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Research Article

EFFECT OF HERBICIDES ON CARBOHYDRATE, PROTEIN AND ELECTROPHORETIC PROTEIN BANDS CONTENT IN *TRITICUM AESTIVUM* L

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ABSTRACT
The effect of two herbicides, singly or in combination, was studied on the three wheat varieties (HUW 234, HUW 468 and HUW 533). The wheat seeds were grown for 72h in different concentrations (0, 50, 100, 200, 400, 800, 1200 ppm) of herbicides 2, 4-Dichlorophenoxy acetic acid (2, 4-D) and Isoproturon (IPU) at normal laboratory temperature (25±2°C) and light condition. The parameters like carbohydrate (mg/g/FW), protein (mg/g/FW) content and seedling protein profile content were analyzed for the effect of two herbicides after 72h treatment and compared with control. The results indicated that both herbicides were able to reduce the carbohydrate and protein content gradually from lower to higher concentration of herbicides. It was observed the effect of herbicides in reduction of band intensity on electrophoretic protein band analysis. The electrophoretic protein band analysis showed the low molecular weight protein bands. The overall result showed that both herbicides had dose dependant effects on carbohydrate contents, protein contents and electrophoretic protein bands of wheat varieties.

Key Words: *Triticum aestivum L., 2, 4-D, IPU, Dose-dependant, Carbohydrate, Protein.*

INTRODUCTION

The life cycle of wheat (*Triticum aestivum* L.) is of short duration but the crop has multipurpose utility owing to its richness in carbohydrates, proteins, starch and vitamins. Wheat seeds represent an important source of food and energy.

The seed storage protein pattern is considered as the genotypic fingerprint. It is, therefore, used for several purposes such as plant variety protection, registration, certification, patents and as a breeding tool especially in flour quality breeding programs (Smith and Smith, 1992). Seed proteins are polymers, consisting of two or more subunits with different molecular weights, linked by inter or intra molecular hydrogen and disulphide bonds (Radic *et. al.*, 1997; Wall, 1979; Arslan and Ertugrul 2010). Shuaib et. al., (2007) reported that gluten proteins (>80%) occupy the endosperm in different wheat varieties and has two types of subunits: the low molecular weight (10,000-70,000 Da) and the high molecular weight (80,000-130,000 Da). The proteins of low molecular weight (LMW) and high molecular weight (HMW) are highly polymorphic and usually used for the cultivar identification in hexaploid and tetraploid wheat (Payne *et. al.,* 1984). Wheat seed storage proteins might be classified and recognized on the basis of water and ethanol solubility: albumins (soluble in water and dilute buffers), globulins (not soluble in water but soluble in saline solutions), major protein groups prolamins (soluble in hydrated ethanol 70-90%) and glutelins (soluble in dilute acids and dilute alkaline solutions).

The wheat varieties differ in quantity, quality and groups of proteins. The gliadins and Low Molecular Weight (LMW) glutenins are responsible for dough viscosity while High Molecular Weight (HMW) glutenins are responsible for dough elasticity and strength (Radic *et. al.*, 1997).

The widespread application of herbicides for protecting crops against weeds attack is an important part of modern agriculture. The repeated use of herbicides for controlling the weeds may act on the crop in field side by side. However it depends on the period of growth, doses level as well as environmental factors. A number of investigations had showed the side effects of the herbicides on the hereditary material of different cells. Other investigations were carried out to indicate the relation between mitotic changes in nucleic acid and protein content as a result of treatment with pesticides and herbicides (Ebad et. al., 1993; Soliman and Ghoneam, 2004).

Herbicides are unintentionally toxic substances because of their utilization purpose. The most obvious side effects of these toxicants are: more or less effects in growth and metabolism in plant species, changes in abundance of species, diversity in ecosystem and reduction in productive potential of natural resources and non-target species. These effects range from a slight growth inhibition to deleterious morphological disturbances for whole plant or at particular organ (Khan *et al.*, 2006). It may cause deleterious alteration in cell division, cell enlargement and tissue differentiation (Yin et al 2008).

