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Possible Mechanism Associated with Herbicidal Activity of Soybean Meal Hydrolysate (SMH)

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Abstract

Soybean meal hydrolyzate (SMH) has been proposed for use as a natural preemergence herbicide. However, the mechanism by which SMH exerts its herbicidal activity remains unclear. In this paper, the herbicidal activities of SMH against perennial ryegrass (*Lolium perenne* L.) under non-sterile and sterile conditions were evaluated and the relationship between the molecular weight of the ultrafiltration (UF) fractions of SMH and their herbicidal activities were investigated. Besides, the ammonia content changes of the media of SMH treatments 7 d after incubation were also analyzed. The results showed that SMH inhibited the radicle growth of germinating *L. perenne* seeds in a dose-dependent manner under non-sterile condition. However, SMH in the concentrations investigated increased the radicle length and shoot length by 14-17% and 11-15% respectively under sterile condition. The ammonia contents in the SMH media at all treatments increased greatly from less than 0.01 mg L^{-1} to up to 11.86-41.37 mg L^{-1} after 7 d incubation under non-sterile condition. However, ammonia content did not change under sterile condition, proposing that the herbicidal activity might be caused by the free ammonia released from SMH by microbial activity. There was no difference on the perennial ryegrass radicle inhibition among the UF fractions of SMH on an equivalent N basis. It could be concluded that SMH exerted its herbicidal activity through the free ammonia released under non-sterile condition instead of by specific peptide(s) in SMH.

Key words: soybean meal hydrolysate, herbicidal active mechanism, ammonia

INTRODUCTION

Synthetic herbicides are widely used to control weeds in crop fields and turf lawns. Concern over the long-term ecological effects of synthetic agricultural chemicals has led to increased efforts in searching for natural products (Christians 2005). Plant-derived compounds as an environmentally sound approach for weeds control have been used for years in practices (Lydon and Duke 1987; Vaughn and Berhow 1998; Kuk *et al*. 2001; Xuan *et al*. 2003). Corn gluten meal (CGM), a by-product of corn from the wet milling process, has been identified and patented as a natural preemergence herbicide for use in turfgrass and other crops (Christians 1993; Nonnecke and Christians 1997). Further studies showed that corn gluten hydrolyzate (CGH), produced by the action of a bacterial proteinase, inhibited radicle growth more effectively under controlled environment than CGM (Liu *et al*. 1994; Liu and Christians 1997). Five dipeptides and a pentapeptide were identified as the active components of the hydrolyzate (Liu and Christians 1994, 1996). Subsequent research showed that the herbicidal activity of CGH was largely reduced in field, and this reduction was attributed to microbial degradation (Dilley *et al*. 2002).

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Soybean meal is a by-product of milling soybeans to extract soybean oil. Soybean meal has been used to supplement plant protein for animal, chicken and pet feeds. Soybean meal contains an average of 7% nitrogen, 1.2% phosphorous, 1.5% potassium, and various micronutrients (Zublena *et al*. 1997). The product is also used as a slow releasing organic fertilizer for organic vegetable production (Gangon and Berrourad 1994; Hafez and Sundaraj 1999). US patent No. 5290749 reported that soybean meal hydrolysate (SMH), a water-soluble product derived from soybean meal through enzyme hydrolysis, also had the herbicidal activity (Christians *et al*. 1994). However, the mechanism by which SMH exerts its herbicidal activity remains unclear. SMH and CGH, containing mainly soluble peptides, are organic materials with high nitrogen contents. It is well known that proteins and soluble peptides are easily degraded by microbes and inorganic nitrogen, such as ammonium, is often released during this process. Ammonium is either directly taken up from exogenous sources or the organisms employ reaction pathways that generate ammonium from other nitrogenous compounds. On the other hand, ammonium (in this paper, the term ammonium denotes NH_3 and NH_4^+) is known to be toxic to plants, especially inhibit the seed germination and seedling establishment (Britto and Kronzucker 2002). In previous studies, the herbicidal activities of CGH and SMH were observed under only non-sterile condition. Under this condition, peptides in CGH and SMH were converted into inorganic forms of N (ammonium and nitrate) by microbial degradation (Okamoto and Okada 2004). Hence, it is difficult to determine if the peptides in CGH and SMH are the direct herbicidal components. To confirm whether the herbicidal activity of SMH is contributed by peptides, it is necessary to determine the herbicidal activity of SMH under sterile condition. The purpose of the present study is to determine if the observed herbicidal property of SMH is contributed by specific peptide(s) such as those found in CGH or due to a more generic reaction such as ammonium toxicity.

