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Chlorsulfuron-induced Phytotoxicity in Canola (*Brassica napus* L.) Seedlings

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캐놀라 식물체내에서 클로르설푸론의 약해 유발 요인

金晟文* · 許長鉉 · 韓大成** · 윌리암 반덴본*****

ABSTRACT

Chlorsulfuron, an acetolactate-synthase-inhibiting sulfonylurea herbicide, induces many metabolic and physiological changes in susceptible plants. The objective of this study was to determine to what extent chlorsulfuron-induced phytotoxicity was due to a shortage of final products(the branched-chain amino acids valine, leucine, and isoleucine) or to an accumulation of a toxic metabolite(2-ketobutyrate), or both, in a susceptible species. Chlorsulfuron-treated canola seedlings showed growth inhibition and injury symptoms that included chlorosis, downward leaf rolling, and accumulation of anthocyanins. Supplementation with valine, leucine, and isoleucine prevented the chlorsulfuron-induced growth inhibition and injury symptoms only partially, suggesting that factor(s) other than a shortage of the branched-chain amino acids also are involved in the phytotoxicity. Canola seedlings treated with 2-ketobutyrate showed reduced growth, but they showed different changes in metabolites than seedlings treated with chlorsulfuron. The results suggest that 2-ketobutyrate is not involved in chlorsulfuron-induced phytotoxicity. We conclude that chlorsulfuron-induced phytotoxicity is due at least in part to a shortage of branched-chain amino acids.

Key words : Chlorsulfuron, Canola, Mechanism of action, Branched-chain amino acids, 2-Ketobutyrate.

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INTRODUCTION

Biosynthesis of branched-chain amino acids (BCAA) has been of interest to many weed scientists as well as to herbicide developers, not only because it is unique in plants but also because it includes several enzymes that are possible herbicide targets.[18] Of these enzymes, acetolactate synthase only(ALS ; E.C. 4.1.3.18) is the target of several commercial herbicides : sulfonylureas, imidazolinones, triazolopyrimidines, and pyrimidinyl oxobenzoates. The interaction between the herbicides and ALS, and characteristics of ALS, have been studied in detail.[3,10] It is not known, however, how the growth of ALS-inhibitor-treated plants is inhibited and why the treated plants eventually die. It has been suggested that the interaction between ALS and its inhibitors results in a shortage of BCAA[9] and an accumulation of 2-ketobutyrate(2-KB) and 2-aminobutyrate(2-AB).[6] These primary changes would induce many other secondary metabolic and physiological changes including an inhibition of cell division[9,12] and of assimilate translocation[1,5] and an increase in free amino acid and sugar content.[1,13]

Chlorsulfuron-treated plants had smaller amounts of BCAA than control plants.[14] When the treated plants were supplemented with BCAA, herbicide-induced phytotoxicity was prevented.[7,10] However, in a follow-up experiment, Giardina et al.[4] found that supplementation with Val and Ile did not prevent the chlorsulfuron-induced inhibition of root growth in maize and pea. These results suggest that factors other than a shortage of BCAA also are involved in the phytotoxicity.

An accumulation of 2-KB and its metabolic product 2-AB also has been suggested as a possible cause of toxic effects in ALS-inhibitor-treated microorganisms and plants.[6,11] Recent work by Shaner and Singh[15], however, indicated that, in higher plants, ALS-inhibitor-induced phytotoxicity is not due to an accumulation of 2-KB and/or 2-AB.

The objective of this research was to determine whether chlorsulfuron-induced phytotoxicity in canola seedlings was due to a shortage of BCAA and/or an accumulation of 2-KB. Canola was selected as the experimental plant on the basis of high sensitivity to chlorsulfuron and uniform rapid growth. The questions were examined by observing the growth of chlorsulfuron-treated plants that were pre-treated with BCAA and by comparing metabolic changes in plants treated with 2-KB with those in plants treated with chlorsulfuron.