Earlier studies on carbohydrates in treated leaves and exudates from excised leaves suggested that carbohydrate accumulation in leaves might be caused by an impairement of phloem loading (Kim et. al., 1997; Zabalza *et al.*, 2004). Royuela *et al.*, (2000) suggested that carbohydrate accumulation in leaves might be caused by decrease in sink strength. Coble and Slife, (1971) showed the carbohydrate reduction in *Ampelamus albidus* Nutt. Britt. after three days foliar application of 2, 4-D. Alvi *et al.*, (2003) reported the reduction in carbohydrate and protein of *Vigna radiata* L. after application of atrazine herbicide.

The application of 2, 4-D at jointing stage of wheat and barley showed rapid reduction in sugar and proteins (Pellett and Saghir, 1971). Bovey and Meyer, (1981) reported that 2,4-D and 2,4,5-T affected the protein content of the wheat. Chlorsulfuron and sulfometuronmethyl reduced the synthesis of amino acids valine, leucine and isoleucine by blocking the acetolactate synthase pathway in *Pisum sativum*, *Phaseolus vulgaris*, *Vicia faba*, soyabean cell suspension culture and *Salmonella typhimurium* (Fayez and Kristen, 1996; Larossa and Schloss, 1984; Scheel and Casida, 1985). The herbicide EL-107 or isoxaben (N-[3-(1-ethyl-1-methylpropyl)-5-isoxazolyl]-2, 6-dimethoxybenzamide) at higher concentration (10^{-4}M) inhibited the protein synthesis , glucose biosynthesis, cytotoxic and cell wall biosynthesis in *Acer pseudoplatanus* L. (Lefebvre et. al., 1987). The N-(2,6-Diethylphenyl)-N- nutoxymethyl 2-chloro-acetamide inhibits the protein synthesis in the rice plant (Omokawa et. al., 1988). A extreme higher concentration (10^{-3}M) of 2,4-D or 2,4,5-T leads to the death of bean and sunflower plants or decrease growth in maize plants by occurrence of abnormalities in the nucleic and protein synthesis (Shaddad et. al., 1990). The herbicides like benifin, dinitroamine and nitralin decrease the total protein content in soybean under field condition (Durgesha, 1994). Khan et. al., (2004) suggested that herbicides when applied indiscriminately had variable effects on the legume production. The above mentioned morphological changes and hazardous impact resulting herbicides to disturb in physiological processes and metabolic pathway in plants.

Among biochemical techniques SDS-PAGE is widely used for its simplicity and effectiveness for describing the genetic structure of crop germplasm (Siddiqui and Naz, 2009). The electrophoretic analysis of the protein provides information concerning structural genes and their regulatory system that control the biosynthetic pathway of that protein (Hassan, 2000). Electrophoretic SDS protein profiles were successfully used to establish biochemical and genetic finger printing of many plants (Badr, 1995; Soliman and Ghoneam, 2004). Payne et. al., (1981) reported that the HMW glutenin subunits related to the wheat quality. The LMW glutenin protein subunits might be improved by use of one dimensional gel electrophoresis (Gupta and Shepherd, 1990; Singh et. al., 1991).

The main component of the cereal crop wheat has carbohydrates and proteins. Therefore, the wheat seeds were treated with the herbicides 2, 4-D and IPU singly or in combination for 72 h and studied the effect on carbohydrate content, protein content and protein profiling bands.

MATERIALS AND METHODS

The seeds of wheat varieties (HUW 234, HUW 468 and HUW 533) were surface sterilized with 0.5% of $HgCl_2$ (w/v) for 10 min followed by washing with distilled water thrice to remove the traces of $HgCl_2$. The filter papers were cut into round strips (90 mm). The strips were kept in petridishes (size 90 mm dia.) and soaked with different concentrations (0, 50, 100, 200, 400, 800, 1200 ppm) of herbicides 2, 4-D and IPU singly or in combination. In each, petridish 10 seeds were kept and maintained normal laboratory light condition and temperature (25±2^{o}C) for 72 h. After 72 h of incubation the amount of carbohydrate and protein content in the seedlings were studied. The experimental design was completely randomized and all the experiments were repeated thrice.

Total soluble Carbohydrate analysis

The carbohydrate content was measured by the anthrone reagent method with some modifications (Drywood, 1946). 0.2% anthrone reagent was prepared in conc. sulphuric acid and standard glucose solution was prepared (1mg/ml). 1g seedlings from each treatment and control were grinded in chilled mortar and pestle with 80% ethanol (chilled), the solution were filtered by whattman No. 1 filter paper. The residues on the filter paper were washed with 80% ethanol (chilled) and regrinded. Again filtered and filtrates were combined (residue is discarded) and the volume was made upto the 50 ml by adding 80% ethanol (chilled). The centrifugation was done at 3000rpm for 15 minutes to obtain a clear supernatant.

