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Mixing Pyroligneous Acids with Herbicides to control Barnyardgrass (*Echinochloa crus-galli*)

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ABSTRACT. Alternatives to commercial chemical herbicide are currently being searched and tested due to the numerous adverse effects of commercially available herbicides to the environment. Barnyardgrass (*Echinochloa crus-galli*) is an important weed species around the world, especially in paddy rice fields. This study focuses on the favorable effects of mixing pyroligneous acids with commercial liquid herbicides. Seedlings were transplanted and grown under greenhouse conditions. The effect of treatment time or leaf-stage on herbicide-pyroligneous acid efficacies was checked, coupled with isolation and quantification of biochemical compounds. Results revealed that herbicide treatment at early post emergence (2~3 leaf stage) of *Echnochloa crus-galli* leads to effective control. Both liquid herbicides affected fatty acid, protein, and amino acid syntheses as reflected on their contents. The influence of wood vinegar (WV) or rice vinegar (RV) on these compounds was not thoroughly verified due to lack of information on the pyroligneous products. We observed that mixing WV or RV with BCB (bentazone + cyhalof-butyl) gives more favorable results than BUC (butachlor + clomazone), mixed with WV or RV. The result would indicate the potential of mixing pyroligneous acid in reducing herbicide application rate.

Key words: Barnyardgrass (*Echinochloa crus-galli*), Fatty acid synthesis, Rice Vinegar, Wood Vinegar

Introduction

Barnyardgrass (*Echinochloa crus-galli*) is a major weed species in almost all rice fields, causing yield and grain quality losses worldwide (Smith, 1983). Widespread distribution of this species may be attributed to prolific seed production, with a slight degree of dormancy, and most of all adaptability to adverse environmental stress (Maun and Barrett, 1986). The use of chemical herbicides remains the most practical approach to control this noxious weed, especially at intensive rice farming areas. While the use of herbicides has enabled intensive agriculture to progress, excessive application and high application rates cause hazards to the environment and pose threats to the human food supply (Bernard et al., 2005; Jiraungkoorskul et al., 2002). In this regard, the public has increasingly concerned and supports sustainable agriculture movement, particularly in Korea, leading to intensive search for natural compound(s) that can be used in crop production with the goal of minimizing agrochemical use.

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Alternate and integrated methods of weed control have continuously been sought. Bio-herbicides and/or allelochemicals may have potential to replace chemical herbicide or reduce chemical herbicide use. Morgan (1989) listed a few alternatives to chemical herbicide, which are weed (species) specific. The use of pyroligneous acids in agricultural production is also becoming popular in Asian countries. Pyroligneous acids are by-products of making charred-materials (“*biochar*”) containing numerous amounts of carboxylic/organic acids and other organic components.

Pyroligneous acid from wood (or wood vinegar) has been reported to have a promoting effect on growth and nutrient absorption of rice roots (Ichikawa and Ota, 1982). It also showed a degree of herbicidal activity (Mu et al., 2003) although according to Esguerra et al. (2009), it is impractical to use wood vinegar alone as a replacement for chemical herbicide. Pyroligneous acids can increase herbicide efficacy due to vast amounts of organic compounds present in it; most of which are organic acids, phenols, carbonyls, and alcohol (Kim et al., 2001). Acetic acid, the most potent component of wood vinegar has been used for weed control (Coffman et al., 2005). It was also found to be effective in controlling or killing below-ground vegetative propagules of fresh water plants e.g. *Hydrilla*, *Spartina alterniflora* and *Potamogeton pectinatus* (Anderson, 2007; Spencer and

Ksander, 1997, 1999).

The purpose of this study is to determine the contribution of using pyroligneous acid in combination with commercial chemical herbicide for effective control of barnyardgrass (*E. crus-galli*), and specifically determine the effect(s) of treatment time/leaf stage on herbicidal efficacies of pyroligneous acid.