MATERIALS AND METHODS

Plant Materials

Canola(*Brassica napus* L. cv Westar) seeds were placed in a petri dish containing two sheets of Whatman No. 1 filter paper moistened with distilled water and kept at 21℃ for 2 days. In some experiments(2-KB treatment and BCAA supplementation), seedlings were transferred to Holiday® vermiculite(Vil Vermiculite Inc.) in 8 by 8 by 8cm containers with perforated bottoms. The containers were placed in larger trays that contained nutrient solution. The nutrient solution was made by combining 5ml 1M $CaCl_2$, 2ml $MgSO_4$, 5ml 1M KNO_3, 1ml Fe-EDTA, 1ml micronutrient solution containing 1M H_3BO_3, 1M $MnCl_2 \cdot 4H_2O$, 1M $ZnSO_4 \cdot 7H_2O$, 1M $CuSO_4 \cdot 5H_2O$, and 1M $H_2MoO_4 \cdot H_2O$, in 1 liter. Fe-EDTA was prepared according to Stegner.[17] In another experiment(chlorsulfuron treatment), seedlings were transferred to Terra Lite Metro-Mix growth medium(W.R. Grace & Co. of Canada Ltd.) in 8 by 8 by 8cm container. Seedlings were grown in a growth cabinet with 21/18℃ day/

night temperatures, 16-h photoperiod, and 400 ($\mu E/m^2/s$ light intensity. The relative humidity was 50%.

Supplementation with BCAA

Seedlings were grown in nutrient solution in large trays until they were at the four-leaf stage. Uniform seedlings then were transferred to trays (28 by 54 by 6.5cm) that contained nutrient solution with or without added 5 mM of each of L-valine, L-leucine, and L-isoleucine. Each experimental unit(total four) consisted of 16 seedlings. Two days after the transfer, seedlings in two trays were treated with chlorsulfuron. The procedure for chlorsulfuron application is described in the following section. Leaf area and dry weight of the third and fourth leaves, the actively growing tissues, of seedlings were measured 0, 2, 5, and 8 days after herbicide treatment. Visible injury symptoms of controls and chlorsulfuron-treated seedlings were recorded. The nutrient solution with and without BCAA was changed every 2 days. Two replications were used. The experiments were conducted three times ; however, the data for only one representative experiment are presented.

Treatment with Chlorsulfuron

Seedlings at the four-leaf stage were treated with chlorsulfuron {(2-chloro-N-[(4-methoxy-6-methyl-1,3,5-triazin-2-yl)aminocarbonyl] benzenesulfonamide ; Glean™, 75% DF)}. The herbicide was dissolved in distilled water with 0.1% surfactant(Agral 90 ; 90% nonylphenoxy polyethoxythanol) ; 10(l of solution containing 1(g chlorsulfuron was applied as six droplets to each seedling. The droplets were placed on the first true leaf of the seedlings, with a micropipet, and were spread over the entire surface using the column of the micropipet. Control plants received six droplets(total volume 10 (l) of application solution without chlorsulfuron. Each experimental unit consisted of 36 uniform seedlings. The first leaves of seedlings were harvested 12, 24, and 48h after treatment. The harvested leaves were frozen with liquid nitrogen and stored at -20℃ until extraction. Six replications with two seedlings each were used for the different measurements.

Treatment with 2-Ketobutyrate

In a preliminary experiment, the dose response of canola seedlings to 2-ketobutyrate was examined. Seedlings were grown in vermiculite with nutrient solution in a large tray until they were at the four-leaf stage. Roots of seedlings were washed gently with nutrient solution to remove vermiculite from the roots. Each seedling was placed in a styrofoam support. Seedlings were placed in large trays with nutrient solution for 2 days and then were transferred to trays(25 by 54 by 6.5cm) that contained nutrient solution with 0, 5, 10, 25, or 50mM 2-KB. Each experimental unit consisted of five uniform seedlings. After 2h, seedlings in each unit were transferred to trays that contained nutrient solution without 2-KB. Three days later, visual observations on growth were recorded and seedling dry weights were determined. Only the highest concentration caused a reduction in dry weight(20%), and this concentration was used in further experiments.