In 1 ml supernatant, 4 ml reagent was added mixed well and kept for 10 m at room temperature (RT) and optical density was taken at (620 nm) against reagent blank. Standard graph plotted with glucose standard was used to measure the amount of carbohydrate and recorded. The carbohydrate content was expressed in mg/g/FW.

Total soluble Protein analysis

In second part 1g growing seedlings from each treatment and control were grinded in chilled mortar and pestle with phosphate buffer (0.1M, pH 6.8). Samples were centrifuged at 5000 rpm for 15 min and supernatant were collected. 5 ml TCA (10%) was added in the supernatant and boiled in the water bath for 3 min. Again centrifugation was done at 5000 rpm for 15 min. The supernatant was collected and added with 5 ml of NaOH (0.1N). The homogenate was used for protein estimation by the Lowry et. al., (1951) method.

In 1 ml of the homogenate, 5 ml of the alkaline solution (5% Na_2CO_3 and 0.5% CuSO4 in 1% Sodium Potassium Tartarate) was added. The mixture was incubated for 10 min at RT. The Folin's reagent (50% diluted) was added with equal amount of the mixture and incubated at 37 ^{0}C for 30 min. The OD was measured at 650 nm.

The standard protein (BSA, 1mg/ml) was used for standard curve. The protein content was measured and expressed in mg/g/FW.

SDS-PAGE Protein profiling

The seedlings of wheat varieties were selected from the treatment concentrations (0, 400, 800, 1200 ppm) of herbicides after 72 h of incubation. The seedlings were weighed (1 g) and the amount of seedling storage protein was analyzed by one dimensional vertical mini gel electrophoresis. The experimental design was completely randomized.

RESULTS

Total soluble carbohydrate content

The maximum amount of total soluble carbohydrate (Control) in three varieties HUW 234, HUW 468 and HUW 533 was 250.17±0.888mg, 245.00±0.567mg and 248.09±0.871mg after 72h growth respectively. The total soluble carbohydrate exhibited a declining trend corresponding to the increasing concentration of the herbicides and their combinations (Fig. 1-3).

2,4-D treatment:

In comparison to the control, at lower concentration, soluble carbohydrate declined to about 1 fold and value obtained were for the HUW 234 (170.58±0.900mg) followed by the HUW 533 (158.77±0.829mg) and HUW 468 (147.00±0.793mg) at 50 ppm concentration while the maximum inhibition in carbohydrate was recorded in HUW 533 (54.58±0.949mg) followed by the HUW 468 (58.83±0.761mg) and HUW 234 (62.55±0.713mg) at 1200 ppm respectively. The difference in values between lowest and highest concentration was 3 fold decreases in carbohydrate content (Fig. 1).

IPU treatment:

In comparison to the control, the maximum value of soluble carbohydrate treated was obtained for the HUW 234 (192.28±0.907mg) followed by the HUW 533 (185.58±0.883mg) and HUW 468 (172.49±0.700mg) at 50 ppm concentration while the rate of inhibition enhanced and was recorded in

HUW 468 (50.22±0.697mg) followed by the HUW 533 (52.58±0.541mg) and HUW 234 (55.02±0.808mg) at 1200 ppm (Fig. 2).

2,4-D+IPU treatment:

In comparison to the control, in treated plants inhibition of soluble carbohydrate was apparent and values for HUW 234 (189.00±0.746mg) followed by the HUW 533 (180.52±0.766mg) and HUW 468 (172.77±0.645mg) at 50 ppm concentration was obtained while the maximum inhibition in values were recorded in HUW 468 (58.93±0.938mg) followed by the HUW 533 (64.31±0.825mg) and HUW 234 (71.28±0.821mg) at 1200 ppm (Fig. 3).

Total soluble Protein content

In control the maximum mean total soluble protein content in three varieties HUW 234, HUW 468 and HUW 533 obtained after 72 h was 172.03±0.554mg, 169.45±0.927mg and 170.19±0.858mg respectively. In general with increase in herbicide concentrations (both singly and in combination) the total soluble protein exhibited a declining trend (Fig. 4-6).

