EFFECTS OF BUTACHLOR ON SEED GERMINATION AND SEEDLING GROWTH OF BARNYARDGRASS[1]

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Abstract: The effects of butachlor (2-chloro-2′, 6′-diethyl-*N*-(butoxymethyl) acetanilide) on seed germination of barnyardgrases (*Echinochloa crus-galli* (L.) Beauv.) and subsequent seedling growth were investigated. Results showed butachlor treatment did not affect seed germination up to a concentration of 200 ppm, but it delayed the time of radicle emergence through the pericarp of seed for 12 hours. Futher seedling growth was severly inhibited. Emergence of both the primary leaf from the coleoptile and the root were inhibited by a 6 ppm concentrtaion of butachlor. Time elapse studies of the effect of butachlor on the development of amylase and protease activities revealed that the inhibition these activities observed during the early period of seed germination prevented the degradation of reserve carbohydrates and proteins in the endosperm. However butachlor treatment did not affect the polyribosome level in the initial 36 hours period of seed germination

INTRODUCTION

Barnyardgrass (*Echinochloa crus-galli* (L.) Beauv.) is an annual grass that causes severe losses in rise fields[20,25,26]. Butachlor (2chloro-2′, 6′-dithyl-N-(butoxymethyl) acetanilide) is a herbicide used alone or in combination with other herbicides. It effectively controls most annual grasses and certain broadleaved weeds when applied at the time of pre-emergence or early post-emergence. This herbicide is widely used to control barnyardgrass and other weeds in rice fields in Taiwan. Although immature rice seedlings are sensitive to butachlor before the coleoptile emerges, barnyardgrass is sensitive from germination to the two-leaf stage. Butachlor belongs to the class of α-chloroacetamide herbicides[12]. This class includes alachlor (2-chloro-2, 6′-diethyl-N-(methoxylmethyl)acetanilide); metochlor(2-chloro-N-(2-ethyl-6-methyl -phenyl)-N-(2-methoxyl-1-methylethyl) acetamide); propachlor (2-chloro-N-isopropylacetanilide); prynachlor (2-chloro-N-(1-methyl-2-propynyl) acetanide); and CDAA (2-chloro-N, N-diallyl-acetamide) etc. Little is known about the mode of action of α-chloroacetamide, especially in butachlor; however, there have been several reportes on enzyme activity[2,15] and enzyme synthesis[6,9,17,19,23]. Jawarski[15] reported that CDAA inhibits certain sulfhydryl containing enzymes involved in respiration. Devlin and Cunningham[6] reported that propachlor inhibited gibberellic acid-induced hydrolytic enzyme production. Rao and Duke[23] also reported alachlor, propachlor and prynachlor inhibited gibberellic acid-induced production of protease and α-amylase in de-embryonated barley seeds (*Hordeum vulgare* L., "Schuyler"). Propachlor has been shown to inhibit the incorporation of ^{14}C-leucine into protein in young seedlings. Time-elapse studies indicated that the inhibition of protein synthesis preceeded the inhibition of

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nucleic acid synthesis and consequent growth[8]. Eshel reported that alachlor caused a decrease in the weight of cotton seeclings accompanied by a severe reduction in root growth[11]. Since butachlor is a widely used herbicide in control of barnyardgrass and other weeds in Taiwan, studies reported herein were conducted to determine the morphological and biochemical effect of butachlor on barnyardgrass seed germination and subsequent growth.

MATERIALS AND METHODS

Seeds of barnyardgrass (*Echinochloa crus-galli* (L.) Beauv.) were sterilized in 1% sodium hypochlorite for 20 minutes, rinsed with tap water for one hour, and planted in 20 cm petri dishes containing two layers of filter paper moistened with distilled water (as control) or butachlor solution (as treated). Technical grade of butachlor (90%) was obtained from the Agricultural Division of Monsanto Company. Concentrations of 6 ppm, 25 ppm, 50 ppm, 100 ppm, and 200 ppm were used in this study. For morphological studies, barnyardgrass seeds were planed on perforated filter paper standing in a 100 ml beaker (Fig. 4). Seedlings were grown in a 24°C growth chamber under 12-hour daylength at 6,000 lux light intensity. For biochemical analysis, samples were harvested at 48 hour intervals.