Uniform seedlings at the four-leaf stage were placed in styrofoam holders in trays that contained nutrient solution with 50mM 2-KB. Each experimental unit consisted of 16 seedlings. Seedlings were harvested every 24 hours. Four replications, with one seedling each, were used for measurements. Fresh weight of the seedlings was determined, and the first leaf of each seedling was frozen with liquid nitrogen and stored at -20℃ until it was assayed for total sugar and free amino acid content.

Extraction and Separation of Metabolites

Leaves were frozen in liquid nitrogen and stored at -20℃ until extraction.[2] Each leaf was homogenized with 2ml MCW solution(methanol : chloroform : water, 12 : 5 : 3, v/v/v) in a mortar and pestle. The homogenate was put in a disposable culture tube(A). The mortar and pestle were washed twice with 1.5ml MCW solution, and the washings were added to tube A. The tube was centrifuged at 156*g* for 10 min, and the supernatant was saved in another centrifuge tube. The pellet in tube A was washed with 5ml MCW solution, and the tube was centrifuged at 156*g*. This procedure was repeated until the supernatant was clear. Three milliliters water was added to every 5ml supernatant. The centrifuge tubes with the supernatant and water mixture were centrifuged at 156*g* for 10 min. The upper phase (methanol/water) was transferred to a 100-ml round-bottom flask and evaporated in a rotary evaporator at 35℃. The final volume of concentrate was diluted to 1 ml with water, stored at -20℃, and used to determine total sugars and free amino acids.

Analysis of total sugar

The amount of total sugar was assayed using an anthrone reagent.[16] Absorbance was measured at 620 nm.

Analysis of free amino acids

The amount of free amino acids was assayed using ninhydrin reagent.[8] Absorbance was measured at 570 nm.

RESULTS AND DISCUSSION

Changes in leaf area and dry weight of the third and fourth leaves of canola plants grown in nutrient solution supplemented with BCAA paralleled each other in all instances, and only the dry weight data are reported(Fig. 1).

During the first 4 days, i.e., 2 days before and 2 days after chlorsulfuron treatments were applied to half of the plants, there were no visible effects of BCAA supplementation on any of the plants, and leaf dry weights of the third and fourth leaves were not affected(Fig. 1). Thereafter, dry weight of leaves of control plants, in the absence of added BCAA, increased rapidly and linearly, at about 0.1g/plant/day. In the presence of added BCAA, the growth rate was reduced temporarily, between 4 and 7 days(2 to 5 days after chlorsulfuron treatment). Thereafter, it was similar to the rate for unsupplemented plants.

The third and fourth leaves of chlorsulfuron-treated plants increased in weight slightly between 2 and 5 days, and then ceased to grow. Corresponding leaves of plants supplemented with BCAA continued to grow after 5 days but remained much smaller than leaves of plants not treated with herbicide and not supplemented with BCAA(Fig. 2).

Five days after treatment, chlorsulfuron-treated plants showed chlorosis in the third and fourth

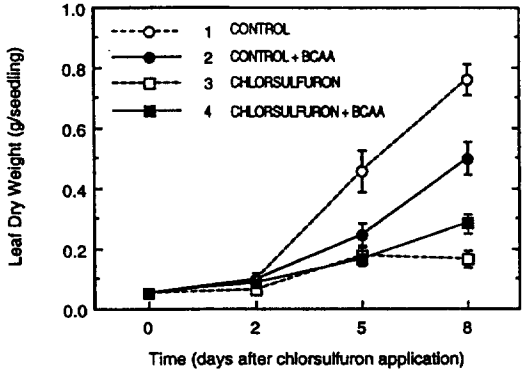

Fig. 1. Effect of supplementation with branched-chain amino acids on the dry weights of the third and fourth leaves of chlorsulfuron-treated canola seedlings. Means and standard errors are based on two experiments with two replicates each.

leaves(Fig. 2), downward leaf rolling in the second leaf, and anthocyanin accumulation in the first and second leaves. Supplementation with BCAA prevented downward leaf rolling but did not prevent the other injury symptoms from occurring.

