Int.J.Curr.Microbiol.App.Sci (2017) *6*(11): 2045-2064

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 6 Number 11 (2017) pp. 2045-2064**
Journal homepage: http://www.ijcmas.com

Original Research Article https://doi.org/10.20546/ijcmas.2017.611.243

Evaluation of Oxidative Stress on Soybean (*Glycine max* L. *Merrill*) and Wheat (*Triticum aestivum*) Crops in Response to Chlorpyrifos

Nutan Singh*, Pratibha Katiyar and Ajay

Department of Plant Physiology, Indira Gandhi Krishi Viswavidyalaya, Raipur-492012, Chhatishgarh, India
**Corresponding author*

Keywords

Oxidative stress on soybean, Wheat, Crops.

Article Info

Accepted:
17 September 2017
Available Online:
10 November 2017

A B S T R A C T

Study aim to evaluate the effect of chlorpyrifos on several metabolic and stress related parameters associated with pesticide resistance. The experiment was conducted at Indian Institute of Soil Science (ICAR), Bhopal (MP) during 2015-2016, comprising four concentrations of pesticide chlorpyrifos viz. 0%, 0.25%, 0.75% and 1.25%. The commencement of foliar treatment applied on vegetative phase *i.e.*, 30, 45 and 60 days after sowing on soybean (Kharif) and wheat (rabi). The morphological, physiological and biochemical characteristics of soybean and wheat were recorded after various doses of pesticide treatment application. It was observed that the increased rate of pesticide treatment significantly decreased the morphological parameters *i.e.*, plant height, number of leaves, leaf area leaf dry weight, stem dry weight, root dry weight. The rate of photosynthesis, transpiration, stomatal conductance decreased with increased concentration of pesticide in soybean and wheat. The peroxidase, polyphenol oxidase activity and structural carbohydrate decreased with increasing level of pesticide treatment in both the experimental crops. Whereas, nitrate reductase, soluble protein and proline content increased with increasing levels of pesticide treatments. However, the increase in soluble protein and protein content was more in wheat as compared to soybean and also shown more resistance towards the oxidative stress.

Introduction

The use of synthetic pesticides as crop protection chemicals has become the most accepted ecological weapon for assured crop production. With the restricted use of most of the organochlorine insecticides, the organophosphorus compounds are taking the major share of insecticide consumption in India (Aditya *et al.*, 1997). Chlorpyrifos [*O,O*diethyl *O*-(3,5,6-trichloro-2-pyridyl) phosphorothioate] is a broad-spectrum organophosphate insecticide being used for more than a decade to control foliar insects that affect agricultural crops, to reduce pod damage (Khan *et al.*, 2009, Kumar *et al.*, 2010), and subterranean termites (Venkateswara Rao *et al.*, 2005). Chlorpyrifos produces hazardous effects on the environment when it is applied directly on plants or mixed with soil (Howard 1991). Soybean (*Glycine max L. Merrill*) is an important pulse as well as oilseed crop. It has become wonder crop of twentieth century and is often designated as "Golden bean." And Wheat (*Triticum* spp.), the world's most

widely cultivated crop, in 2000, world wheat production was approximately 572 million metric tons on 205 million hectares (Anonymous 2002, Stoskopf 1985). Wheat is the staple food for about 40% of the world's population (Wiese 1987). Common bread Wheat (*T. aestivum*, L.) and durum Wheat (*T. durum* Desf.) make up 90% of the world's Wheat crop. Wheat is further classified as winter or spring, hard or soft, red or white, and by protein content (Briggle and Curtis 1987). In the process of development of agriculture, pesticides have become an important tool as a plant protection agent for boosting food production.

Materials and Methods

A pot experiment will be laid out in control condition in completely Randomized Block Design at Indian Institute of soil science, Bhopal. Lab work will be conducted in laboratory of Indian Institute of Soil Science.

Description of crop: Soybean and Wheat
Soybean genotype: JS 9305
Wheat genotype: HD 2987

Physiological parameters were measured using standard procedures. The following parameters were studied in the present study.

Morphological parameters

Plant height

Plant height was recorded from base of the plant to the uppermost node of main shoot of plant at 30 days after showing (DAS) and 45 days (DAS) and 60 days (DAS). In each site, one plant was selected and height was expressed in cm.

Plant biomass

The selected plant was removed from the plastic container. The whole plant was divided into leaves, stem and roots and then weight of leaves and stem was measured for fresh weight. The samples were dried in oven for 72 hrs at 65°C and total dry weight (expressed in gram) of leaves and stem was recorded for dry weight.

Root biomass

The whole root was weighed immediately for fresh weight and dried in oven for 72 hrs at 65°C and total dry weight (expressed in gram) of root was recorded for dry weight.

Leaf area

Leaf area was measured by leaf area meter LICOR make (Model 3100) and expressed in cm^2.

Physiological parameters

Estimation of gaseous exchange parameters

Gas exchange parameters viz. Photosynthesis rate (μMCO_2 m^{-2} s^{-1}), transpiration rate (MmH_2O m^{-2} s^{-1}) and stomatal conductance (μM m^{-2} s^{-1}) were recorded in the morning (9 to 11:30 AM) in the experimental plant leaves using Photosynthesis system (make: PP systems and model: CIRAS-3).

Biochemical parameters

Peroxidase (POX) activity assay (ΔODμg protein^{-1} min^{-1})

POX activity was assayed by following the method of summer and Gjessing, (1943). The extract from SOD Assay was used for POX assay. Added 1ml O-dianisidine (0.01M in methanol), 0.5 ml H_2O_2 (0.02M), 1 ml phosphate buffer, 2.4 ml distilled water and 0.2 ml enzyme and incubated at 30^0 C for 5 min. The reaction was stopped by adding 1ml 2N H_2SO_4. Blank tube excluding H_2O_2 was

prepared by adding 0.5 ml distilled water. The colour intensity was measured by spectrophotometer at 430 nm wavelength and POX was expressed in unit (ΔODμg protein^{-1} min^{-1}).

Estimation of nitrate reductase activity (μgNO_2/g fw/h)

Nitrate reductase can be estimated by the in vivo assay method given by Nicholas and Nason (1957), by incubating pieces of plant tissues as such in KNO_3 solution. The amount of nitrite formed is estimated as a measure of the enzyme activity. Leaves were cut into 2-3mm fragments and suspended in 5ml of phosphate buffer containing 0.1M KNO_3 placed in 25ml beakers. The beakers were placed in a vacuum desiccator and tissues were vacuum infiltrated. The beakers were incubated in a water bath at 30°C for 30 min. 2.5ml of sulphanilamide-HCl was added to estimate the nitrite formed in suspending medium.

The mixture was filtered through Whatman No.1 filter paper to remove the leaf fragments and 2.5ml of NEDH was added. The absorbance of solution was measured at 540 nm in a colorimeter. The results were expressed as μmol NO_2- formed per 30 min per g fresh tissue.

Estimation of polyphenol oxidase (ΔODμg protein^{-1} min^{-1})

Polyphenol oxidase was measured by following method given by Fujita (1979).

0.2 M Acetate Buffer pH 4.5
0.2 M phosphate buffer pH 6.0
1% pyrogallol solution

Acetate buffer: for 1 liter = 3.5ml CH_3COOH + 3.2g Sodium Acetate Phosphate Buffer: For 1 liter = KH_2PO_4 11.93g + K_2HPO_4 2.14g

Method: leaves sample 0.2g + 10ml acetate buffer → 10min incubation.1 ml supernatant 2 ml phosphate buffer 0.5ml pyrogallol→ OD at 495nm.