Determination of fresh weight and dry weight:

Fresh weight of seedling was determined after blotting to remove excess water; dry weight was determined after drying in a 60°C oven for 48 hours.

Effect of concentration of butachlor and timing of treatment on seedling growth:

In order to determine the effect of various concentrations of butachlor on barnyardgrass seed germination and seedling growth, seeds were imbibed and grown in 25, 50, 100, and 200 ppm butachlor solution for 8 days. The influence of time of butachlor addition on seedling growth was also studied. Butachlor solution at a concentration of 100 ppm was added to the seeds and seedling at 0, 2, and 4 days. Seedling growth was examined at the end of the 8 day experimental period.

Quantitative analysis of soluble sugar and amino acids:

Fifty entire seedlings or excised endosperms were boiled in 20 ml of 50% ethanol for 30 minutes, homogenized with a polytron, and then boiled again for another 5 minutes. After centrifuging at 7,700 × g for 20 minutes, the supernatant was collected. This fraction was used for quantitative determination of soluble sugars and amino acids. Soluble sugars were determined according to the method of Yemn[28] using glucose as a standard. The method of Moore and Stein[18] was used for the quantitative determination of amino acids using leucine as a standard.

Enzyme assay of amylase:

The amylase was extracted according to the method of Bernfeld[4]. The tissue was homogenized in cold 0.01 M acetate buffer (pH 4.8) containing 0.01 M $CaCl_2$ with a polytron. After centrifugation at 4,500 × g for 10 minutes, the supernatant was used for the enzyme assay of amylase. The procedures for amylase assay were similar to that Chrispeels and Varner[5]. Amylase activity was measured by adding 1 ml of enzyme solution to 1 ml of 0.15% potato starch solution (containing 2 mM $CaCl_2$ and 0.6% KH_2PO_4), mixed in a test tube and then incubated 30°C water bath for 3 minutes. At zero time and after 3 minutes of reaction, 1 ml of I_2-KI solution was added to each sample. The samples were diluted with 5 ml of distilled water; optical density was determined at 620 nm. The amylase activity was expressed as the decrease in optical density at 620 nm (ΔOD_{620}/mg protein) at zero time and after 3 minutes of enzyme reaction.

Enzyme assay of protease:

Tne method of Angelo *et al.*[1] was used to prepare the crude protease enzyme. The tissue was homogenized in cold 0.01 M Na-phosphate buffer (pH 7.4) containing 10 mM β-mercaptoethanol with a polytron. After centrifuging at 20,000 × g for 30 minutes, the supernatant was used for the assay of protease activity. Two different methods were used to assay the protease activity: (A) The Erlanger *et al.* method[10]: Protease activity was measured by adding 0.2 ml of enzyme solution to 2.5 ml 4 mM BAPA (N-benzoyl-L-arginine-4-nitroanilide) and 0.3 ml of 0.3 M K-phosphate buffer (pH 8.0) in a test tube, mixed, and then incubated in 30°C water bath for 30 minutes. After dilution with 6 ml of distilled water, the optical density was determined at 405 nm. The protease activity was expressed as the increase in optical density at 405 nm (ΔOD_{405}/mg protein). (B) The Kunitz method[16]: Protease activity was measured by adding 1 ml of enzyme solution to 5 ml of 0.6% casein solution, mixed, and then incubated in 37°C water bath for 3 hours. At the end of reaction, 0.7 ml of 0.5 N perchloroacetic acid was added and then centrifuged at 20,000 × g for 10 minutes. The optical density of the supernatant was determined at 275 nm using a Gilford Model 250 spectrophotometer. The enzyme activity was expressed as the increase in optical density at 275 nm (ΔOD_{275}/mg protein).