MATERIALS AND METHODS

Preparation of SMH

Soybean meal was grinded and then sieved through a 100-mesh sieve. The powder under the sieve was collected and suspended in deionized water to make a 10% (w/v) solution. Then, the mixture was hydrolyzed with alkaline protease (Novo Nodisk, Denmark) in the substrate/enzyme ratio 100:1.5 (w/v), temperature 60°C and pH 8.0 for 6 h. After hydrolysis, the solution was boiled for 10 min to inactivate the enzyme and then centrifuged at 4 500 r/min for 20 min. The supernatant was lyophilized and stored at -20°C until use.

Fraction of SMH with ultrafiltration membranes

To confirm whether the herbicidal activity of SMH was contributed by specific peptide(s) or was related to its nitrogen content, SMH was fractionated through 4 ultrafiltration membranes (Pellicon™-2, Millipore, USA) with MWCO of 10, 5, 3, and 1 kDa in sequence. The 4 fractions were designated as F1 with molecular weight (MW) between 10 and 5 kDa, F2 with MW between 5 and 3 kDa, F3 with MW between 3 and 1 kDa, and F4 with MW less than 1 kDa. All the fractions were lyophilized and stored at -20°C until use (Tsai *et al*. 2008). The nitrogen contents of the 4 fractions were 12.3, 11.2, 11.0, and 9.4%, respectively, according to the Kjeldahl method.

Herbicidal activity of SMH under non-sterile condition

30 perennial ryegrass (*Lolium perenne* L.) seeds were placed on one layer of 7-cm-diameter Whatman No. I filter paper in a 100-mL conical flask. Then 2 mL of each test solutions, containing 1, 1.5, 2, and 3 mg mL^{-1} SMH respectively in distilled water, was added to each conical flask. The control was treated in the same method, except that the distilled water contained no SMH. The flasks were sealed with Parafilm and placed in a growth chamber. The radiation intensity in the growth chamber was at 70 μmol s^{-1} m^{-2} with the temperature of 25°C/15°C (day/night) and a 16-h photoperiod. Germinated seeds were counted and the length of radicles and shoots were measured 7 d after incubation using a vernier caliper. The measurements of established seedlings in each flask were averaged before statistical analysis. The germination rate, radicle inhibition rate and shoot inhibition rate were calculated

according to the following equations:

Germination rate (%) = Number of germinated seeds in treatment/Number of germinated seeds in control × 100

Radicle (shoot) inhibition rate (%) = (Length of control - Length of treatment)/Length of control × 100

Herbicidal activity of SMH under sterile condition

30 perennial ryegrass (*L. perenne* L.) seeds were immersed in 100 mL 5% sodium hydrochlorite solution for 30 min for surface sterilization and then washed with sterilized water for three times. The seeds were placed on the top of 7-cm-diameter Whatman No. I filter paper in a 100-mL conical flask. The filter papers and flasks were sterilized in 121°C for 20 min before use. SMH solutions in the concentrations of 1, 1.5, 2, and 3 mg mL^{-1}, containing 10 g L^{-1} ampicillin (Sigma, St. Louis, MO, USA), were filtered through the 0.2-μm membrane filter (Sterradisk, Kurabo) and the filtrates were then applied to each flask. Ampicillin was used to inhibit bacterial growth (Chapin *et al*. 1993). The seeds were cultivated in the same conditions as those under non-sterile condition.