Total soluble protein assay

Total soluble protein was measured by following method given by Bradford (1976). Used the extract for assay and added 0.2 ml sample, 4 ml 0.1% protein reagent (100 mg Coomassie Brilliant Blue) and 0.8 ml distilled water. The colour intensity was measured by spectrophotometer at 595nm wavelength. Calculation of the total soluble protein content was done by creating a standard curve using a standard bovine serum albumin (25 mg in 0.15 M NaCl and made up to volume 25 ml stock and working stock was made by diluting 10 times) and was expressed in mg per gram fresh weight (mg g^{-1} FW).

Estimation of non-structural carbohydrate (mg/g)

Non-structural carbohydrate was calculated as sum of total soluble sugar and starch and expressed as percentage of dry weight.

Estimation of proline content (μmg^{-1})

Proline content was measured following the method of Bates *et al.,* (1973). 0.5 gram of fresh plant sample (leaves) was taken and 10ml of 3% aqueous sulphosalicylic acid added and ground in pestle and mortar, and then filtered through whatman No. 42 filter paper.

Statistical analysis

CRD (Completely randomized design) was used to statistical analysis. The analysis was performed online in OPSTAT (Online statistical analysis tool) analysis. The critical difference (C.D.) was calculated at 5% levels.

Results and Discussion

Morphological parameter

Plant height (cm)

The plant height of soybean, and wheat were evaluated at 30 days, 45 days and 60 days after sowing the pesticide chlorpyrifos were sprayed (by Trigger type hand spryer) and having the nozzle (soild cone) one week before the observation recorded. Hence the oxidative stresses were applied three days in all experimental crops. It was observed that the plant heights were not affected significantly in all the experimental crops except in wheat at 60 DAS. However, the plant heights of all the experimental crops were higher in control (no chlorpyrifos). The plant height is decreased with increasing concentraiont of chlorpyrifos in soybean and wheat. However, there was non-significant variation within treatments (Table 1; Fig. 1-2).

The similar impact were observed by Kumar and Kumar (1993) in *Vicia faba* by the use of metasystox @50-100 mg/l. The higher concentration 200-300mi/l were found to have inhibitory effect on plant height, no. of branches, no of leaves per plants, total leaf area and plant biomass. The similar results were confirmed by Bashir *et al.,* (2007) in *Lens culinaris* L. with mancozeb. Stevens *et al.,* (2008) in rice with use of imidacloprid. They observed the lower dose of dimethoate (50ppm) proved stimulant in growth of rice.

The suppression in morphological parameters might be due to the retarded cell growth and division in root, cell elongation and conversion of Indole-3- acetic acid IAA into various photooxidative products. Tevini and Teramura (1989) suggested that these compounds function as strong auxin antagonists. Another reason could be explained on the basis of inhibition in the activity of 4- hydroxyl phenyl pyruvate dioxygenase (HPPD), an enzyme needed for the growth and development of meristematic tissue as suggested by Luscombe *et al.,* (1993) following pesticide isoxaflutole treatment in maize and sugarcane.

Leaf area (cm^2)

Leaf area of experimental crops was significantly affected with chlorpyrifos doses. However, the maximum increase in leaf area was observed at lower dose of pesticide in wheat (41.66) over control (39.03) where as in soybean increased concentration of pesticide decreased the leaf area gradually (Table 2).

Kasyap and Kumar (2013) also reported that the lower concentration (50ppm) of chlorpyrifos significantly proved highly effective, non-phytotoxic and seemed to elevate growth parameters. Kumar and Kumar (1993) also confirmed the similar impact were observed by on *Vicia faba* by the use of metasystox @50-100 mg/l. The higher concentration 200-300mi/l were found to have inhibitory effect on plant height, no. of branches, no of leaves per plants, total leaf area and plant biomass.

Leaf dry wt. (g)

Significant impact of chlorpyrifos treatment was observed in dry weight of leaf in soybean and wheat at 30 and 60 DAS. The increased concentration significantly decreased the dry weight of leaves (Table 3).

Siddiqui *et al.,* (1997) have reported that usage of low dosage of Methyl thiophenate (fungicide) treatment in *Sesbania sesban* has been found to cause an increase in fresh and dry weight, where higher dosages have been found to be deleterious. Though most of the

systemic fungicides are used as seed dressing, yet there are several reports showing phytotoxicity on various plants. Similar facts were observed by Hack, 1994: Holderness, 1990; Kaspers *et al.*, 1987).

Stem dry weight (g)

The stem dry weight was non-significantly affected by various concentration of chlorpyrifos treatment in soybean and wheat. However, it was effective at later phases of growth when applied hence in wheat increased concentration decreased the dry matter of stem gradually over control. This might be due to the control of pest infestation resulted more leaf area and better dry matter production. However, the response of higher dose was found negative due to higher oxidative stress automatically reduced the photosynthetic rate and resulting reduced dry matter partitioning and stem weight (Table 4).

Kumar and Kumar (1993) also confirmed the similar impact were observed by on *Vicia faba* by the use of metasystox @50-100 mg/l. The higher concentration 200-300mi/l were found to have inhibitory effect on plant height, no. of branches, no of leaves per plants, total leaf area and plant biomass. Iqtidar *et al.*, (1995) also confirmed the similar effect of morpholine systemic fungicide in wheat and maize plants. And Elbashir *et al.*, (2016) also confirmed the sevin (carbaryl) recommended dose relatively had a positive effect on plant height, dry weight, and no. of leaves per plant.

Root dry weight (g)

The impact of pesticide treatment was observed on root dry weight of soybean and wheat At 30, 45 and 60 DAS. The root dry weight was significantly higher at control (0.213) in soybean. The increased concentration of pesticide decreased the dry weight of root. The similar trend was observed in wheat also. However, at 60 DAS the difference in root dry was non-significant with increased rate of pesticide application. The might be due to the plant growth physiology. Plant attained maximum root growth upto that phase (Table 5).

Boutin *et al.*, (2004) also reported that the phytotoxic and inhibitory effects of herbicides on germination, root and shoot growth. Basher *et al.*, (2007) also confirmed the similar impact of seed treatment by mancozeb on various morphological parameters in *Lens culinaris* L. in different developmental stage and concluded that all the morphological parameters increased significantly only lower dose (0.1%) whereas a liner decrease with increasing concentrations of mancozeb was observed.

Photosynthesis rate (µ mole CO_2/ m^2/ sec)

The photosynthesis rate was measured in 3rd fully expended leaves from the top. The rate was highest in control and gradually decreased with increase concentration of treatment. The mean value was greater in control (8.32) in soybean. Whereas in wheat the average value of photosynthesis rate was highest at T_3 0.75% of chlorpyrifos (9.28) followed by control (8.53) and at T_1 0.25% (7.58) the minimum photosynthesis rate was observed in T_4 1.25% concentration of chlorpyrifos (6.58). However, the result was non-significant at 30 DAS (Table 6; Fig. 3-4).

Decreased photosynthetic efficiency of the high concentration Chlorpyrifos treated seedlings may decrease the production of dry matter. The studies of Kumar *et al.*, (1993) indicate that the use of Metasystox on *Vicia faba* is promontory for seed germination and growth when used in lower concentrations (50-100 mg l^{-1}). The higher concentrations (200-300 mg l^{-1}) were inhibitory for

photosynthesis rate stomatal conductance and transpiration rate. Similar results have been obtained by Coskun *et al.,* (2015) in maize reported by Olszewski (2004) in pea and Singh *et al.,* (1970) in Citrus.