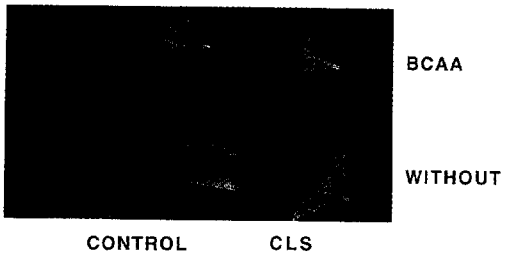

Fig. 2. Effect of supplementation with branched-chain amino acids(BCAA) on injury symptoms in chlorsulfuron-treated canola seedlings. The third and fourth leaves of chlorsulfuron-treated seedlings with and without supplementation show chlorosis 5 days after treatment. CONTROL, without chlorsulfuron treatment ; CLS, chlorsulfuron treatment ; WITHOUT, without supplementation with BCAA.

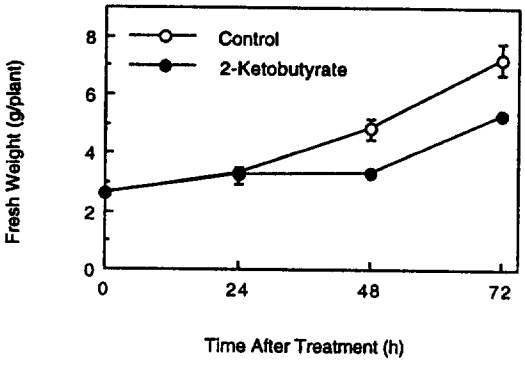

Fig. 3. Fresh weight of canola seedlings grown in nutrient solution containing 50mM 2-ketobutyrate. Means and standard errors are based on data from four replicates.

A cause of injury or death, as a result of chlorsulfuron treatment, could be the accumulation of some toxic compounds in treated plants.[6,11] If this is correct, then one might expect to observe the same injury symptoms and/or metabolic responses after treatment of plants with such toxic metabolites as after treatment with chlorsulfuron.

The addition of 2-KB to the nutrient solution resulted in growth inhibition. Fresh weight increase of plants between 24 and 48h after placing the plants in solution containing 2-KB was inhibited ; after that time, growth resumed, and final fresh weight after 72h was 73% of that of control plants(Fig. 3).

Total sugar content in the first leaf fluctuated during the 72-h experiment but was not affected significantly by the presence of 2-KB in the growth medium(Table 1). Free amino acid content, on the other hand, began to decrease after 48h.

Contrary to the results with 2-KB(Table 1), chlorsulfuron treatment resulted in an increase in total sugars in the first leaf after 48h(Table 2). Free amino acid content in leaves of chlorsulfuron-treated plants was consistently higher than in leaves of control plants.

Our observations do not support the view that 2-ketobutyrate is directly involved in the phyto-

Table 1. Effect of 2-ketobutyrate on total sugar and free amino acid content in the first leaves of canola seedlings. Data, means and standard errors(in parentheses), are based on the results from four replicates.

Time	Total Sugar		Effect	Free Amino Acids		Effect
	Control	Treated		Control	Treated	
(h)	(nmoles glucose/mg FW)		(% of control)	(nmoles glucose/mg FW)		(% of control)
0	27.8(1.4)	27.8(1.4)	100	15.7(0.8)	15.7(0.8)	00
24	33.6(3.3)	43.8(2.8)	128	16.1(1.6)	16.6(0.8)	103
48	29.7(1.7)	33.3(2.5)	112	12.1(9.9)	10.9(0.5)	90
72	23.2(2.4)	24.6(4.7)	106	9.9(0.6)	4.3(0.8)	43

Table 2. Effect of chlorsulfuron on total sugar and free amino acid content in the first leaves of canola seedlings. Data, means and standard errors(in parentheses), are based on the results from six replicates.