Sucrose density gradient analysis of polyribosomes:

6 grams (fresh weight) of 36-hour imbibed seeds were homogenized with a polytron in 9 ml of buffer A (containing 0.2 M tris-HCl buffer, pH 8.5, 0.25 M sucrose, 50 mM KCl, 25 mM $MgCl_2$). The homogenate was filtered through two layers of miracloth. The filtrate was centrifued at 17,000 × g for 30 minutes. The supernatant was layered over 5 ml of 60% (w/v) sucrose in buffer B (containing 40 mM tris-HCl buffer, pH 8.5, 20 mM KCl, 10 mM $MgCl_2$) and centrifuged at 220,000 × g for 90 minutes in type 65 rotor of MSE model 65 ultracentrifuge. The ribosome pellets were suspended in buffer B and layered over a linear sucrose density gradient from 15% to 50% (w/v) for centrifugation in SW 30 rotor at 25,000 rpm for 150 minutes. The sucrose density gradient was fractionated by an ISCO density fractionator (model 185) and scauned at 254 nm with an ISCO model UA-4 absorbance monitor.

RESULTS

Effect of butachlor treatment on barnyardgrass seed germination and seedling growth:

Butachlor treatment did not affect seed germination up to a concentration of 200 ppm, but delayed the time of radicle emergence through the pericarp of seed for 12 hours. The time required for radicle emergence through the pericarp from the untreated seeds was 36 hours after imbibition of water, but the butachlor-treated seeds required 48 hours for radicle emergence. Furthermore protrusion of the primary leaf from the coleoptile and root elongation (including lateral roots and root hairs) were severely inhibited by as low a concentration as 6 ppm of butachlor treatment (Fig. 1, Fig. 2, and Fig. 3). The treated seedlings were less than 2 cm long after 6 days of germination; there was no further elongation during the 16-day experimental period. Figs. 2 and 5 show the results of barnyardgrass seed which were germinated in concentration of 6 ppm and 100 ppm butachlor for 12 hours, and then transferred to filter paper with distilled water for 6 days. Fig. 4 shows the results of seeds which were germinated in water for 2 or 4 days and then transferred to 100 ppm butachlor solution for another 2 to 4 days. In the initial 12-hour germination period, the 6 ppm concentration of butachlor was as effective as the 100 ppm concentration. Moreover, treatment during the initial 12-hour germination period caused severe retardation of subsequent growth and proved to be as effective as the 8 day treatment. The imbibed herbicied remained effective even after 8 hours of continuous rinsing with tap water.

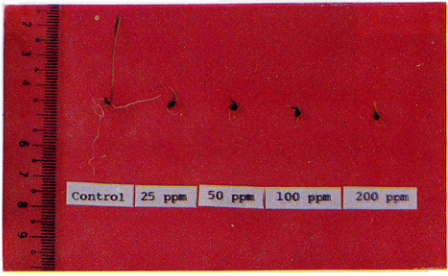

Fig. 1. The effect of various concentration of butachlor on barnyardgrass seed germination and seedling growth.

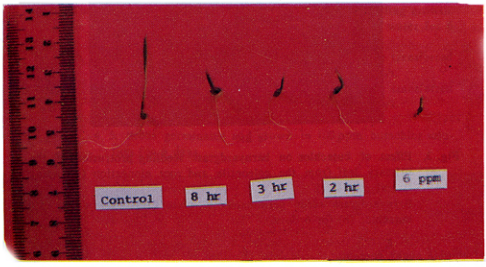

Fig. 2. Results of barnyardgrass seeds in 6 ppm butachlor solution for 12 hours, rinsed with tap water for 2, 3 or 8 hours, and germinated in distilled water for 6 days.

Changes in fresh weight and dry weight during germination:

Fig. 6 shows a steady increase in the fresh weight during the 8 day experimental period. The fresh weight of the butachlor treated seedlings showed a steady increase similar to that of the control for the first 2 days of germination, but after 4 days incremental growth was considerably less than that of the control. At the end of the 8 day experimental period, the fresh weight of the treated seedling was 28% less than that of the control seedlings. Fig. 7 shows a decrease in the dry weight. The difference of the dry weights at the end of 8 days, revealed that the dry weight of the treated seedlings was 13% more than the control. The decrease of dry weight may be due to the respiratory metabolism which was more active in the control seedlings than in the treated.