Materials and Methods

Herbicides and pyroligneous acids tested

Pyroligneous acids were tested in combination with chemical herbicides to evaluate the herbicidal activity and its possible herbicidal effect for the control of barnyardgrass. Two commercial mixed-formulation herbicides namely: bentazone + cyhalof-butyl (BCB) and butachlor + clomazone (BUC), common herbicides used to control annual (broadleaf, sedges, and grasses) weeds in paddy rice fields of Korea were selected as chemical herbicide. Pyroligneous acids from wood (*Quercus* sp.) and rice husk/hull were obtained from the Korean Forest Research Institute (KFRI) and Daewon Global System Integration (Daewon GSI), respectively. The former denoted as wood vinegar (WV) and the latter rice hull vinegar (RV). RV was found to be more acidic (lower pH) than WV (Table 1).

Treatments and experimental set-up

The experiment was conducted under greenhouse conditions at the Agricultural Experiment Station and Research Facility, College of Agriculture and Life Sciences, Kyungpook National University, Daegu, Korea from November 2010 to October 2011. Seeds of barnyardgrass (*Echinochloa crus-galli)* were obtained from Gyeongbuk Agricultural Research and Extension Service, Korea. The seeds (2 g) were first sown on a disposable petri dish with 15 ml water and incubated in a plant growth chamber (30ºC, 24 h white light) for 3 days. The seedlings were later transplanted in a 0.01 m^3 (0.41 m×0.24 m ×0.11 m) plastic tray, containing paddy soil. Recommended herbicide or full rate (100%) and half (50%) were used as references. Pyroligneous acids were diluted at 1 ml per 500 ml of water before applying into the soil with 50% of the recommended herbicide rate. Control (untreated) seedlings were also grown. Growth progression and herbicide symptoms were evaluated every day upon treatment.

Table 1. Physical and chemical components of pyroligneous acids.

Items	Wood vinegar	Rice hull vinegar
pH	3.03	1.86
Amount of acid (%)	3.75	3.60
Acetic acid (%)	3.52	3.33
Propionic acid (%)	0.23	0.27
Sediment (%)	0.05	0.06
Brix (%)	3.5	__
Organic matter:N ratio	__	0.42
Transparency	Transparent	Transparent
Color	Reddish Brown	Golden Yellow

Source: A & F Research Co., Ltd.

Effect of treatment time/seedling stage

Seeds of barnyardgrass were germinated and transplanted as above mentioned. For this experiment set-up, BCB and BUC-pyroligneous acid treatments were applied at 2-, 3- and 4-leaf stages of *E. crus-galli*. The tray was kept flooded (3 cm water depth) by regular watering throughout the test period. Plant height (cm), fresh, and dry weights (g) were recorded at 7, 14, and 21 DAT of BCB and BUC-pyroligneous acids. Efficacy was reported as percent (%) reduction in dry weight relative to the untreated control.

Fatty acid extraction and concentration

To evaluate the effects of pyroligneous acids-herbicide mixture on the fatty acid biosynthesis, the fatty acid composition of lipid extracts from *E. crus-galli* shoots was determined according to the method described by Chung (1991). Samples were finely ground and 1 g of the sample was mixed with chloroform:methanol (2:1 v/v) and then vortex/mixed for 10 minutes. The supernatant was collected after the solution was centrifuged (5 min, 3000 rpm) and the oil was concentrated using rotary evaporator (15~20 min, 45ºC). The concentrated oil went through acid catalyzed esterification which was adopted from the protocol of Ruibal-Mendieta et al. (2004). Concentrated oil was mixed with 0.1 ml butylated hydroxyl toluene (BHT) in hexane solution. Samples were subjected to GC-MS analysis. The gas chromatograph (6890 plus, Hewlett Packard, Co., USA) was equipped with a DB-225 column (30 m×0.25 mm×0.25 μm). The column temperature was held at 140ºC for 2 min while the injector was 250ºC, then gradually increased up to 200ºC at 5ºC min^{-1}, and finally increased to 220ºC at a rate of 10ºC min^{-1}. The injection volume was 1 μl injected at a flow rate of 1 ml min^{-1}.