Herbicidal activity of different UF fractions of SMH under non-sterile condition

The herbicidal activities of the 4 UF fractions of SMH were compared in the same nitrogen content. Four concentrations of each fraction, corresponding to nitrogen contents 0.68, 0.136, 0.204, and 0.272 mg mL^{-1}, were investigated in this study. The herbicidal activities of the UF fractions were measured in the same conditions as those under non-sterile condition.

Determination of pH and inorganic nitrogen concentrations in the media of SMH treatments

An experiment was conducted simultaneously to determine pH and inorganic nitrogen changes of SMH media in both non-sterile and sterile conditions. The treatments were the same as those described in the corresponding herbicidal activity assay experiments, except that no seeds were added.

The pH of the media after 7 d cultivation was measured with a pH meter (Orion 710A, Orion, USA). Before the measurement, the medium was centrifuged at 3 000 r/min for 20 min and the supernatant was collected for pH determination.

The concentrations of nitrate and nitrite nitrogen were determined as follows: mix 10 mL culture fluid after 7-d cultivation with 1 mL of 10% zinc sulfate. Adjust the pH to 10.5 with 25% sodium hydroxide solution. Add 1 g active carbon to the solution to remove pigments. Centrifuge at 8 000 r/min for 20 min and collect the supernatant. The nitrate and nitrite contents were measured in the ion chromatographic method by using an ion chromatography system (ICS-90, DIONE, USA). The concentration of ammonia nitrogen, including NH_4^+ and NH_3, was measured in the Nesster's reagent colorimetric method (Editorial board of Methods of Monitoring and Analyzing Water and Wastewater of State Environmental Protection Administration 2002). The concentration of free NH_3 was calculated according to the following equation (Zhu and Wen 1990):

$$\text{Free } NH_3 = \frac{\text{Ammonia nitrogen concentration}}{1 + 10^{0.09018 + 2727.92/T - pH}}$$

in which, T = 273 + 25

Data analysis

Data were represented in mean ± SD. Differences between mean values were determined using the analysis of variance using SAS ver. 8.0. *P* value less than 0.05 was considered statistically significant and *P* value less than 0.01 was considered extremely significant. The linear regression was performed with Microsoft Office Excel 2003.

RESULTS

Preemergence herbicidal activity of SMH

Table 1 showed the effects of SMH on the germination of *L. perene* seeds under non-sterile and sterile conditions. Under non-sterile condition, SMH showed a dose-dependent inhibition on the germination and early development of *L. perene* seedlings. When the SMH

Table 1 Effect of SMH on the germination, shoot length and radicle length of *L. perenne* seeds under non-sterile and sterile conditions

Cultivation condition	SMH concentration (mg mL^{-1})			
	1.0	1.5	2.0	3.0
Radicle length inhibition (%)				
Non-sterile	14±2 d	38±4 c	57±5 b	100±0 a
Sterile	-14±3 a	-17±3 a	-14±2 a	-16±3 a
Shoot length inhibition (%)				
Non-sterile	-5±2 c	6±2 b	8±2 b	14±2 a
Sterile	-12±4 a	-11±3 a	-13±2 a	-15±2 a
Germination rate (%)				
Non-sterile	99±2 b	97±6 b	92±4 b	0±4 a
Sterile	98±16 a	101±3 a	103±0 a	102±4 a

In the same rows, values with same letters mean no significant difference ($P > 0.05$), with adjacent letters mean significant difference ($P < 0.05$) and with apart letters mean extremely significant difference ($P < 0.01$). The same as below.

concentration reached 3 mg mL^{-1}, the radicle growth was completely inhibited and the shoot growth was reduced by 14% compared with control. In contrast, under sterile condition, SMH increased the radicle length by 14-17% and the shoot length by 11-15% in the concentrations investigated, but the promoting effects were not significantly different ($P < 0.05$) among the 4 concentrations. The results demonstrated that SMH had no preemergence herbicidal activity under sterile condition. The promoting effect under sterile condition was possibly due to its nutrition function.

