2,4-D to field grown soybeans, less than 20% of the original application rate was detected by gas chromatography/mass spectrophotometry (GC/MS). By 12 d after application, only 3% of the 2,4-D applied was detected (Andersen et al. 2004). The 2,4-D was extracted by alkaline extraction and then plant extracts were derivatized to a more volatile form so parent 2,4-D could be detected using GC and quantified using MS. This extraction method would not detect the more soluble metabolites reported in this study. Based on this research and evidence from the literature, the 2,4-D in the soybean plant was being conjugated into more water-soluble forms, either glucose or amino acid conjugates, or both. These reactions would decrease the 2,4-D concentration over time and are similar in time-step to what Andersen et al. (2004) observed. These data would explain why, at the end of the season, no or very little 2,4-D was detected in stems or leaf tissue, although injury symptoms were very evident. An acidic extraction that may break the glucose or amino acid bond, and change the metabolite back to parent acid, may yield a higher amount of 2,4-D in these plant tissues if further degradation does not occur.

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