Stomatal conductance (µmol /m^2/sec)

Stomatal conductance was measured at 30, 45 and 60 DAS at various concentration of pesticide. It was observed that the pesticide have significant impact on this physiological parameter of gaseous exchange. The stomatal conductance was increase with increased level of pesticide in soybean and wheat. In soybean maximum stomatal conductance was observed at T_4 1.25% treated (111.33) over control (100.00). Whereas, in wheat value was maximum in treatment T_2 0.25% (3.31) followed by T_3 0.75% (3.09) and T_4 1.25% (2.86). (Table 7).

Xia *et al.,* (2006) also reported that the The phytotoxicities of nine pesticides at practical dosages on photosynthesis were investigated in cucumber (*Cucumis sativus* L.) Plants treated with paraquat showed the severest phytotoxic symptom with the highest reduction in net photosynthetic rate, stomatal conductance (Gs) and intercellular CO_2 concentration (Ci). Dias M. C. (2012) also confirmed the similar impact of these compounds on the photosynthetic apparatus.

Table.1 Impact of pesticide on plant height (cm) of soybean and wheat at various growth phases *i.e.,* 30, 45 and 60 DAS

Plant height (cm)	**DAS 30**	**DAS 45**	**DAS 60**	Total	Mean
Soybean					
T_1- Control (No chlorpyrifos)	27.00	28.50	31.00	86.50	**28.83**
T_2- 0.25%(low)	25.00	27.50	30.00	82.50	**27.50**
T_3- 0.75% (medium)	25.50	26.00	27.00	78.50	**26.17**
T_4- 1.25%(High)	23.50	24.50	26.00	74.00	**24.67**
Mean	**25.25**	**26.63**	**28.50**		
CD(P=0.05)	NS	1.75	3.49		
CD(P=0.01)	NS	NS	NS		
SE(m±)	0.79	0.43	0.87		
C.V.	4.43	2.30	4.30		
Wheat					
T_1- Control (No chlorpyrifos)	44.00	61.00	68.00	173.00	**57.67**
T_2- 0.25%(low)	37.00	59.00	62.50	158.50	**52.83**
T_3- 0.75% (medium)	40.50	54.00	67.00	161.50	**53.83**
T_4- 1.25%(High)	46.00	57.00	58.00	161.00	**53.67**
Mean	**41.88**	**57.75**	**63.88**		
CD(P=0.05)	5.79	4.03	3.02		
CD(P=0.01)	NS	NS	4.88		
SE(m±)	1.44	1.00	0.75		
C.V.	4.85	2.45	1.66		

DAS (Days after sowing)

Table.2 Impact of pesticide on leaf area (cm^2) of soybean and wheat at various growth phases *i.e.*, 30, 45 and 60 DAS

Leaf area(cm2)	**DAS 30**	**DAS 45**	**DAS 60**	Total	Mean
Soybean					
T_1- Control (No chlorpyrifos)	118.60	153.26	204.76	476.61	**158.87**
T_2- 0.25%(low)	116.50	119.25	191.46	427.21	**142.40**
T_3- 0.75% (medium)	110.55	128.15	151.18	389.88	**129.96**
T_4- 1.25%(High)	104.38	114.35	153.00	371.73	**123.91**
Mean	**112.51**	**128.75**	**175.10**		
CD(P=0.05)	7.43	13.42	26.53		
CD(P=0.01)	NS	21.66	42.83		
SE(m±)	1.84	3.33	6.58		
C.V.	2.32	3.66	5.31		
Wheat					
T_1- Control (No chlorpyrifos)	37.35	44.48	35.27	117.09	**39.03**
T_2- 0.25%(low)	33.88	34.63	56.46	124.97	**41.66**
T_3- 0.75% (medium)	29.60	31.50	37.32	98.41	**32.80**
T_4- 1.25%(High)	34.84	39.69	35.76	110.29	**36.76**
Mean	**33.92**	**37.57**	**41.20**		
CD(P=0.05)	4.67	4.57	6.06		
CD(P=0.01)	NS	7.37	9.78		
SE(m±)	1.16	1.13	1.50		
C.V.	4.83	4.26	5.16		

DAS (Days after sowing)

Table.3 Impact of pesticide on leaf dry weight (g) of soybean and wheat at various growth phases *i.e.*, 30, 45 and 60 DAS

Leaf dry wt. (g)	**DAS 30**	**DAS 45**	**DAS 60**	Total	Mean
Soybean					
T_1- Control (No chlorpyrifos)	0.860	0.960	1.525	3.345	**1.115**
T_2- 0.25%(low)	0.650	0.855	1.120	2.625	**0.875**
T_3- 0.75% (medium)	0.580	0.780	0.960	2.320	**0.773**
T_4- 1.25%(High)	0.510	0.750	1.015	2.275	**0.758**
Mean	**0.650**	**0.836**	**1.155**		
CD(P=0.05)	0.144	0.086	NS		
CD(P=0.01)	0.234	0.130	NS		
SE(m±)	0.036	0.021	0.1420		
C.V.	7.769	3.612	17.354		
Wheat					
T_1- Control (No chlorpyrifos)	0.250	0.215	0.410	0.875	**0.292**
T_2- 0.25%(low)	0.210	0.280	0.405	0.895	**0.298**
T_3- 0.75% (medium)	0.230	0.250	0.380	0.860	**0.287**
T_4- 1.25%(High)	0.190	0.185	0.360	0.735	**0.245**
Mean	**0.220**	**0.233**	**0.389**		
CD(P=0.05)	0.040	0.032	0.036		
CD(P=0.01)	NS	0.054	NS		
SE(m±)	0.010	0.008	0.009		
C.V.	6.428	4.809	3.279		

DAS (Days after sowing)

Table.4 Impact of pesticide on stem dry weight (g) of soybean and wheat at various growth phases *i.e.*, 30, 45 and 60 DAS

Stem dry wt. (g)	**DAS 30**	**DAS 45**	**DAS 60**	Total	Mean
Soybean					
T_1- Control (No chlorpyrifos)	0.375	0.390	0.740	1.505	**0.502**
T_2- 0.25%(low)	0.235	0.410	0.710	1.355	**0.452**
T_3- 0.75% (medium)	0.340	0.425	0.560	1.325	**0.442**
T_4- 1.25%(High)	0.230	0.345	0.610	1.185	**0.395**
Mean	**0.295**	**0.393**	**0.655**		
CD(P=0.05)	0.082	NS	0.103		
CD(P=0.01)	NS	NS	NS		
SE(m±)	0.020	0.015	0.025		
C.V.	9.737	5.553	5.505		
Wheat					
T_1- Control (No chlorpyrifos)	0.175	0.165	0.795	1.135	**0.378**
T_2- 0.25%(low)	0.165	0.150	0.675	0.990	**0.330**
T_3- 0.75% (medium)	0.135	0.145	0.460	0.740	**0.247**
T_4- 1.25%(High)	0.130	0.120	0.760	1.010	**0.337**
Mean	**0.151**	**0.145**	**0.673**		
CD(P=0.05)	0.027	0.025	0.071		
CD(P=0.01)	NS	NS	0.111		
SE(m±)	0.007	0.006	0.018		
C.V.	6.185	5.973	3.718		

DAS (Days after sowing)

Table.5 Impact of pesticide on root dry weight (g) soybean and wheat at various growth phases *i.e.*, 30, 45 and 60 DAS

