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Pesticides induced alterations in biochemical responses in ashwagandha (*Withania somnifera*)

Nutan Singh, Ajay and Pratibha Katiyar

Abstract
Phytotoxicity is defined as the detrimental effect of chemical products used for the eliminating or growth regulating purpose on some morphological, anatomical and physiological processes of plants. We examined the effect of chlorpyrifos, which is used to crop against fleas, insects, termites, pests and mosquitoes to determine whether it adversely affect ashwagandha plant. 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 transplanting on ashwagandha. The biochemical characteristics of ashwagandha were recorded after various doses of pesticide treatment application. The peroxidase and catalase activity decreased with increasing level of pesticide treatment in ashwagandha. Chlorophyll a content and chlorophyll b content decreased with higher dose of pesticide treatment in the experimental crops.

Keywords: Pesticides induced, biochemical responses, ashwagandha

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) [1]. 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) [5-6], and subterranean termites (Venkateswara Rao *et al.* 2005) [8]. Chlorpyrifos produces hazardous effects on the environment when it is applied directly on plants or mixed with soil (Howard 1991) [3].
Ashwagandha (*Withania somnifera*) known commonly as ashwagandha, Indian ginseng, poison gooseberry, or winter cherry, is a plant in the Solanaceae or nightshade family. Several other species in the genus Withania are morphologically similar. It is used as an herb in ayurvedic medicine. This species is a short, tender perennial shrub growing 35–75 cm (14–30 in) tall. Tomentose branches extend radially from a central stem. Leaves are dull green, elliptic, usually up to 10–12 cm long. The flowers are small, green and bell-shaped. The ripe fruit is orange-red. Ashwagandha 3rd important prioritized medicinal plant listed by NMPB is also known as Indian Ginseng. Ashwagandha is highly popular herb and widely used in lot of ayurvedic formulations, nutracitical product and other herbal product. The annual demand of this herb was estimated to be 9127.5 tons per annum in the year 2005 (Annual demand 2012, Shinde 2014). Based on the trend the current demand of ashwagandha per annum would be around 12500 tons (Shinde 2014).

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: Aswagandha

Treatments

Table 1

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.

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

Biochemical parameters

Assay of antioxidant enzyme

Enzyme extraction

For enzyme assay leaf sample were collected from plant and kept in ice box. 0.5gram of fresh leaf was homogenized in 3 ml of extraction buffer of 0.1 M phosphate buffer (7.8) containing 0.5 mM EDTA in pre chilled mortar and pestle. The homogenized tissue was centrifuged at 13,000g for 10 min at 4˚C and the collected supernatant was used for enzyme assay. Total process was carried at 4˚C.

Super oxide dismutase (SOD) activity assay (unit/ g fw)

SOD activity was assayed by following the method of Dhindsa *et al.* (1981). Added 0.3 ml supernatant to reaction mixture containing 1 3µM Riboflavin 63µM of NBT and 200mM of methionine. Tubes were covered with aluminum foil to prevent light. Prepared blank without enzyme supernatant as control. Exposed tubes to light in light box for 3 min. The colour intensity was measured by spectrophotometer at 560 nm wavelength and SOD activity, was expressed in unit g^{-1}fw.

Catalase (CAT) activity assay (mmolH_2O_2min^{-1}g^{-1})

CAT activity was assayed by following the method given by Barber (1980). The extract from SOD Assay was used for CAT assay. Added 1.5 ml phosphate buffer, 1 ml H_2O_2 (0.005M) and 0.5 ml enzyme. Incubated at 20^0C for 1 min and stopped reaction by 5ml 0.7 N H_2SO_4.Titrated reaction mixture against 0.01N KM_nO_4 until a faint /light purple colour persists for at least 15 second. Prepared blank by adding enzyme extract reaction mix without incubation and CAT was expressed in unit mmolH_2O_2min^{-1}g^{-1}.

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})

Chlorophyll content estimation (mg/g fw)

The chlorophyll content was estimated at 30, 45 and 60 DAT. Total chlorophyll, chlorophyll a and chlorophyll b contents were determined by following the method of Hiscox and Israelstom (1979). 500 mg of fresh leaf tissues were cut into small pieces and incubated in 5.0 ml of DMSO (dimethyl sulfoxide) at 50°C for 2.30 hours. At the end of incubation period the supernatant was decanted and leaf tissues discarded. The absorbance was read at 645 and 663 nm in UV-vis spectrophotometer (ELICO, 159). Total chlorophyll, chlorophyll a and chlorophyll b content were calculated using the formula given by Arnon (1949) and expressed in mg per gram fresh weight.

Result Discussion

Super oxide dismutase (unit/g fw)

The super oxide dismutase (SOD) was measured at various growth stage after application of pesticide. The activity of SOD was higher at 60 DAS irrespective of level of treatments in experimental crops. In ashwagandha the maximum SOD activity was observed in T_2 (0.25%) 10.362 over control 0.322. This might be due to the activity of oxidative enzyme to release the chemical stress associated with resistance (Table no 1 fig no 1).

Abdul *et al.* (2006) also informed that the increased antioxidant enzymes activities like superoxide dismutase, ascorbate peroxidase and catalase activities in response to triadimefon treatment in *Catharanthus roseus*. Similar results have been obtained by Tabg *et al.* (2006) in bitter gourd reported by Wang *et al.* (2006) [10].