Table 2 showed the changes of pH and inorganic nitrogen concentrations in the media after 7 d cultivation. No obvious changes were observed in media pH or inorganic nitrogen compared with the media before cultivation under sterile condition. Nevertheless, under non-sterile condition, the pH of SMH media increased to 8.65-8.90 after 7 d of incubation, compared to the original media pH 6.87-7.03 prior to incubation. The media contained initial ammonia nitrogen at 9.19-30.17 mg L^{-1}, free ammonia below 0.01 mg L^{-1}, nitrate nitrogen at 0.26-0.30 mg L^{-1} and nitrite nitrogen at 0.039-0.049 mg L^{-1}. After 7 d of non-sterile incubation, the nitrate and nitrite nitrogen contents changed slightly, whereas the ammonia nitrogen and free ammonia contents increased significantly in a dose-dependent manner. For example, the ammonia content in the SMH media at all treatments increased greatly from very low initial content ($\leqslant$0.01 mg L^{-1}) to 11.86-41.37 mg L^{-1}. Because the concentrations of nitrate nitrogen and nitrite nitrogen did not change under both sterile and non-sterile conditions, it could be deduced that the inhibitory effect of SMH was contributed by ammonia nitrogen or free ammonia.

Herbicidal activity of the UF fractions of SMH

Table 3 showed that all the 4 UF fractions of SMH inhibited the radicle growth of geminated *L. perenne* seeds in a dose-dependent manner in non-sterile condition. As the concentrations of the 4 fractions increased, all the fractions showed the same effect on radicle inhibition at the same nitrogen content. Hence, the inhibitory effect of SMH on radicle development of *L. perenne* seeds was not caused by specific peptide(s) in SMH. Instead, this effect depended on nitrogen content. All the SMH UF fractions stimulated shoot growth at 0.5 mg mL^{-1} under non-sterile condition, but

Table 2 changes of SMH media pH and inorganic nitrogen concentration after 7 d cultivation under non-sterile and sterile conditions

Item		SMH concentration (mg mL^{-1})				Control
		1.0	1.5	2.0	3.0	
pH [1)]	Before	6.87	7.01	7.03	7.03	6.98
	Sterile	7.02	7.01	7.02	7.03	
	Non-sterile	8.65	8.72	8.82	8.90	
Ammonia nitrogen (mg L^{-1})	Before	9.19±0.71 a	14.72±1.76 a	18.91±1.68 a	30.07±3.21 a	0.17±0.02 a
	Sterile	10.31±0.98 a	15.03±1.51 a	19.03±1.53 a	30.23±3.61 a	
	Non-sterile	56.87±5.35 b	71.40±3.21 a	102.79±11.21 b	151.26±17.09 b	
Free ammonia (mg L^{-1})	Before	< 0.010	< 0.010	< 0.010	< 0.010	< 0.01
	Sterile	< 0.010	< 0.010	< 0.010	< 0.010	
	Non-sterile	11.86	16.88	28.82	41.37	
Nitrate nitrogen (mg L^{-1})	Before	0.26±0.03 a	0.28±0.04 a	0.30±0.03 a	0.29±0.04 a	0.303±0.04 a
	Sterile	0.27±0.05 a	0.29±0.02 a	0.28±0.02 a	0.31±0.03 a	
	Non-sterile	0.29±0.04 a	0.25±0.05 a	0.29±0.05 a	0.29±0.05 a	
Nitrite nitrogen (mg L^{-1})	Before	0.041±0.010 a	0.043±0.007 a	0.042±0.009 a	0.040±0.007 a	0.038±0.005 a
	Sterile	0.042±0.007 a	0.049±0.004 a	0.044±0.006 a	0.042±0.005 a	
	Non-sterile	0.039±0.005 a	0.039±0.006 a	0.042±0.017 a	0.042±0.002 a	

[1)] Each concentration was tested in 9 replicates and the fluids in the 9 flasks were combined and centrifuged for pH determination.