Root dry wt. (g)	**DAS 30**	**DAS 45**	**DAS 60**	Total	Mean
Soybean					
T_1- Control (No chlorpyrifos)	0.440	0.095	0.105	0.640	**0.213**
T_2- 0.25%(low)	0.155	0.125	0.110	0.390	**0.130**
T_3- 0.75% (medium)	0.245	0.065	0.060	0.370	**0.123**
T_4- 1.25%(High)	0.195	0.085	0.080	0.360	**0.120**
Mean	**0.259**	**0.093**	**0.089**		
CD(P=0.05)	0.048	0.020	0.036		
CD(P=0.01)	0.070	0.035	NS		
SE(m±)	0.012	0.005	0.009		
C.V.	6.553	7.644	14.363		
Wheat					
T_1- Control (No chlorpyrifos)	0.135	0.146	0.345	0.626	**0.209**
T_2- 0.25%(low)	0.126	0.141	0.275	0.542	**0.181**
T_3- 0.75% (medium)	0.091	0.095	0.245	0.431	**0.144**
T_4- 1.25%(High)	0.084	0.116	0.245	0.445	**0.148**
Mean	**0.109**	**0.125**	**0.278**		
CD(P=0.05)	0.011	0.006	0.053		
CD(P=0.01)	0.016	0.003	NS		
SE(m±)	0.003	0.001	0.013		
C.V.	3.513	1.633	6.742		

DAS (Days after sowing)

Table.6 Impact of pesticide on photosynthesis rate (µmoleCO_2/m^2/sec) of soybean and wheat at various growth phases *i.e.*, 30, 45 and 60 DAS

Photosynthesis rate	DAS 30	DAS 45	DAS 60	Total	Mean
Soybean					
T_1- Control (No chlorpyrifos)	7.80	9.10	8.05	24.95	**8.32**
T_2- 0.25%(low)	8.20	5.90	8.35	22.45	**7.48**
T_3- 0.75% (medium)	7.05	4.10	9.65	20.80	**6.93**
T_4- 1.25%(High)	4.05	3.55	4.90	12.50	**4.17**
Mean	**6.78**	**5.66**	**7.74**		
CD(P=0.05)	1.00	0.86	1.30		
CD(P=0.01)	1.61	1.40	2.10		
SE(m±)	0.25	0.21	0.32		
C.V.	5.17	5.34	5.91		
Wheat					
T_1- Control (No chlorpyrifos)	7.85	11.15	6.60	25.60	**8.53**
T_2- 0.25%(low)	8.35	7.05	7.35	22.75	**7.58**
T_3- 0.75% (medium)	9.40	9.95	8.50	27.85	**9.28**
T_4- 1.25%(High)	5.15	7.25	7.35	19.75	**6.58**
Mean	**7.69**	**8.85**	**7.45**		
CD(P=0.05)	1.49	1.55	0.55		
CD(P=0.01)	NS	2.50	0.80		
SE(m±)	0.37	0.38	0.14		
C.V.	6.81	6.14	2.60		

DAS (Days after sowing)

Table.7 Impact of pesticide on stomatal conductance (µmol/m^2/sec) of soybean and wheat at various growth phases *i.e.*, 30, 45 and 60 DAS

Stomatal conductance	DAS 30	DAS 45	DAS 60	Total	Mean
Soybean					
T_1- Control (No chlorpyrifos)	79.0	105.5	115.5	300.0	**100.0**
T_2- 0.25%(low)	98.0	116.0	116.5	330.5	**110.2**
T_3- 0.75% (medium)	100.5	120.0	96.0	316.5	**105.5**
T_4- 1.25%(High)	162.5	86.0	85.5	334.0	**111.3**
Mean	**110.0**	**106.9**	**103.4**		
CD(P=0.05)	15.2	16.0	18.9		
CD(P=0.01)	24.5	NS	NS		
SE(m±)	3.8	4.0	4.7		
C.V.	4.8	5.3	6.4		
Wheat					
T_1- Control (No chlorpyrifos)	2.94	3.60	2.55	9.09	**3.03**
T_2- 0.25%(low)	3.02	3.40	3.50	9.92	**3.31**
T_3- 0.75% (medium)	2.62	3.65	3.00	9.27	**3.09**
T_4- 1.25%(High)	2.07	3.50	3.00	8.57	**2.86**
Mean	**2.66**	**3.54**	**3.01**		
CD(P=0.05)	0.49	NS	0.58		
CD(P=0.01)	NS	NS	NS		
SE(m±)	0.12	0.14	0.14		
C.V.	6.39	5.74	6.74		

DAS (Days after sowing)

Table.8 Impact of pesticide on transpiration rate (µmol $H_2O/m^2/sec$) of soybean and wheat at various growth phases *i.e.*, 30, 45 and 60 DAS

Transpiration rate	DAS 30	DAS 45	DAS 60	Total	Mean
Soybean					
T_1- Control (No chlorpyrifos)	2.81	2.60	2.94	8.35	**2.78**
T_2- 0.25%(low)	3.50	2.93	3.02	9.45	**3.15**
T_3- 0.75% (medium)	3.60	3.15	2.62	9.36	**3.12**
T_4-1.25%(High)	5.17	2.59	2.26	10.01	**3.34**
Mean	**3.77**	**2.82**	**2.71**		
CD(P=0.05)	0.54	NS	0.42		
CD(P=0.01)	0.87	NS	NS		
SE(m±)	0.13	0.40	0.11		
C.V.	5.01	20.06	5.48		
Wheat					
T_1- Control (No chlorpyrifos)	2.94	3.60	2.55	9.09	**3.03**
T_2- 0.25%(low)	3.02	3.40	3.50	9.92	**3.31**
T_3- 0.75% (medium)	2.62	3.65	3.00	9.27	**3.09**
T_4-1.25%(High)	2.07	3.50	3.00	8.57	**2.86**
Mean	**2.66**	**3.54**	**3.01**		
CD(P=0.05)	0.49	NS	0.58		
CD(P=0.01)	NS	NS	NS		
SE(m±)	0.12	0.14	0.14		
C.V.	6.39	5.74	6.74		

DAS (Days after sowing)

Table.9 Impact of pesticide on peroxidase activity (ΔODµgprotein^{-1}min^{-1}) in soybean and wheat at various growth phases *i.e.*, 30, 45 and 60 DAS

POXunit/gm	DAS 30	DAS 45	DAS 60	Total	Mean
Soybean					
T1- Control (No chlorpyrifos)	122.4	47.55	108.4	278.35	92.78
T2- 0.25%(low)	102.75	66.35	86.05	255.15	85.05
T3- 0.75% (medium)	72.1	79.2	71.85	223.15	74.38
T4- 1.25%(High)	65.5	88.25	60.1	213.85	71.28
Mean	**90.69**	**70.34**	**81.60**		
CD(P=0.05)	19.898	18.765	16.215		
CD(P=0.01)	32.13	NS	26.18		
SE(m±)	4.935	4.655	4.022		
C.V.	7.696	9.358	6.97		
Wheat					
T1- Control (No chlorpyrifos)	103.07	117.175	143.565	363.81	121.27
T2- 0.25%(low)	103.76	137.445	122.145	363.35	121.12
T3- 0.75% (medium)	89.455	142.29	146.37	378.115	126.04
T4- 1.25%(High)	134.64	148.92	140.76	424.32	141.44
Mean	**107.73**	**136.46**	**138.21**		
CD(P=0.05)	21.125	16.13	13.773		
CD(P=0.01)	NS	NS	NS		
SE(m±)	5.24	4.001	3.416		
C.V.	6.878	4.146	3.496		

DAS (Days after sowing)

Table.10 Impact of pesticide on polyphenol oxidase (µgProtein^{-1}min^{-1}) in soybean and wheat at various growth phases *i.e.*, 30, 45 and 60 DAS