2,4-D treatment:

In comparison to the control, the maximum soluble protein was obtained for the HUW 234 (112.49±0.801mg) followed by the HUW 533 (106.89±0.638mg) and HUW 468 (102.45±0.877mg) at 50 ppm concentration that showed slight decrease than control, while the maximum inhibition in protein amount was recorded in HUW 468 (30.75±0.973mg) followed by the HUW 234 (35.22±0.996mg) and HUW 533 (36.39±0.948mg) at 1200 ppm (Fig. 4).

IPU treatment:

In comparison to the control, the maximum soluble protein was obtained for the HUW 533 (116.73±0.569mg) followed by the HUW 468 (111.52±0.826mg) and HUW 234 (103.34±0.887mg) at 50 ppm concentration and showed almost parallel decrease to previous one, while the maximum decrease was recorded in HUW 468 (34.44±0.741mg) followed by the HUW 533 (43.85±0.691mg) and HUW 234 (49.50±0.598mg) at 1200 ppm (Fig. 5).

2,4-D+IPU treatment:

In comparison to the control, the amount of soluble protein was obtained for the HUW 234 (132.49±0.811mg) followed by the HUW 533 (127.50±0.945mg) and HUW 468 (123.32±0.536mg) at 50 ppm concentration, While the maximum decrease was recorded in HUW 468 (41.04±0.564mg) followed by the HUW 533 (47.24±0.786mg) and HUW 234 (52.12±0.654mg) at 1200 ppm (Fig. 6).

Overall, result indicates that the herbicides (2, 4-D and IPU) alone or in combination have mixture effect on the wheat varieties in terms of total soluble protein and carbohydrate content (Fig. 1-6). The Duncan test ($p \leq 0.05$) was performed for the total soluble protein and carbohydrate content and F-values were found significant (Table 1).

SDS-PAGE Protein profiling

The electrophoretic analysis of protein extract from treated (400, 800 and 1200 ppm) and control samples after 72 h treatment with herbicides were illustrated in Figure 7. The varieties were used to see the effect of herbicides 2, 4-D on the banding patterns of protein in the seedlings. SDS-protein molecular weight marker (PMWM) for 12% gel was used to determine the molecular weights of the protein band analysed. Most of the protein bands were of low molecular weights (LMW) and ranged from 10-65 kDa.

Electrophoretic analysis of protein extracts showed variations among the studied samples. Each sample exhibited a distinctive electrophoretic pattern. The observed changes were both qualitative and quantitative and may be illustrated by disappearance of some bands and changes in peak and amount intensity of some bands (Fig. 7).