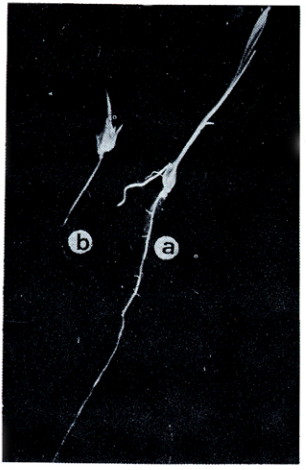

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Fig. 3. Effect of butachlor on barnyardgrass seedling protrusion of the primary leaf from the coleoptile and root elongation (including lateral root hair).
Barnyardgrass seeds germinated in water (a) and in 100 ppm butachlor (b) for 6 days.

Changes of soluble sugar contents:

Changes of soluble sugar contents in each part of control and treated seedling are shown in Fig. 8. During the 8-day period, the total soluble sugar concentration in the control seedlings increased gradually after imbibition of water, reached its maximnm concentration on the 6th day, and thereafter decreased gradually. Changes of soluble sugar content in excised endosperm was similar to that observed in control seedlings, but decreased rapidly after 6th day. In butachlor-treated seedlins, the total soluble sugar also increased gradually during the 8 day period but at a slower incremental than the control seedlings. On the 8th day of germination, the total sugar content of the butachlor-treated seedings was only 50% of that the control seedlings. The soluble sugar in endosperms excised from treated seedlings was also much less than that in the control endosperm. These results indicate that butachlor treatment caused a severe inhibition of degradation of reserve carbohydrates during early seed germination and seedling growth. After 6 days of germination, the control seedlings revealed that the soluble sugar content decreased rapidly in the endosperm and increased rapidly in the embryonic axis.

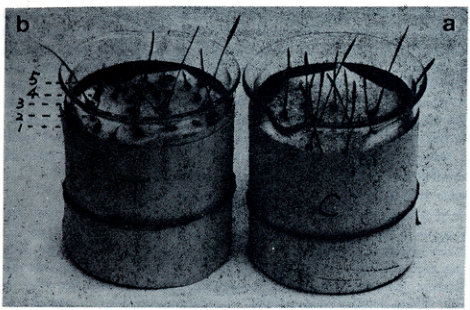

Fig. 4. The influence of time of butachlor addition on seedling growth.
a. Control seeds germinated in water and grown in water for 6 days.
b. 1. Seeds germinated in water for 2 days and then transferred to 100 ppm butachlor solution for another 4 days.
2. Seeds germinated in water for 4 days and the transferred to 100 ppm butachlor solution for another 2 days.
3. 6-day seedlings from control.
4, 5. Seeds germinated and grown in 100 ppm butachlor solution for 6 days.

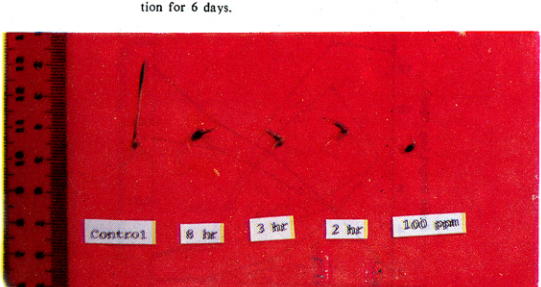

Fig. 5. Bardyardgrass seeds imbibed in 100 ppm butachlor solution for 12 hours, rinsed with tap water for 2, 3 or 8 hours and germined in distilled water for 6 days.

Changes in soluble amino acid contents:

The soluble amino acid contents in whole seedlings, endosperms, and embryonic axes during germination are shown in Fig. 9. In the control seedlings, the amino acid content increased gradually after imbibition of water, reached its maximum level after 4 days, and then decreased rapidly. The changes of soluble amino acids in control endosperm and embryonic axis were similar to the changes found in the whole seedlings; however the decrease of amino acid content occurred after 6 days as opposed to 4 days. In treated seedlings, the change in amino acid content was similar to that of the control seedling the first 4 days, but after 6 days, it was much higher. These results indicate that the mobility of soluble amino acids from the endosperm to the embryonic axis in the control seedlings was more active than in the treated seedlings.