Polyphenol oxidase (µg Protein^{-1} min^{-1})	**DAS 30**	**DAS 45**	**DAS 60**	Total	Mean
Soyabean					
T_1- Control (No chlorpyrifos)	0.0241	0.0311	0.0289	0.0841	**0.0280**
T_2- 0.25%(low)	0.0204	0.0301	0.0304	0.0809	**0.0270**
T_3- 0.75% (medium)	0.0266	0.0288	0.0295	0.0849	**0.0283**
T_4- 1.25%(High)	0.0193	0.0328	0.0253	0.0774	**0.0258**
Mean	**0.0226**	**0.0307**	**0.0285**		
CD(P=0.05)	0.0040	0.0020	0.0030		
CD(P=0.01)	NS	NS	NS		
SE(m±)	0.0010	0.0010	0.0010		
C.V.	7.4940	2.8400	4.1330		
Wheat					
T_1- Control (No chlorpyrifos)	0.0096	0.0162	0.0102	0.0359	**0.0120**
T_2- 0.25%(low)	0.0114	0.0155	0.0116	0.0385	**0.0128**
T_3- 0.75% (medium)	0.0079	0.0129	0.0152	0.0360	**0.0120**
T_4- 1.25%(High)	0.0102	0.0134	0.0129	0.0365	**0.0122**
Mean	**0.0098**	**0.0145**	**0.0124**		
CD(P=0.05)	0.0008	0.0007	0.0010		
CD(P=0.01)	NS	0.0002	0.0006		
SE(m±)	0.0002	0.0002	0.0003		
C.V.	2.9417	1.8162	2.9763		

DAS (Days after sowing)

Table.11 Impact of pesticide on nitrate reductase (µgNO_2/g fw/h) in soybean and wheat at various growth phases *i.e.*, 30, 45 and 60 DAS

Nitrate reductase (µg NO_2/g fw/h)	**DAS 30**	**DAS 45**	**DAS 60**	Total	Mean
Soyabean					
T_1- Control (No chlorpyrifos)	0.0946	0.0661	0.0180	0.1786	**0.0595**
T_2- 0.25%(low)	0.0528	0.0734	0.0719	0.1981	**0.0660**
T_3- 0.75% (medium)	0.0731	0.0601	0.0267	0.1599	**0.0533**
T_4- 1.25%(High)	0.1390	0.0198	0.0663	0.2251	**0.0750**
Mean	**0.0899**	**0.0548**	**0.0457**		
CD(P=0.05)	0.0010	0.0008	0.0011		
CD(P=0.01)	0.0060	0.0060	0.0060		
SE(m±)	0.0003	0.0002	0.0003		
C.V.	0.4123	0.5055	0.8626		
Wheat					
T_1- Control (No chlorpyrifos)	0.0096	0.0162	0.0102	0.0359	**0.0120**
T_2- 0.25%(low)	0.0114	0.0155	0.0116	0.0385	**0.0128**
T_3- 0.75% (medium)	0.0079	0.0129	0.0152	0.0360	**0.0120**
T_4- 1.25%(High)	0.0102	0.0134	0.0129	0.0365	**0.0122**
Mean	**0.0098**	**0.0054**	**0.0054**		
CD(P=0.05)	0.0008	0.0007	0.0010		
CD(P=0.01)	NS	0.0030	0.0060		
SE(m±)	0.0002	0.0002	0.0003		
C.V.	2.9417	1.8162	2.9763		

DAS (Days after sowing)

Table.12 Impact of pesticide on proline content (µmole/g) in soybean and wheat at various growth phases *i.e.*, 30, 45 and 60 DAS

Proline (µmole/g)	**DAS 30**	**DAS 45**	**DAS 60**	Total	Mean
Soybean					
T_1-Control (No chlorpyrifos)	0.0160	0.0250	0.0170	0.0580	**0.0193**
T_2- 0.25%(low)	0.0160	0.0210	0.0200	0.0570	**0.0190**
T_3- 0.75% (medium)	0.0150	0.0190	0.0220	0.0560	**0.0187**
T_4- 1.25%(High)	0.0150	0.0190	0.0230	0.0570	**0.0190**
Mean	**0.0155**	**0.0210**	**0.0205**		
CD(P=0.05)	0.0010	0.0010	0.0010		
CD(P=0.01)	0.0010	0.0080	0.0040		
SE(m±)	0.0000	0.0000	0.0000		
C.V.	1.6140	0.8890	1.5110		
Wheat					
T_1-Control (No chlorpyrifos)	0.1200	0.0060	0.0070	0.1330	**0.0443**
T_2- 0.25%(low)	0.3250	0.0100	0.0100	0.3450	**0.1150**
T_3- 0.75% (medium)	0.0300	0.0090	0.0090	0.0480	**0.0160**
T_4- 1.25%(High)	0.0450	0.0090	0.0090	0.0630	**0.0210**
Mean	**0.1300**	**0.0085**	**0.0088**		
CD(P=0.05)	0.0140	0.0010	0.0020		
CD(P=0.01)	0.0200	0.0030	NS		
SE(m±)	0.0040	0.0000	0.0000		
C.V.	3.8470	6.0610	7.3120		

DAS (Days after sowing)

Table.13 Impact of pesticide on total soluble protein (mg/g fw) in soybean and wheat at various growth phases *i.e.*, 30, 45 and 60 DAS

TSP (mg/gm fw)	**DAS 30**	**DAS 45**	**DAS 60**	Total	Mean
Soybean					
T_1- Control (No chlorpyrifos)	1.750	1.450	1.680	4.880	**1.627**
T_2- 0.25%(low)	1.595	1.360	1.575	4.530	**1.510**
T_3- 0.75% (medium)	1.645	1.540	1.680	4.865	**1.622**
T_4- 1.25%(High)	1.545	1.355	1.910	4.810	**1.603**
Mean	**1.634**	**1.426**	**1.711**		
CD(P=0.05)	0.117	0.046	0.058		
CD(P=0.01)	NS	0.075	0.095		
SE(m±)	0.029	0.011	0.014		
C.V.	2.514	1.136	1.186		
Wheat					
T_1- Control (No chlorpyrifos)	1.680	1.720	1.430	4.830	1.610
T_2- 0.25%(low)	1.740	1.675	1.485	4.900	1.633
T_3- 0.75% (medium)	1.645	1.780	1.530	4.955	1.652
T_4- 1.25%(High)	1.755	1.735	1.630	5.120	1.707
Mean	**1.705**	**1.728**	**1.519**		
CD(P=0.05)	0.051	0.062	0.076		
CD(P=0.01)	NS	NS	0.128		
SE(m±)	0.013	0.015	0.019		
C.V.	1.057	1.262	1.758		

DAS (Days after sowing)

Table.14 Impact of pesticide on non structral carbohydrate (mg/g) in soybean and wheat at various growth phases *i.e.*, 30, 45 and 60 DAS