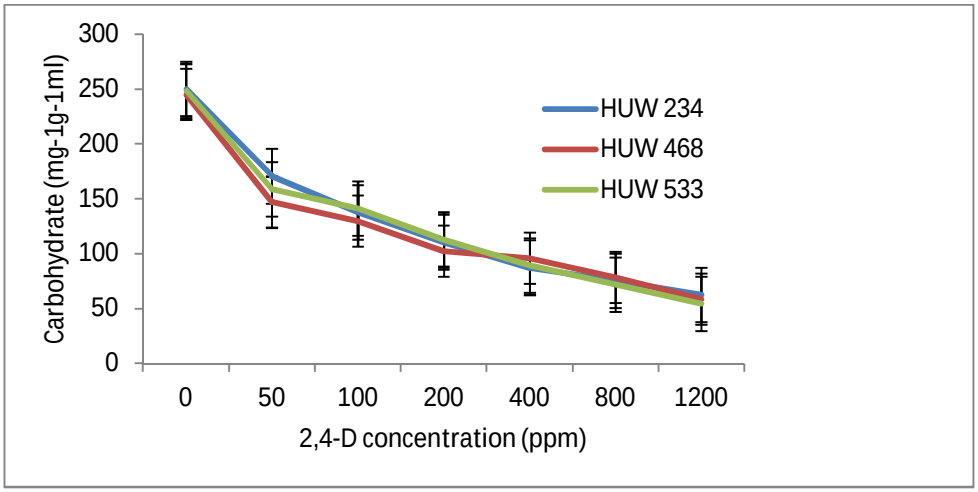

Figure 1: Effect of herbicide (2, 4-D) on Carbohydrate content of wheat

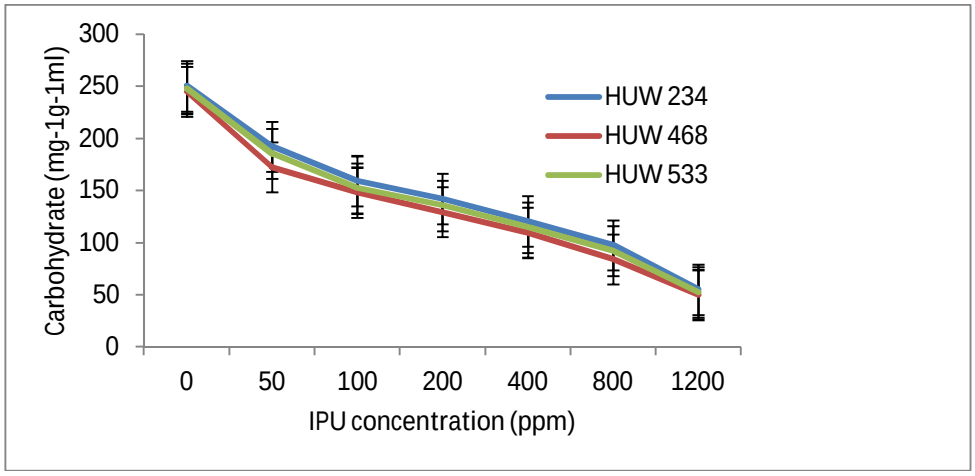

Figure 2: Effect of herbicide (Isoproturon) on Carbohydrate content of wheat

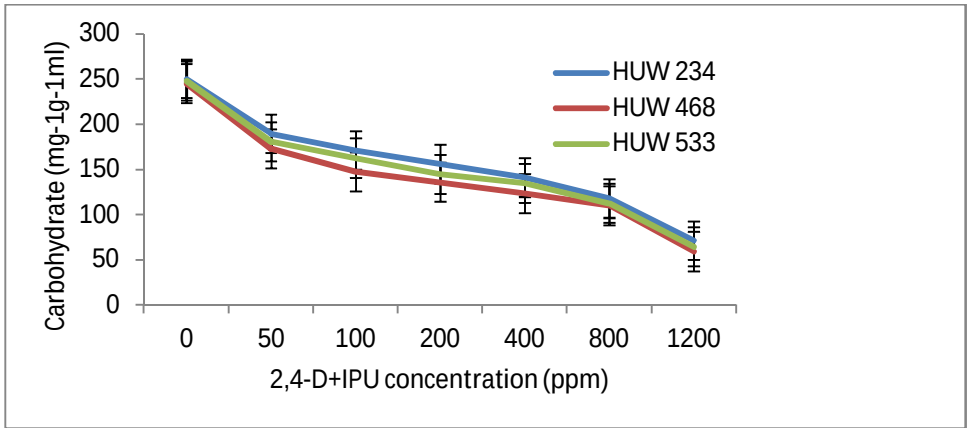

Figure 3: Effect of herbicides combination (2, 4-D+ Isoproturon) on Carbohydrate content of wheat