Changes in soluble protein contents:

The soluble protein contents in whole seedlings are shown in the Fig. 10. The soluble protein content in the control seedlings increased during the 6 days period. Data showed that the period of rapid increase occurred between 2 and 4 days. In the treated seedlings, the change in soluble protein content was similar to that of the control seedlings during the first 4 days. After 4 days, the soluble protein in treated seedling was much less than that of the control seedling. These results indicated that the protein synthesis in treated seedling was much less than in the control seedling.

Changes of amylase activity in the germinating barnyardgrass seeds:

In order to determine the effect of butachlor treatment on the breakdown of starch, the amylase activity in the early stages of germination was studied. These results are shown in Fig. 11. The amylase activity in the control seedlings could be detected after the 8 hours of germination. The amilase activity in the butachlor-treated seeds could not be detected until 12 hours of germination. After the 24 hour germination period, the amylase activity in the

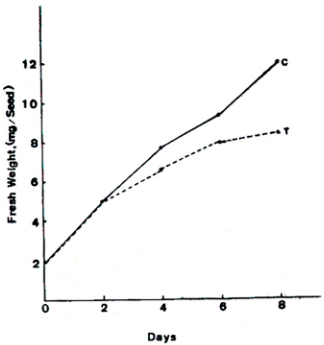

Fig. 6. Change of fresh weight following germination.
C: Control; T: Butachlor-treated.

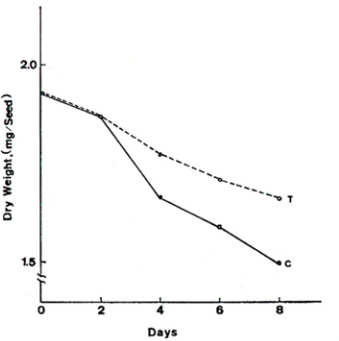

Fig. 7. Change of dry weight following germination.
C: Control; T: Butachlor-treated.

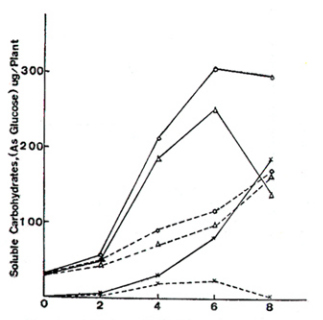

Fig. 8. Change of soluble sugar content following germination.

∘——∘ Control (Whole seedling)	∘- - -∘ Treated (Whole seedling)
△——△ Control (Endosperm)	△- - -△ Treated (Endosperm)
×——× Control (Embryonic axis)	×- - -× Treated (Embryonic axis)

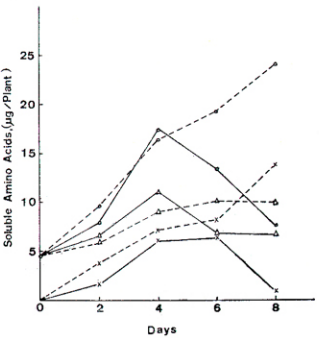

Fig. 9. Changes in amino acid content following germination.

o——o Control (Whole seedling) o- - - -o Treated (Whole seedling)
△——△ Control (Endosperm) △- - -△ Treated (Endosperm)
×——× Control (Embryonic axis) ×- - - -× Treated (Embryonic axis)

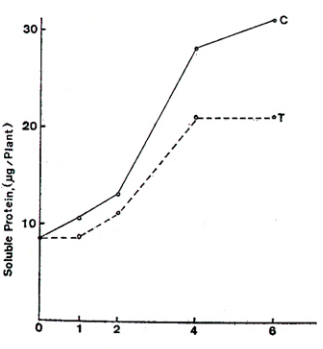

Fig. 10. Changes in soluble protein content following germination.
C: Control; T: Butachlor-Treated.

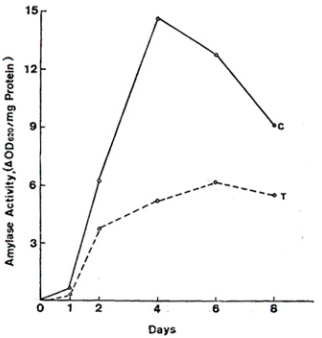

Fig. 11. Change of amylase activity following germination.
C: Control; T: Butachlor-treated.