Protein extraction and quantification

To evaluate the effects of pyroligneous acids-herbicide mixture on amino acid and protein biosynthesis, shoots of *E. crus-galli* were collected 7 days after herbicide-pyroligneous acids treatment. Extraction was carried out based on the methods of Damerval et al. (1986) with some modifications. Samples (1 g) were frozen and ground through a mortar and pestle with liquid nitrogen then suspended in 5 ml pre-cooled (-20ºC) solution of 10% trichloroacetic acid (TCA) in acetone with 0.07% dithiothreitol (DTT). The suspension was incubated (-20ºC, 1 h) and centrifuged (15 min, 19,500 rpm, 4ºC); this procedure was repeated twice. The supernatant containing photosynthetic pigments was discarded. Total protein content of the supernatant was determined through spectrophotometric methods suggested by Bradford (1976).

For the amino acid analysis, extraction was done by digesting 1.0 g oven-dried shoot samples in 10 ml 6 N HCl at 110ºC, then vacuumed dried to removed HCl. Afterwards, 10 ml of 0.2 M sodium citrate loading buffer (pH 2.2) was added. The mixture was filtered in 0.22 μm membrane filter and was subjected to Sep-pak plus C18 Cartridge to completely remove pigment and other high molecular material. Analysis was done using an amino acid auto analyzer (Biochrom 30, Biochrom Ltd., UK).

Data analyses

The experiments were laid out in a Completely Randomized Design (CRD) with 3 replicates. Data were analyzed using the Statistical Analysis System ver. 9.1 (SAS Institute, Cary, North Carolina, USA). The mean separation method used was Duncan Multiple Range Test (DMRT).

Results and Discussion

Effect of treatment time/seedling stage on herbicide-pyroligneous acid efficacy

Timing of application is vital, particularly when applying post-emergence herbicides. Streit et al. (2003) even emphasized that post-emergence is better than the pre-emergence control of weeds in a wheat field. In this study, the resulting dry weight of seedlings from herbicide-pyroligneous acid treatments at two- and three-leaf stages was significantly lower compared to the control. Also, efficacy values were generally higher when treatments were done at two- and three-leaf stages compared with four-leaf stage. At two-leaf stage, we observed low herbicidal efficacies values but not significantly different from the full rate herbicide treatment when *E. crus-galli* seedlings were treated with BCB 50% + pyroligneous acids (Table 2). This response was almost similar across different treatment time/ leaf stages. Additionally, herbicide efficacies were usually unpredictable (or low) when *E. crus-galli* was treated at four-leaf stage. Herbicide application is encouraged at "early" post emergence than later time (Klingman et al., 1992), in this case two- and three-leaf stages of *E. crus-galli*. In general, BUC treatments appear to be less effective than BCB, even if treated in mixture with pyroligneous acids.

Effect on fatty acids

Eight (8) fatty acids were detected on *E. crus-galli* leaves after treatment with herbicide-pyroligneous acid combinations (Tables 3 & 4) namely: myristic (C14), palmitic (C16), palmitoleic (C16:1), stearic (C18), oleic (C18:1), linoleic (C18:2), linolenic (C18:3), and arachidic acid (C20). Based on the results, palmitoleic acid was the least dominant fatty acid followed by myristic, while linolenic and linoleic were found to be the most dominant (higher percentage/proportion). In the other crops e.g. rice, maize, grain sorghum, linoleic acid was the main unsaturated fatty acid found in control and herbicides treated plants (Wu et al., 2000). In this study, application of BCB and BUC mixed with pyroligneous acids resulted to slight variations on fatty acid composition relative to the control. Treatment with BCB at full rate resulted to an increase of myristic acid, palmitic acid and arachidic acid but reduced the linolenic acid content as compared with the control and those treated with half rate of BCB. On the other hand, the mixture of 50% BCB diluted with wood or rice vinegar (500×) resulted to higher oleic acid content but lower linolenic acid as compared to the control and those treated with half rate of

Table 2. Effect of treatment time/leaf-stage on herbicide-pyroligneous acid efficacies 21 days after treatment[a].