Non structral carbohydrate(mg/g)	**DAS 30**	**DAS 45**	**DAS 60**	Total	Mean
Soybean					
T_1-Control(No chlorpyrifos)	2.931	7.615	11.006	21.553	**7.184**
T_2- 0.25%(low)	3.474	9.144	12.513	25.130	**8.377**
T_3- 0.75% (medium)	3.063	7.508	10.271	20.841	**6.947**
T_4- 1.25%(High)	3.190	6.906	8.963	19.059	**6.353**
Mean	**3.164**	**7.793**	**10.688**		
CD(P=0.05)	0.081	0.737	0.325		
CD(P=0.01)	0.136	1.220	0.530		
SE(m±)	0.021	0.188	0.083		
C.V.	0.920	3.404	1.094		
Wheat					
T_1-Control(No chlorpyrifos)	3.275	5.719	5.719	14.713	**4.904**
T_2- 0.25%(low)	4.550	10.250	10.250	25.050	**8.350**
T_3- 0.75% (medium)	3.775	6.463	6.463	16.700	**5.567**
T_4- 1.25%(High)	2.571	4.975	4.975	12.521	**4.174**
Mean	**3.543**	**6.852**	**6.852**		
CD(P=0.05)	0.404	0.911	1.031		
CD(P=0.01)	0.668	1.510	1.700		
SE(m±)	0.103	0.232	0.263		
C.V.	4.108	4.788	4.059		

DAS (Days after sowing)

Treatments

Treatments	**Treatment details**
T_1	Control (No chloropyrifos)
T_2	Foliar application below normal (Low)-0.25%
T_3	Foliar application recommended dose (Medium)-0.75%
T_4	Foliar application supra-optimal dose (High)-1.25%

T_1- Control (no pesticide use)

T_2– The spray solution is prepared from stock solution. Take 0.25 ml of stock solution and make up the volume 100 ml. So 0.25% low chloropyrifos.

T_3- The spray solution is prepared from stock solution. Take 0.75 ml of stock solution and make up the volume 100 ml. So 0.75% medium chloropyrifos.

T_4- The spray solution is prepared from stock solution. Take 1.25 ml of stock solution and make up the volume 100 ml. So 1.25% high chloropyrifos.

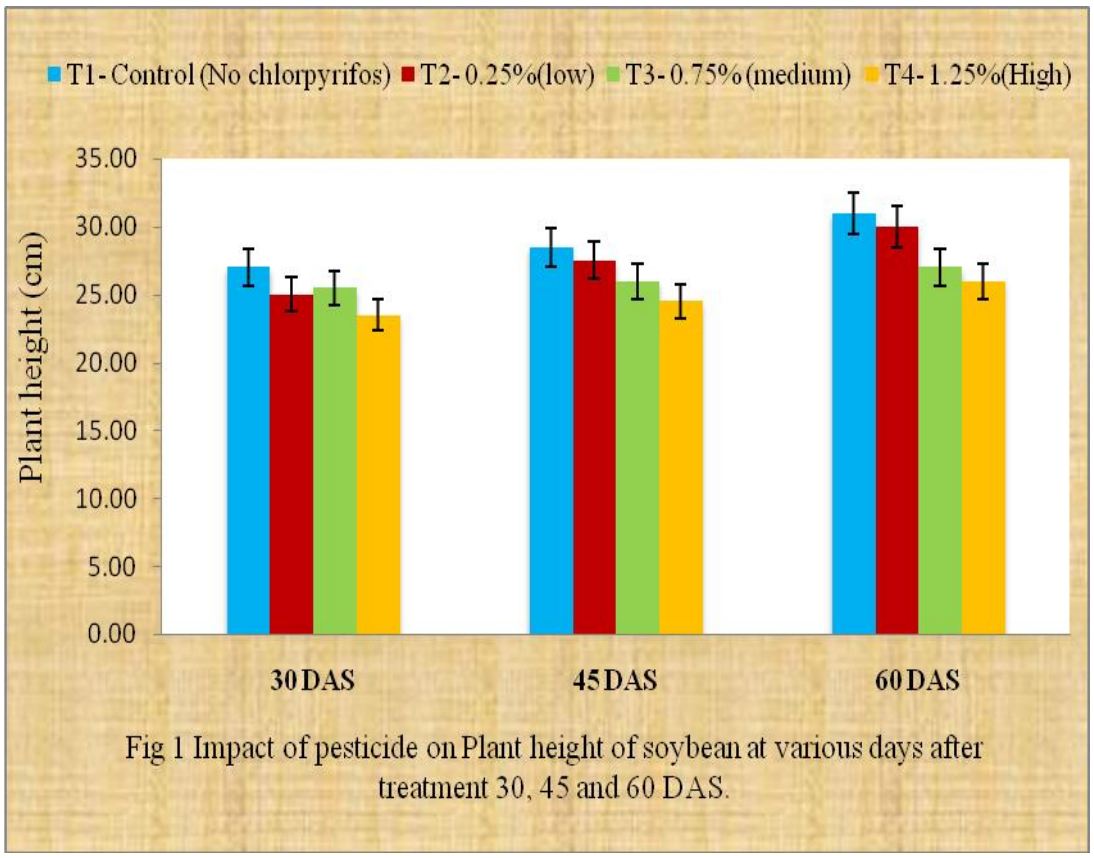

Fig 1 Impact of pesticide on Plant height of soybean at various days after treatment 30, 45 and 60 DAS.

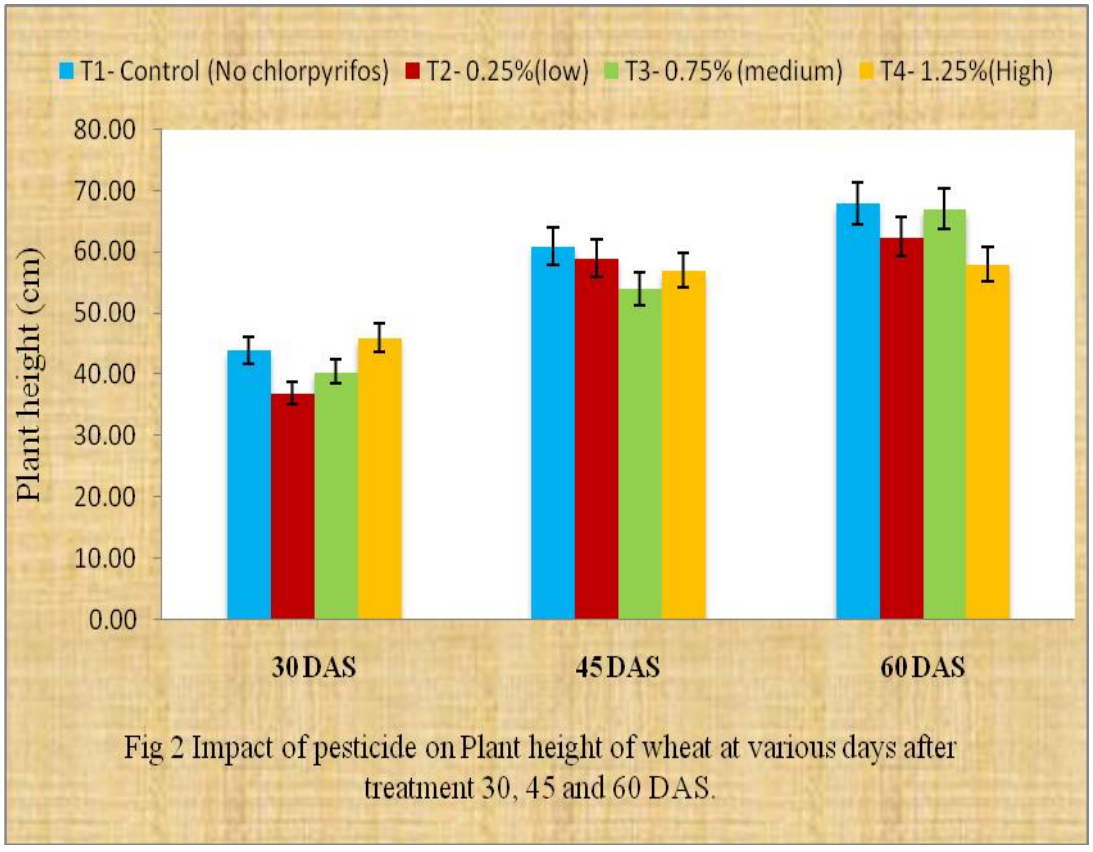

Fig 2 Impact of pesticide on Plant height of wheat at various days after treatment 30, 45 and 60 DAS.