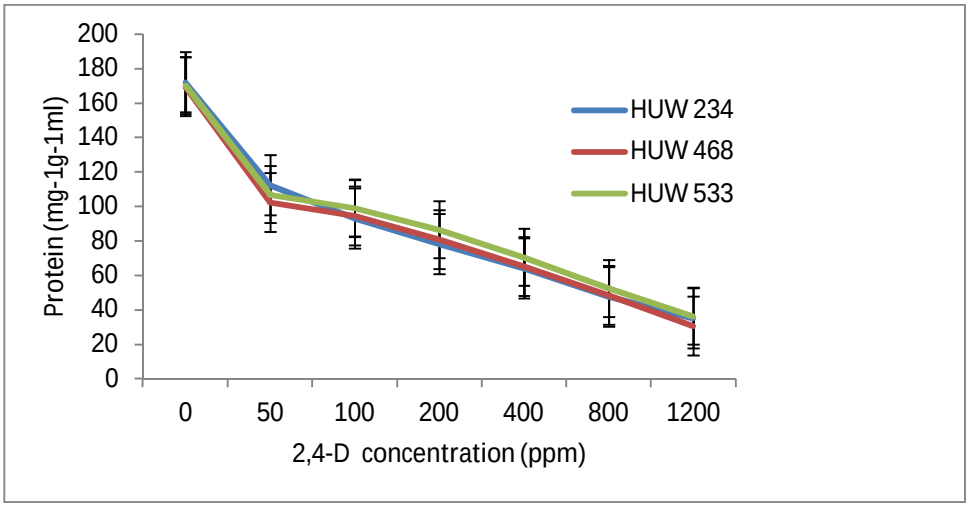

Figure 4: Effect of herbicide (2, 4-D) on Protein content of wheat

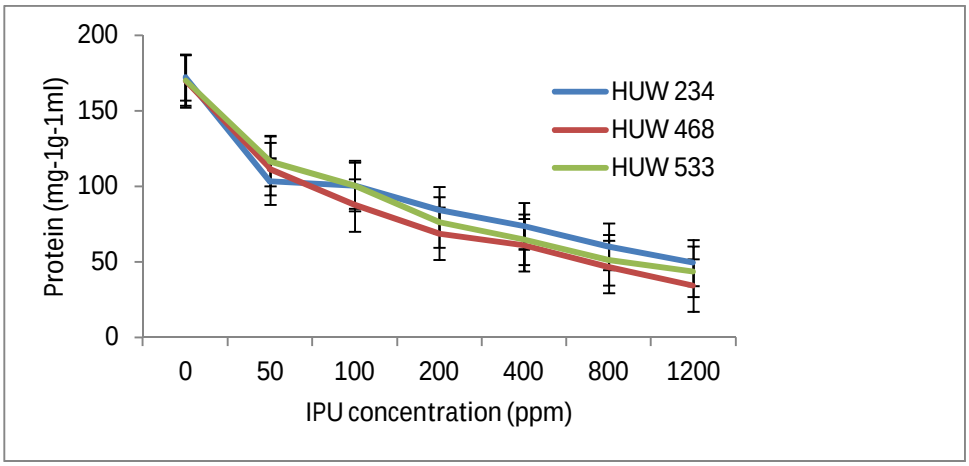

Figure 5: Effect of herbicide (Isoproturon) on Protein content of wheat

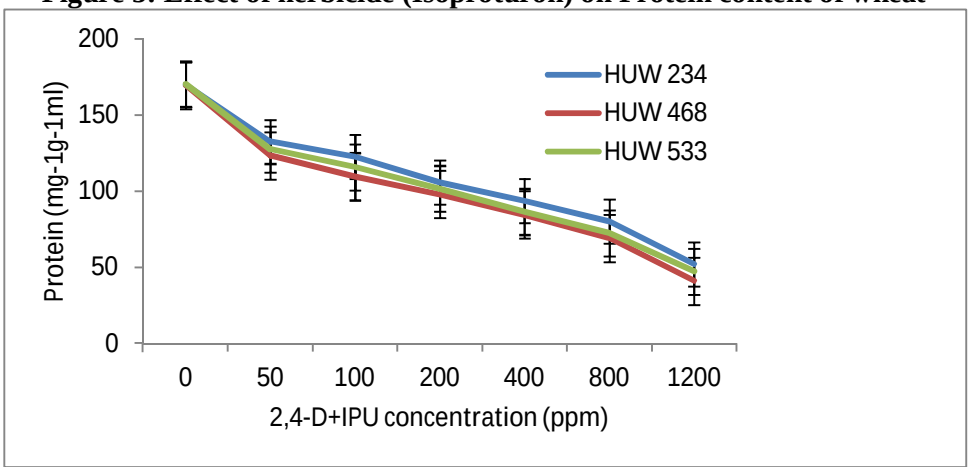

Figure 6: Effect of herbicides combination (2, 4-D+Isoproturon) on protein content of wheat

Table 1: Analysis of one way Anova for gradual decrease in Carbohydrate and Protein content by application of different herbicides

ANOVA (F-value, P≤0.05)						
Triticum aestivum L. var.	Carbohydrate			Protein		
	2,4-D	IPU	2,4-D+IPU	2,4-D	IPU	2,4-D+IPU
HUW 234	5382.378	7484.664	5651.499	3130.800	3101.971	2844.603
HUW 468	6000.518	8637.165	6193.633	2597.440	2951.004	2927.519
HUW 533	6788.405	6471.704	5840.071	2492.640	3050.425	2378.576