Time	Total Sugar		Effect	Free Amino Acids		Effect
	Control	Treated		Control	Treated	
(h)	(nmoles glucose/mg FW)		(% of control)	(nmoles glucose/mg FW)		(% of control)
12	43.6(3.2)	36.8(1.8)	85	23.6(1.8)	31.8(1.5)	134
24	47.7(6.3)	43.7(1.9)	92	30.8(2.5)	54.8(3.7)	178
48	46.7(3.7)	71.5(6.9)	153	9.8(1.0)	34.6(7.0)	353

toxic action of chlorsulfuron. The metabolic responses of the canola plants and the visual growth responses differed enough to make such a conclusion unacceptable.

Despite all the reported information about plant responses to ALS-inhibiting herbicides, we still do not have a clear understanding of how susceptible plants die after treatment with such herbicides. A simple explanation might be the production of inadequate amounts of valine, leucine, and isoleucine but, unless there are serious secondary consequences of such shortages, growth stoppage might be all that happens, without the plant dying soon after treatment. In addition, supplying treated plants with exogenous amino acids should overcome the growth inhibition, provided that the amino acids enter the plants and reach the necessary destinations.

Several reports[7,10,15] have indicated that supplementation of chlorsulfuron-treated plants with BCAA could overcome growth inhibition. In our 8-day experiments, however, an exogenous supply of amino acids prevented only part of the chlorsulfuron-induced injury symptoms in young canola plants, and restored only a small part of the growth of the youngest two leaves. We conclude, therefore, that the shortage of branched-chain amino acids does play a role in inhibiting plant growth and causing death of susceptible plants, but that other factors, still unidentified, also are important.

Not the least of these is likely the inhibiting effect of chlorsulfuron on export of assimilate from photosynthesizing source leaves to young growing leaves or roots.[5] A shortage of carbohydrates in such young growing tissue almost certainly is as important as a shortage of certain amino acids, in terms of causing plant growth to stop. If the synthesis of certain essential enzyme proteins also is inhibited, such a combination of detrimental consequences could be enough to bring about the death of treated plants.

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摘 要

Acetolactate synthase를 저해하는 sulfonylurea계 제초제 chlorsulfuron은 감수성 식물 체내에서 많은 생리, 생화학적 변화를 유도한다. 이 연구의 목적은 chlorsulfuron이 유발하는 독성이 감수성 식물체내에서 branched-chain 아미노산 생합성경로의 최종산물인 leucine, valine과 isoleucine의 결핍에 의한 것인지 혹은 branched-chain 아미노산 생합성경로중 독성 대사산물의 축적에 의한 것인지를 결정하는 것이다. Chlorsulfuron에 처리된 캐놀라의 성장은 저해되었으며, 처리된 식물은 백화 현상, 잎말이 현상 그

리고 안토시아닌 축적과 같은 약해증상을 보였다. Branched-chain 아미노산이 첨가된 영양배양액에서 생장하는 캐놀라에 chlorsulfuron을 처리하였을 경우 생장저해와 약해는 단지 부분적으로 완화되었다. 이와 같은 사실은 chlorsulfuron에 처리된 캐놀라의 약해는 branched-chain 아미노산의 결핍 이외에 또 다른 요인이 있음을 시사하는 것이다. 독성 대사산물로 알려진 2-ketobutyrate에 처리된 캐놀라 식물체내에서의 대사산물 변화와 chlorsulfuron에 처리된 캐놀라 식물체내에서의 대사산물 변화는 서로 다른 양상을 보였다. 본 연구에서 얻어진 결과들은 chlorsulfuron에 처리된 감수성 식물이 나타내는 독성은 부분적으로 branched-chain 아미노산의 결핍에 의한 것이라는 점을 시사한다.

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