Treatments	2-Leaf stage			3-Leaf stage			4-Leaf stage		
	FW	DW	Efficacy (%)	FW	DW	Efficacy (%)	FW	DW	Efficacy (%)
Control	0.82a	0.25 a	-	1.21 a	0.57 a	-	1.45 a	0.43 a	-
BCB 100%	0.03 bc	0.02 ed	94.0 ab	0.01 c	0.01 c	99.2 a	0.12 d	0.07 d	82.9 a
BCB 50%	0.06 bc	0.04 de	86.5 cd	0.06 bc	0.04 c	93.0 abc	0.33 cd	0.12 cd	70.4 ab
BCB 50%+500 WV	0.02 bc	0.01 e	98.4 a	0.03 bc	0.02 c	97.3 a	0.13 d	0.08 d	80.9 a
BCB 50%+500 RV	0.00 c	0.00 e	100.0 a	0.05 bc	0.03 c	95.6 ab	0.08 d	0.05 d	87.6 a
BUC 100%	0.06 bc	0.05 cd	81.7 d	0.05 bc	0.03 c	95.2 abc	0.91 b	0.32 b	23.9 c
BUC 50%	0.20 b	0.11 b	61.8 e	0.18 b	0.12 b	79.4 d	1.14 b	0.40 ab	6.5 c
BUC 50%+500 WV	0.04 bc	0.03 ed	90.9 bc	0.10 bc	0.06 c	89.3 c	1.07 b	0.34 ab	18.6 c
BUC 50%+500 RV	0.18 bc	0.09 bc	67.6 e	0.08 bc	0.05 c	90.5 bc	0.44 c	0.19 c	55.6 b

[a] Means within the same column having the same letter are not significantly different at 5% level by DMRT.
FW=fresh weight (g), DW=Dry weight (g)

Table 3. Percentage of fatty acids detected from shoots of *E. crus-galli* treated with wood vinegar (WV) and rice hull vinegar (RV) mixed with bentazone + cyhalofop-butyl (BCB) 21 days after treatment[a].

	Myristic acid (C14)	Palmitic acid (C16)	Palmitoleic acid (C16:1)	Stearic acid (C18)	Oleic acid (C18:1)	Linoleic acid (C18:2)	Linolenic acid (C18:3)	Arachidic acid (C20)
Control	0.61 b	25.1 b	0.05 c	4.52 a	7.8 b	25.8 a	35.0 a	1.09 c
BCB 100%	1.47 a	31.5 a	1.22 a	5.16 a	6.9 b	26.3 a	23.5 b	3.90 a
BCB 50%	0.44 c	25.0 b	0.20 b	4.40 a	5.2 b	23.0 a	39.9 a	1.91 b
BCB 50%+500WV	0.44 c	22.7 b	0.23 b	4.27 a	15.5 a	27.9 a	28.0 b	0.95 c
BCB 50%+500RV	0.40 c	23.6 b	0.22 b	3.70 a	17.2 a	29.3 a	24.7 b	0.91 c

[a] Means within the same column having the same letter are not significantly different at 5% level by DMRT.

Table 4. Percentage of fatty acids detected from shoots of *E. crus-galli* treated with wood vinegar (WV) and rice hull vinegar (RV) mixed with butachlor + clomazone (BUC) 21 days after treatment[a].