Table 1. Effect of butachlor-preincubation treatment on amylase activity

Treatment	$OD_{660/mg\ protein}$
Control[a]	12.9
Treated[b]	6.2
Treated[c]	12.9

a. Enzyme extracted from 6-day control seedlings.
b. Enzyme extracted from 6-day butachlor (100 ppm) treated seedlings.
c. Enzyme extracted from 6-day control seedlings, preincubated with butachlor to a final concentration at 100 ppm for 1 hour and assayed.

control and in the treated seedlings was not significantly different, however, the difference between the control and treated seedlings after the 48 hour germination period is significant. Approximately 57% of the amylase activity was inhibited in the butachlor-treated seedlings. Also, the peak of amylase activity was delayed for 48 hours. The crude enzyme from the control seedling was pre-incubated with butachlor to a final concentration at 100 ppm for 1 hour. Its activity was assayed to determine whether or not butachlor directly affects the amylase activity in the seedlings after treatment. Results indicate butachlor can not directly affect the amylase activity in seedlings (Table 1).

Changes of protease activity in the germinating barnyardgrass seeds:

The protease was extracted from the whole seedling and its activity was assayed by using BAPA (benzoylarginine-p-nitroanilide) or casein as substrate. The results are shown in Figs. 12 and 13. Fig. 12 shows the results of the casein substrate. The protease activity in the

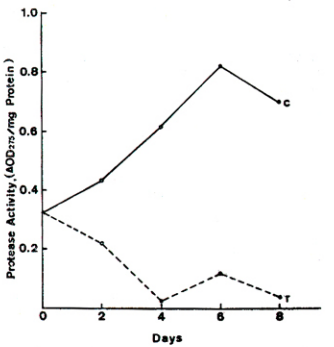

Fig. 12. Change of protease activity following germination with casin substrate. C: Control; T: Butachlor-treated.

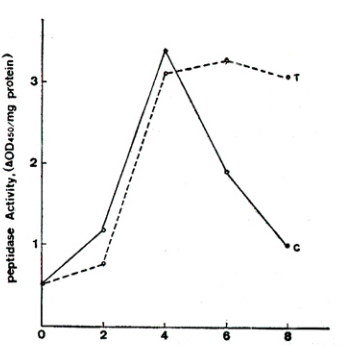

Fig. 13. Change of peptidase activity following germination using a BAPA substrate. C: Control; T: Butachlor-treated.

control seedlings increased gradually, reached its peak after 6 days and thereafter declined. The protease activity in the treated seedlings decreased for 4 days, increased for 4 days, increased slightly from the 4th to the 6th day, and then declined gradually. With BAPA as substrate (Fig. 13), the protease activity in the control seedlings increased rapidly after imbibition, reached its peak on the 4th day and then declined rapidly. The protease activity in the treated seedlings gradually increased for 2 days and then rapidly increased between 2 and 4 days. Activity seemed to level between the 4th and 8th day, showing no significant or decrease. These results indicate that at least two kinds of protease were present in the germinating barnyardgrass seeds, and only one protease using casein as substrate was inhibited by butachlor treatment. These two different kind of protease may play different roles in the early germination of barnyardgrass seed.

Effect of butachlor on polyribosomal level in imbibed seeds:

Ribosomes were preparad from 36 hour-imbibed seeds (both butachlor treated and untreated seeds) and then profiled in a sucrose density gradient. The results shown in Fig. 14 indicated the butachlor treatment had little or no effect on the attachment of ribosomes to mRNA.

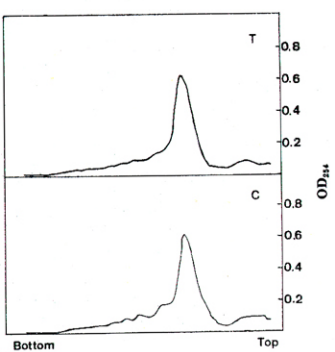

Fig. 14. The OD_{254} profiles of ribosomes on 15 to 50% sucrose density gridient. The ribosomes were isolated from 36-hour water (C) or 100 ppm butachlor (T) imbibed seeds and then layered on 15 to 50% sucrose density gradient. After centrifugation at 25,000 rpm for 150 minutes in a MSE model 65 SW 30 rotor, gradients were fractionated and scanned at 254 nm with an ISCO model UA-4 absorbance monitor.