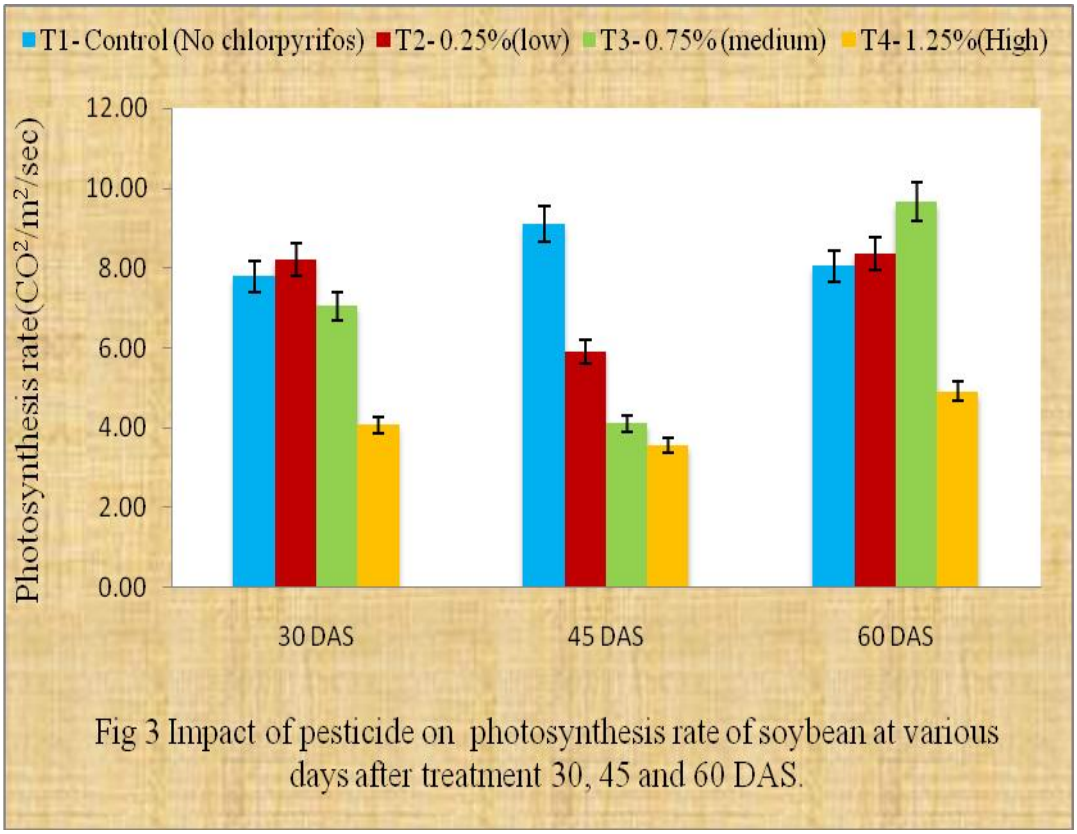

Fig 3 Impact of pesticide on photosynthesis rate of soybean at various days after treatment 30, 45 and 60 DAS.

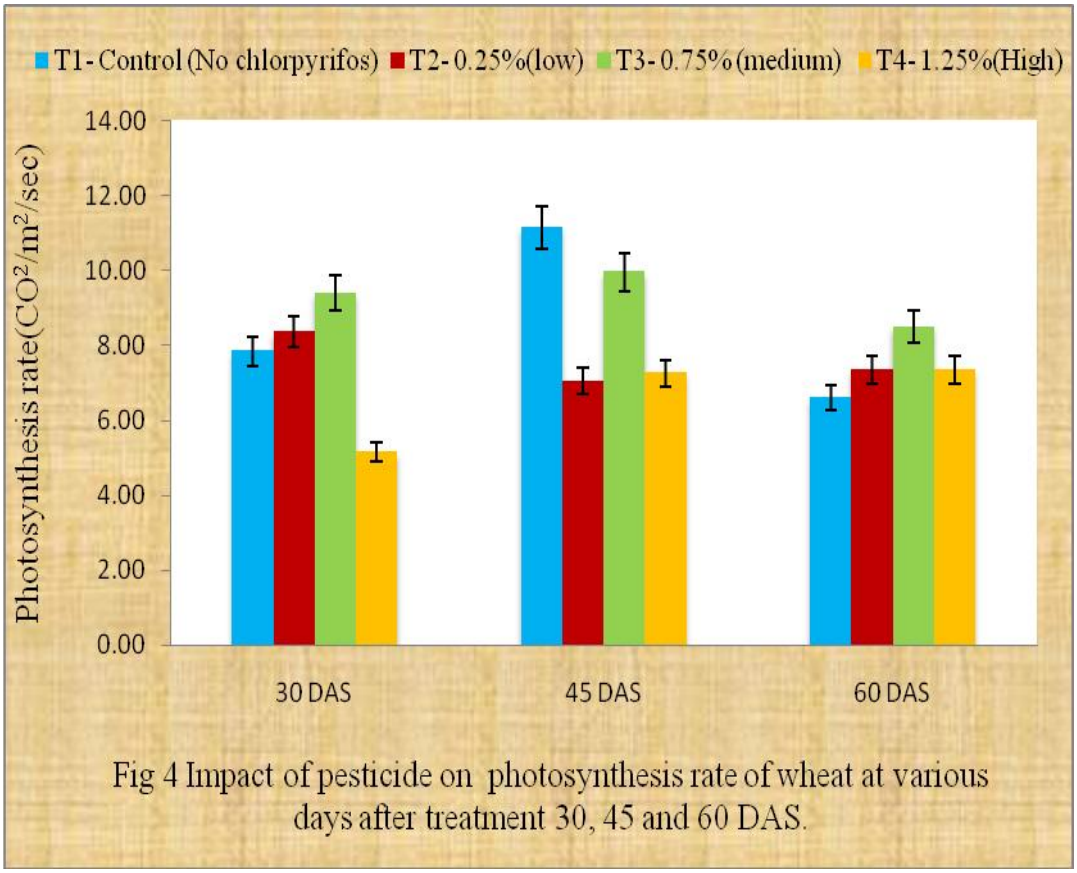

Fig 4 Impact of pesticide on photosynthesis rate of wheat at various days after treatment 30, 45 and 60 DAS.

Transpiration rate (µmol $H_2O/m^2/sec$)

The transpiration rate was measured in 3rd fully expended leaves from the top. The rate was highest in higher concentration and decreased in control. The mean value was greater in T_4 1.25% (3.34) in soybean. Whereas in wheat the average value of transpiration rate was highest at T_2 0.25% of chlorpyrifos (3.31) followed by T_3 0.75% (3.09) and at control (3.03) and minimum rate of transpiration was observed at lower concentration of chlorpyrifos T_4 1.25% (2.86) (Table 8). Transpiration is directly related to stomatal opening, which is essential for leaf intake of carbon dioxide for photosynthesis. Photosynthesis is a more general measurement of carbon dioxide intake and fixation (sugar production). Both processes are interrelated and are directly related to plant growth and productivity.