Table 3 Effects of SMH UF fractions on the germination, radicle length and shoot length of *L. perenne* seeds under non-sterile condition

Fraction	Nitrogen concentration (mg mL^{-1})			
	0.68	0.136	0.204	0.272
Radicle length inhibition rate (%)				
F4	20±8 a	33±10 a	72±15 a	100±0 a
F3	28±11 a	31±4 a	80±8 a	98±3 a
F2	26±5 a	24±8 a	66±8 ab	94±10 a
F1	24±7 a	22±9 a	69±11 a	100±0 a
Shoot length inhibition rate (%)				
F4	-5±1 a	5±0 a	11±3 a	39±9 a
F3	-6±2 a	4±1 a	16±4 a	32±6 a
F2	-7±2 a	6±2 a	14±2 a	36±4 a
F1	-6±1 a	3±1 a	17±3 a	37±3 a
Germination rate (%)				
F4	93±10 a	96±6 a	93±10 a	0±0 a
F3	103±6 a	93±10 a	89±6 a	5±3 a
F2	100±6 a	100±6 a	96±6 a	14±6 a
F1	93±18 a	100±6 a	100±6 a	0±0 a

inhibited shoot growth at higher concentrations. Multiple comparisons showed that there was no significant difference ($P<0.05$) among the SMH UF fractions in the same nitrogen content. The effects of SMH UF fractions on seed germination rate were similar to that on radicle growth. All the fractions showed no difference in inhibitory effects on germination at the concentrations investigated.

DISCUSSION

The seed germination inhibitory activity of ammonia, as well as its antifungal and nematicidal activity, has been known for a long time. Ammonia is often released from organic materials, especially those with a low C/N ratio (Spiegel *et al*. 1987; Oka *et al*. 1993). Candole and Rothrock (1997) reported that free NH_3 released by hairy vetch suppressed the growth of pathogen *Thielaviopsis basicola*. Cottonseed meal mixed into soil at 200 and 300 lbs acre^{-1} (224 and 336 kg ha^{-1}) reduced corn seed germination by 75% compared with soil alone (Sherwin 1923). Free ammonia released from alfafa (*Medicago sativa*) residue during degradation hampered plant seed germination and the early development of seedling (Megie *et al*. 1967; Ells *et al*. 1991). Even low concentration of free ammonia could intoxicate seed germinating and seedling development of several crops. The concentration range that started to show toxicity to severe toxicity was within 0.15-6.0 mmol L^{-1} (Mengel 1982).

In the current study, the contents of both ammonia nitrogen and free ammonia increased significantly under non-sterile condition compared with the media before cultivation. Britto and Kronzucker (2002) had reviewed the toxicity of NH_4^+ in higher plants. It had been reported that, under conditions of high external pH, NH_3 can build up to concentrations large enough to facilitate its entry via passive diffusion (Yin *et al*. 1996; Kosegarten *et al*. 1997; Wilson *et al*. 1998; Gerendas and Ratcliffe 2000; Plieth *et al*. 2000). In this study, the pH reached up to above 8.6 and the free ammonia released from SMH reached 0.70-2.43 mmol L^{-1}. Hence, it was suggested that free ammonia released by microbe degradation of SMH was the major cause of seed germination inhibition. Although the mechanism was not completely clear, it was widely believed that ammonia phytotoxicity was caused by the uncoupling of photophosphorylation (Drath *et al*. 2008).

The growth promotion effect of SMH under sterile condition might result from the absorption of organic nitrogen by plants. Recent studies have shown that plants can absorb organic nitrogen, such as amino acids and soluble peptides (Yamagata and Ae 1996; Okamoto *et al*. 2003; Jones and Shannon 2004). Under sterile condition, organic nitrogen has a greater nutritional effect than mineral nitrogen (Chapin *et al*. 1993; Mo *et al*. 2003).

In the current study, all the UF fractions of SMH showed no significant difference on the radicle inhibition of germinating *L. perenne* seeds at the same nitrogen concentration. Since the fractions were mainly composed of peptides, it was possible that the inhibitory activities were not contributed by one or more specific peptides in the SMH fractions. In conclusion, SMH exerted its herbicidal activity through the free ammonium released by degradation instead of by specific peptide(s) in it. Based on the conclusion, it is suggested that future studies should focus on the release control of free ammonia from SMH for more effective weed control efficacy.

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