	Myristic acid (C14)	Palmitic acid (C16)	Palmitoleic acid (C16:1)	Stearic acid (C18)	Oleic acid (C18:1)	Linoleic acid (C18:2)	Linolenic acid (C18:3)	Arachidic acid (C20)
Control	0.53 c	23.9 b	0.12 d	3.63 b	2.11 b	23.0 a	45.8 a	0.94 c
BCB 100%	1.85 a	33.1 a	0.00 e	8.73 a	3.81 a	19.9 b	28.4 c	4.25 a
BCB 50%	0.46 c	21.6 b	0.39 c	3.65 b	1.43 c	20.6 ab	50.6 a	1.31 c
BCB 50%+500WV	0.95 b	34.1 a	2.34 a	4.77 b	2.54 b	16.2 c	37.1 b	2.03 b
BCB 50%+500RV	0.39 c	23.6 b	0.87 b	4.46 b	1.65 c	25.2 a	42.1 ab	1.72 bc

[a] Means within the same column having the same letter are not significantly different at 5% level by DMRT.

BCB. In the case of BUC treated plants, full rate application of BUC resulted to an increase of myristic acid, palmitic acid, stearic acid and arachidic acid but reduced the linolenic acid content as compared with the control and those treated with half rate of BUC. On the other hand, the mixture of 50% BUC with wood vinegar resulted to higher myristic acid, palmitoleic acid, and arachidic acid content but lower linoleic and linolenic acids as compared to the control and those treated with half rate of BCB. Apparently, half of herbicide treatments (50%) when combined with wood or rice vinegar resulted to alteration of fatty acid contents similarly with full herbicide treatment. This would warrant further investigation on how pyroligneous acids alone could affect biochemical compounds in plants that would in turn result to herbicidal activity.

The changes in the proportion of fatty acids in the leaves of *E. crus-galli* could be attributed in part by the action of different active ingredients of herbicide. For example, BCB contains cyhalofop-butyl, with its mode of action on indirectly-inhibiting the biosynthesis of fatty acids through *acetyl-CoA carboxylase* (ACCase) enzyme (Shaner, 2003). It could inhibit oleic acid production into non-lipid fraction (Usui, 2001) which could result to accumulation in tissues of BCB treated plants. Reduction in linolenic acid could be due to the inhibition of butachlor in BUC. This is parallel with the results of Walker et al. (1988) using another type of phenoxypropanoate herbicide (fluazifop-butyl) resulting to production of short-chain fatty acids rather than C-18 or longer-chain fatty acids on treated barley leaves. BUC contains the chloroacetamide butachlor known to be involved in the enzymatic four-step fatty acid elongation system (Matthes et al., 1998), which explains the reduction in long-chain and/or unsaturated fatty acids when *E. crus-galli* was treated with BUC.

Effect on protein and free amino acid contents

Protein contents in stressed-shoots or treated *E. crus-galli* seedlings were significantly lower than the control (Figure 1). It seems that BUC-treated seedlings have lower protein

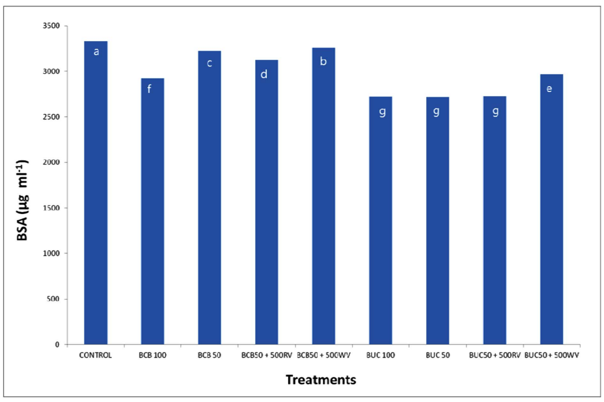

Fig. 1. Protein contents (expressed as Bovine Serum Albumin standard equivalents) in *E. crus-galli* shoots 7 days after herbicide-pyroligneous acid treatments. *Means having the same letter are not significantly different at α=0.05 by LSD.