The higher value of transpiration occurred on insecticide-free plants. Photosynthesis was also higher in the untreated check, suggesting that all insecticide treatments adversely influenced both stomatal opening and over all photosynthesis rates as reported by Sances *et al.,* (1981) similar result were reported by Olszewski. (2004) observed a tendency towards a slight decrease in the rate of photosynthesis under the influence of a fungicidal agent in horse bean and pea. Further, the same author found a significant decline in the levels of photosynthesis and transpiration under the effect of foliar herbicide application. Panduranga *et al.,* (2005) also confirmed the dimethoate causes a reduction in plant growth, photosynthesis pigments and photosynthetic activity of *Glycine max* L.

Peroxidase (ΔODµg protein^{-1} min^{-1})

The peroxidase activity was measured at 30, 45 and 60 DAS in soybean and wheat, and 30, 45 and 60 DAT in ashwagandha. The average rate was decreased with increasing level of pesticide treatment in soybean. However, in wheat it was maximum at T_4 (1.25%) (141.44). While in ashwagandha it was minimum at T_4 (87.64) over control 118.07. However, the enzyme activity was maximum at initial stage of growth and decreased with the passage of time. This might be due to the presence of healthy and efficient H_2O_2 scavenging system during the young stage of plant (Table 9).

The result of present investigation suggest that activity of catalase was gradually decreased in treated plants as compared to control which was similar finding reported by Vidyasagar *et al.,* (2007) in *Sorghum bicolor* L. However, the activity of other oxidative enzymes such as, polyphenol oxidase and peroxidase was increased along with the concentration of chlorpyrifos increased. The increase in peroxidase activity may be due to the metabolic response to environmental stress reported by Fang and Kao (2000). Lee (2002) also reported that the peroxidase activity increased remarkably with Na_2SO_3 treatments. Since peroxidase activity was very high in treated shoots, accumulated H_2O_2 was utilized for various peroxidative polymerization reactions.

Polyphenol oxidase (µg Protein^{-1} min^{-1})

Polyphenol oxidase was measured during the pesticide treatment in soybean and wheat at 30, 45 and 60 DAS and in ashwagandha at 30, 45 and 60 DAT. The content was increased with passage of time in soybean and wheat. Whereas, in ashwagandha it increased maximum at 45 DAT 0.0173 over control. However, it 0.0071 decreased at 60DAT 0.0017. However, the increased concentration of pesticide slightly increased the activity of polyphenol oxidase in all the experimental crops (Table 10).

Fang and Kao (2000) also reported that the activity of oxidative enzymes such as, polyphenol oxidase and peroxidase was increased along with the concentration of chlorpyrifos increased. The increase in peroxidase activity may be due to the metabolic response to environmental stress. Similar results have been obtained by Chauhan *et al.,* (2013) in potato reported by Nanjo *et al.,* (1999).

Nitrate reductase (µg NO_2/g fw/ h)

Nitrate reductase activity was significantly affected by pesticide resistance. However, in general it decreased with passage of time in soybean and wheat. Increased concentration of pesticide has shown the maximum activity of nitrate reductase in soybean and wheat. However, in ashwagandha in general its activity increased with passage of time at 60 DAT it was maximum 0.0081.and increased level of treatment increased the activity *i.e.,* at T_4 1.25% it was 0.0076 (µg NO_2/g fw/ h) over control 0.0075(µg NO_2/g fw/ h) (Table 11).

Nitrate reductase (NR) activity was seen to decrease with concentration of pesticide increase. A result was well supported by Pankaj *et al.,* (2015) also reported significant decrease in activity with increase in concentration of pesticide in Fenugreek.

Proline content (µM g^{-1})

Proline is the amino acid accumulates under stress situation was measured at 30, 45 and 60 DAS after application of chlorpyrifos in soybean, wheat and ashwagandha. The maximum proline content was observed at 60 DAS. However, the increased rate of chemical slightly increased the proline content at T_2 0.25% level in wheat 0.115µm/g over control 0.0443µm/g. It showed more resistant in wheat as compared to soybean and ashwagandha when the control decreased with increasing level of treatment (Table 12).

The proline content also increased in the present studies due to the chlorpyrifos treatment. Similar finding have also been reported by Nasrabadi *et al.,* (2014) in tomato, Parween *et al.,* (2012) in *Vigna radiata* L. and Coskun *et al.,* 2015. Proline accumulated in plants under various stress conditions. The accumulation of proline in plant due to drought and temperature stress is also well documented by Gzik (1996). Proline acts as a hydrophobic protectant for enzymes and sub-cellular organelles Lerudulier *et al.,* (1994). This helps the plant to tolerate or adapt to the stress condition. It is evident from these studies that an increase in proline content may serve as a mean of protection of plant tissue against stress.

Total soluble protein (mg/g fw)

The average total soluble protein was increased with passage of time in all the experimental crops. The treatment of pesticide increased it gradually and significantly in wheat and ashwagandha with increasing level of treatment. Maximum increase was observed at T_4 (1.25%) in wheat 1.707 over control 1.610. Whereas, in ashwagandha maximum soluble protein was obtained at T_3 (0.75%) 1.713 over control 1.600 In soybean crops the content decreased at 45 DAS and the maximum content was observed at T_2 (0.25%) 1.510 over control 1.627. This might be due to the maintain of chemical stress at cellular level. It the content decreased it might be used for synthesis of new proteins under stress and its increase showed the resistance mechanism of stress *viz.* more synthesis of protein (Table 13).

Singh and Tiwari (2003) also found the total protein content in plants decreased with increasing of pesticide concentrations compared to the control plants, respectively.

Protein content in organisms, an important indicator of reversible and irreversible changes in metabolism, is known to respond to a wide variety of stressor such as natural and xenobiotic. It showed that excessive pesticide reduced protein amount of many plant species Zengin *et al.,* (2007) reported by Vidyasagar *et al.,* (2007).

Non-structural carbohydrate (mg/g)

Non-structural carbohydrate (total soluble sugar + starch) was estimated at 30, 45 and 60DAS. It was significantly increased with passage of time in all the experimental crops. It might be due to more anabolic reaction at the later phase of growth band carbohydrate were synthesized more efficiently. However, the lower dose T_2 0.25% of pesticide increased the maximum structural carbohydrate over control in soybean 8.377 over control 7.184. In wheat 8.350 over control 4.904. Whereas, the further increase in dose of pesticide decreased the structural carbohydrate significant in all the experimental crops. However, in ashwagandha the structural carbohydrate was maximum at control over treatments (Table 14).

Siddiqui *et al.,* (2001) also suggested that the carbohydrate content is rapidly decreased with increase the concentration of chlorpyrifos. A similar result was reported by Bhattacharya *et al.,* (2001) in carbendazim treated rice plants.

Chlorpyrifos is a hazardous and important pollutant of the environment. The EU Directive 2008/105/EC lists it as one of the priority water pollutant. Its presence in mainly detected by chemical but, since biological tests have general in importance in the last few years. Chlorpyrifos effect on several metabolic and stress related parameters *i.e.*, morphological physiological and biochemicals. Therefore, the impact of oxidative stress was evaluated on these crop plants to find out the chemical stress tolerance and resistance mechanism. It was observed that as compared to soybean, wheat was found to be same tolerance against the oxidative stress. However, the ashwagandha was initially affected but later it increased its morphophysiological traits.

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How to cite this article:

Nutan Singh, Pratibha Katiyar and Ajay. 2017. Evaluation of Oxidative Stress on Soybean (*Glycine max* L. *Merrill*) and Wheat (*Triticum aestivum*) Crops in Response to Chlorpyrifos. *Int.J.Curr.Microbiol.App.Sci.* 6(11): 2045-2064. doi: https://doi.org/10.20546/ijcmas.2017.611.243