DISCUSSION

Butachlor treatment did not inhibit barnyardgrass seed germination up to a concentration of 200 ppm, but it delayed the time of radicle emergence through the pericarp of seed for 12 hours. The typical morphological effects of butachlor on barnyardgrass seedling were: a) the emergence of primary leaves from the coleoptile; b) inhibition of root growth; c) inhibition

the development of lateral roots and root hairs. (Fig. 1, Fig. 2, Fig. 3, and Fig. 5). The inhibition on barnyardgrass seedling growth by butachlor was different from S-((4-chlorophenyl) methyl) diethylcarbamothioate (benthiocarb). Shibayama and Worley[24] reported that when benthiocarb was applied to barnyardgrass seedlings, it caused severe retardation of shoot growth, but had no effect on root growth and development. Dhillon and Anderson also reported that both the root and the shoot growth of oats and squash were reduced in proportion to propachlor concentration[7]. The development of lateral roots and root hair was also inhibited by butachlor. Poor development of root hair and lateral roots reduced the absorption of water and nutrients of seedling.

Germination is characterized by rapid water uptake which activates the metabolic system and faciliates the mobilization and the ultilization of reserve food for embryonic growth. The major reserve food in the barnyardgrass seed is starch. Starch is normally broken down by amylase. The butachlor treatment delayed the time of seed germination. Butachlor may inhibit the new synthesis of hydrolytic enzyme which are directly involved in the break down of reserve foods. Fig. 10 shows that butachlor treatment caused a significant inhibition on amylase activity by up to 57%. Penner and Ashton[21,22] have reported that the pretreatment of herbicide on barley seeds caused an inhibition of amylase activity of up to 50%; this treatment also cause severe inhibition of the seeding growth. Ashton *et al.*[2] reported that the degree of growth inhibition caused by herbicides was highly correlated with the degree to which they inhibited proteolylic activity. In their study, proteolytic activity in squash seedlings treated with allidochlor, a herbicide related to propachlor, was only 50% as high as the activity of the untreated control seedling. Fig. 12 and Fig. 13 show that butachlor caused a significant inhibition of protease activity, but the activity of peptidase was not affected. Penner and Ashton[21,22] have rorted that at least two proteinases capable of hydrolyzing casein are present in squash cotyledons. Proteinase A accounted for about 30% of the total proteinase activity of 3-day-old squash cotyledons and proteinase B accounted for about 70% of the activity. They suggested that two different types of proteinase may be controlled by two different mechanisms. The butachlor treatment reduced the proteinase activity greater than 70%. Butachlor may be acting at a site common to both[21,22].

It is interesting that the peptidase activity was not affected by butachlor treatment in the barnyardgrass seedlings. Beevers has reported that the proteolytic enzymes of germinating peas showed great diversity in reference to maximal activity in specificity for hydrolysis of casein or BAPA, and in their pH optima[3]. In most cases the extent to which the proteolytic enzymes degrade seed protein has not been investigated. It may be assumed that the proteolytic enzymes degrade storage protein to soluble nitrogenous compounds, which in turn are utilized by various parts of the seedling. Figs. 12 and 13 shows that protease enzymes may play a more important role in the degradation of storage protein than peptidase enzymes. The results shown in Fig. 14 indicate that the butachlor treatment had little or no effect on the attachment of ribosome to mRNA. These results are similar to the results of propachlor reported by Duke *et al.*[8,9]. They found that the primary mechan ism of action of propachlor on protein synthesis has an effect on nascent protein biosynthesis. Van der Wilden *et al.*[27] have reported that protein bodies contain many acid hydrolases and constitute the principal lytic compartment in the storage parenchyma cells. Because some herbicides, such as paraquat, a bipyridylium compound, can affect the perme ability or the entity of cellular membranes[13,14]. Whether or not butachlor can induce the autophagy and the breakdown of cellular orgenelles and macromolecules in the cell of treated seedlings is not known. In order to determine the mode of action af butachlor on the inhibition of growth of seedlings, further ultrastructure studies are necessary.

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