Table 5. Free amino acids (mg/g) in the shoots of *E. crus-galli* 3 days after treatment of bentazone+cyhalofop-butyl (BCB) and butachlor+clomazone (BUC) in mixture with WV and RV[a].

Treatments	Asp	Thr	Ser	Glu	Pro	Gly	Ala	Cys	Val	Met	Ile	Leu	Tyr	Phe	His	Lys	Arg
Control	7.8e	4.8e	4.7e	9.4e	6.3bc	4.8e	6.4e	1.0bcd	4.8e	1.5cd	4.6e	6.4e	4.1cd	5.8d	2.9c	5.7e	5.8c
BCB 100%	11.0b	7.4b	6.8b	13.4b	9.2a	7.0b	9.1b	1.2b	7.3b	2.1b	6.9b	9.4b	5.5b	8.8a	4.4a	8.2b	9.7a
BCB 50%	10.1c	6.3cd	6.1c	12.3c	7.2b	6.3c	8.2c	0.9cde	6.6c	1.5cd	6.2c	8.7c	4.2cd	7.6bc	3.6b	7.4c	7.8b
BCB 50%+500WV	13.5a	8.1a	7.9a	15.6a	10.2a	7.8a	10.5a	1.5a	8.2a	2.6a	7.6a	10.4a	6.4a	9.4a	4.8a	9.0a	10.6a
BCB 50%+500RV	11.0b	6.6c	6.4bc	13.1bc	7.5b	6.5c	8.6bc	1.1bc	6.8bc	1.8bc	6.2c	8.9bc	4.6c	7.8b	3.6b	7.7bc	8.2b
BUC 100%	7.6ef	4.3e	4.3ef	8.8ef	4.6d	4.2f	5.7f	1.0bcde	4.5ef	1.5cd	4.0f	5.8ef	3.4de	5.0e	2.3d	5.0f	5.6c
BUC 50%	6.9fg	4.2e	4.2ef	8.5ef	5.0cd	4.1f	5.6f	0.8de	4.4ef	1.3d	4.0f	5.7f	3.0e	5.0e	2.3d	4.8f	5.3c
BUC 50%+500WV	6.7g	4.1e	4.0f	8.1f	4.5d	4.0f	5.3f	0.7e	4.2f	1.1d	3.8f	5.5f	2.8e	4.8e	2.2d	4.6f	4.9c
BUC 50%+500RV	9.4d	5.8d	5.5d	10.9d	7.7b	5.5d	7.5d	1.2bc	5.8d	1.8bc	5.3d	7.4d	4.7bc	6.8c	3.5b	6.4d	7.8b

[a] Means within the same column having the same letter are not significantly different at 5% level by DMRT.

contents compared to those treated with BCB. This can be explained by the action of butachlor, a component of BUC which directly inhibits protein synthesis (Chang et al., 1985).

A total of 17 free amino acids were detected in *E. crus-galli* shoots (Table 5). The contents of glycine, alanine, isoleucine, phenylalanine, histidine, and lysine were significantly higher in treated leaves compared to the control. Moreover, we observed that amino acid contents were generally higher in BCB treatments relative to the control hence the opposite is true for BUC treatments. Bentazone, a component of BCB is proven to be effective in controlling broadleaf weeds than grasses (Usui, 2001), which poses an explanation why it did not seriously affect the protein contents of *E. crus-galli*. Butachlor, which affects the synthesis of proteins seem also affected amino acid synthesis. However, the direct effects of wood and/or rice vinegar on biochemical compounds of *E. crus-galli* were not investigated in this study. Overall our results showed the potential use of pyroligneous acids to reduce herbicide use to control dominant weed of rice such as *E. crus-galli*. Reducing the application rate to 50% and mixing with 500× dilution of wood vinegar or rice vinegar would result to the same efficacy with that of full rate treatment of BUC and BCB in controlling *E. crus-galli*.

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