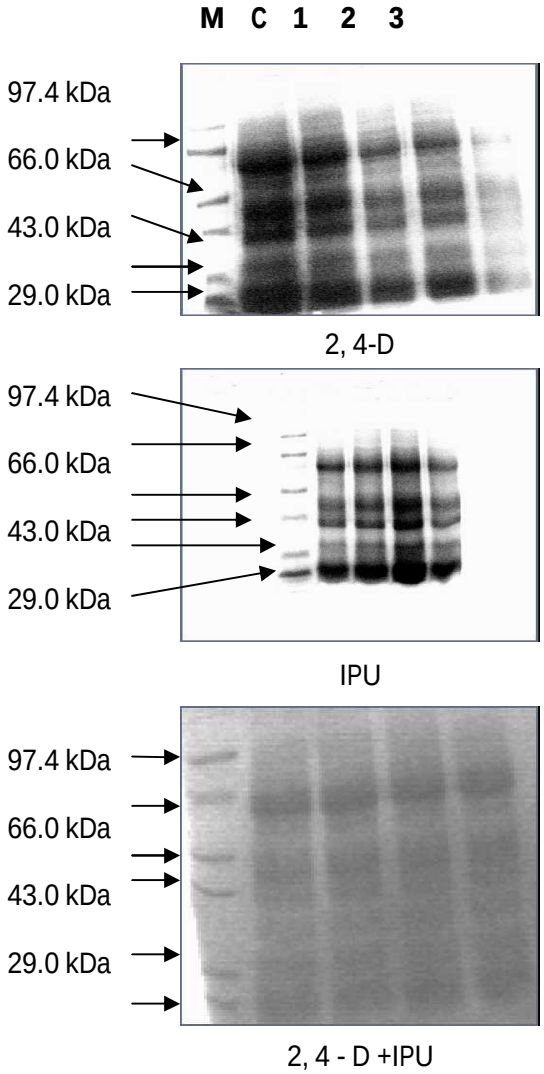

Figure 7: SDS-PAGE for protein bands after application of different herbicides wheat (M= Molecular Marker, C= Control, 1=HUW 234, 2=HUW 468, 3=HUW 533)

DISCUSSION

The effects of herbicides were known by the type and rates of its application, health and stage of plant growth, and other environmental variables. It has been established that there is morphological changes and disturbances in cell division due to impact of both the herbicides and their combinations (Shamsi et al 2006, Qasem 2007, Singh 2007, Kumar 2010, Kumar et al., 2010).

The low value of carbohydrate content at the maturation after application of herbicides indicated the possibility of depletion of carbohydrate reserve in roots (Fig. 1-3). It may be counted major depletion of carbohydrate reserve starts from the 72 h application or 3rd day foliar application of the herbicides (Abo-El-Seoud and Frost 1998).

Egli *et. al.,* (1985) reported that many herbicides interfere with the protein synthesis, for instance, atrazine or diuron inhibits the synthesis of protein in *Solanum nigrum* cell suspension. This might be the unspecific disruption of the cell metabolism by large amounts of the applied exogenous compounds (Fig. 4-6). The direct effect of herbicides on protein or nucleic acid synthesis have not yet been discovered, probably because neither of these sites are primary site of action of any commercial herbicide (Khan et. al., 2006).

Electrophoretic study revealed the appreciable polymorphism in the number and mobility of low and high molecular weight proteins in the wheat (Fig. 7). It has been suggested that damage in protein, deletion or addition of the some proteins in repetitive region might be the reason for this variations (Benmoussa et. al., 2000). The used herbicides caused disappearance of some bands in the *T. aestivum* L. The disappearance of electrophoretic bands could be attributed to the loss of the genetic materials due to fragmentation and laggards found in the present study (Ghareeb, 1998; El-Nahas, 2000; Al-Muraikhi, 2000, kumar 2010). Hassan (2000) reported that absence of some bands to the deletion of their corresponding genes. The herbicides used in this investigation caused changes in band intensity, these changes in band intensity could be explained on the basis of induction of gene mutation at the regulatory system which modulates or enhances transcription rate of a particular structural gene (Grist et. al., 1992). This leads to the production of faint or over expressed protein bands (Barakat and Hassan, 1997). The recorded changes in band intensity could be attributed to the cytological abnormalities induced by herbicides (Shehata *et al.,* 2000). The increase in band intensity could be interpreted on the base of gene duplication which is a result of cytological abnormalities (Soliman and Ghoneam, 2004).

Qualitative and quantitative effects are defined when one band disappears or new one appear and when a noticeable change in the band intensity is observed respectively (Bonfil et. al., 1997). Environmental conditions influence quantitatively the seed storage protein composition of cereals and legumes. These conditions include climatic factors such as precipitation, temperature and heat shock (Benzian and Lane, 1986; Blumenthal *et al.,* 1993) and local conditions such as soil type and structure, water content and mineral nutrition (Bunker et. al., 1989; Kirkman et. al., 1982; Lookhart and Pomeranz, 1985; Moss et. al., 1981; Rahman *et al.,* 1983; Randall *et al.,* 1979; Shewry *et al.,* 1983; Smika and Greb, 1973, Crespo-Corral 2008, Chou *et al.,* 2009, Brutti *et al.,* 2010, fang *et al.,* 2010, Mansilha *et al.,* 2010). Environmental conditions donot usually have a qualitative effect on the expression of the seed storage protein (Bonfil *et al.,* 1997). According to Shewry *et al.,* (1995), many gaps still remains in our knowledge of seed storage protein processes such as folding, assembly, transport and deposition.

The present study indicated the qualitative and quantitative differences of molecular weights as well as in the intensity and number of bands in the wheat varieties (Fig. 7). It might be due to almost all proteins are soluble in SDS and the resulting protein SDS-complexes are of high negative charges. The hydrogen bonds split up and by reducing the sulphur bonds the proteins may be dissolved into low and high molecular weight subunits and the electrophoretic separation of the protein subunits might be depends on only molecular weights. Seed storage protein composition is determined by the genotype (MacRitchie et. al., 1990). The quantitative and qualitative differences in seed storage protein composition were observed upon exposure of wheat to herbicides. All the differences observed were demonstrated by simple one dimensional separation (SDS-PAGE). Therefore, herbicide effects were based on the differences in the

High Molecular Weight (HMW) and Low Molecular Weight (LMW) protein bands. The variations in the protein patterns of herbicides treated seedlings might be assumed to result from changes in the gene expression. Thus, the herbicide treatments applied in the present work are presumed to affect the protein patterns at one or more of the above mentioned points.

The death of many plants treated with herbicides not only with the depletion of carbohydrates and changes in the protein metabolism but many factors are involving like reduction in photosynthetic capacity, ion absorption, phloem proliferation leading to interference with solute transport, rupture of root cortex leading to soil pathogen invasion, abnormal growth in stem axis etc (Laghari et al 2011, Marwat et al 2011, Dawar et al 2011). However, magnitudes of decline depend on harvest time after spraying.

The effects of herbicide application on carbohydrate and protein content and protein profiling of wheat seedlings were demonstrated in Figure 1-7 and Table 1. The protein and carbohydrate content in the seedlings of the wheat was found to be affected by the highest dose rate of the 2,4-D and IPU, suggesting that cell metabolism, nucleic acid synthesis, enzymes, other functional proteins are one of the target sites of herbicide action. This subsequently leads to the alteration in the protein metabolism of the seedlings. The reduction of carbohydrate and protein contents might be the result of reduced synthesis and possibly due to damage of root or vascular tissues. The noticeable decrease in carbohydrates and protein content of wheat seedlings in relation to applied herbicides might be due to the disturbing influence of such toxicants on enzymes involved in metabolic pathway of above mentioned components and supported the various reports (Shams-El-Din *et al.,* 1995; Perveen *et al.,* (2002), Hussain *et al.,* 2003, Zabalza *et al.,* 2004, Song et al., 2007, Yin *et al.,* 2008, Hasaneen *et al.,* 2009, Rosenbom *et al.,* 2010, Wang *et al.,* 2010, Wang *et al.,* 2010, Kumar and Singh 2010, Hala *et al.,* 2011, Morais *et al.,* 2011).

Conclusion

Genetic diversity is the basis for successful crop improvement and can be estimated by different methods such as morphological traits, quality traits and molecular markers (Fufa *et. al.,* 2005). The differences in quality between varieties are correlated with allelic variations in the composition of seed storage proteins (Bonfil *et. al.,* 1997). The seed storage protein profiles could be useful markers in cultivar identification, registration of new varieties, pedigree analysis, studies of genetic diversity, classification of adapted cultivars and improving the efficiency of wheat breeding programmes in cultivar development.

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