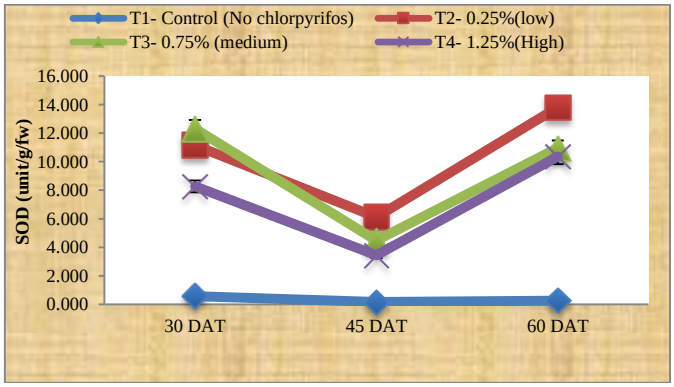

Fig 1: Impact of pesticide on Super oxide dismutase of ashwagandha at various days after treatment 30, 45 and 60 DAT

Table 1: Impact of pesticide on super oxide dismutase (unit/g fw) in ashwagandha leaves at various growth phases *i.e.*, 30, 45 and 60 DAT.

SOD (Unit/g fw) Ashwagandha	DAT 30	DAT 45	DAT 60	Total	Mean
T_1- Control (No chlorpyrifos)	0.575	0.150	0.240	0.965	0.322
T_2- 0.25%(low)	11.155	6.120	13.810	31.085	10.362
T_3- 0.75% (medium)	12.305	4.480	10.955	27.740	9.247
T_4- 1.25%(High)	8.270	3.430	10.355	22.055	7.352
Mean	8.076	3.545	8.840		
CD(P=0.05)	1.979	0.800	1.878		
CD(P=0.01)	3.195	1.299	3.024		
SE(m±)	0.491	0.198	0.466		
C.V.	8.594	7.916	7.451		

DAT (Days after transplanting**)**

Catalase activity (mMol H_2O_2 $min^{-1}g^{-1}$ fw)

Catalase activity was significantly varied at 30 DAT, 45 and 60 DAT in ashwagandha whereas, at later phase of growth 45 and 60 DAS it was not significant at all the stage of growth. except 60 DAT at 1% level of significant. However, in ashwagandha also the impact of pesticide was non significant. The catalase activity was highest in control over treatment in all the experimental crops and it decreased with increasing level of pesticide treatment in ashwagandha. (Table no 2 fig no. 2).

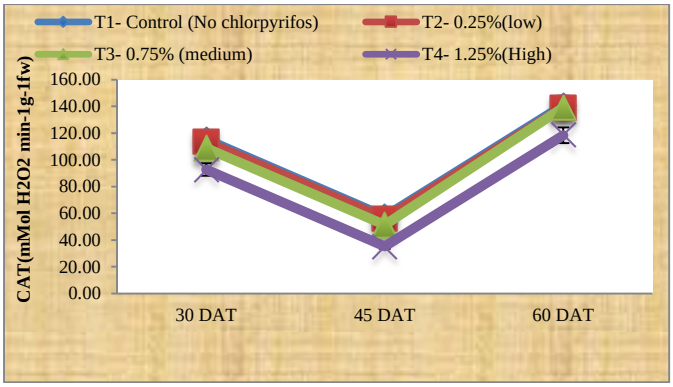

Fig 2: Impact of pesticide on catalase activity of ashwagandha at various days after treatment 30, 45 and 60 DAT

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 e*t al.* (2007) [9] 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.

Table 2: Impact of pesticide on catalase activity (H_2O_2-^{1}$min^{-1}g^{-1}$) in ashwagandha leaves at various growth phases *i.e.*, 30, 45 and 60 DAT.

CAT unit/gm Ashwagandha	30 DAT	45 DAT	60 DAT	Total	Mean
T_1-Control (No chlorpyrifos)	115.20	57.60	140.80	313.60	104.53
T_2- 0.25%(low)	113.60	56.00	139.20	308.80	102.93
T_3- 0.75% (medium)	108.80	51.20	139.20	299.20	99.73
T_4- 1.25%(High)	92.80	35.20	118.40	246.40	82.13
Mean	107.60	50.00	134.40		
CD(P=0.05)	11.63	11.63	10.20		
CD(P=0.01)	NS	NS	16.48		
SE(m±)	2.88	2.88	2.53		
C.V.	3.79	8.16	2.66		

DAT (Days after transplanting)

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

The peroxidase activity was measured at 30, 45 and 60 DAT in ashwagandha. 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 3 & fig 3)

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 e*t al.* (2007) [9] 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) [2]. Lee (2002) [7] 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.

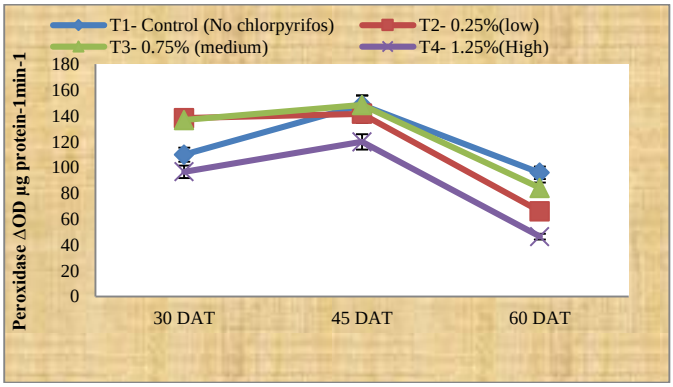

Fig 3: Impact of pesticide on Peroxidase activbity of ashwagandha at various days after treatment 30, 45 and 60 DAT.

Table 3: Impact of pesticide on peroxidase activity (ΔODµgprotein^{-1}min^{-1}) in ashwagandha leaves at various growth phases *i.e.*, 30, 45 and 60 DAT.

POXunit/gm Ashwagandha	DAT 30	DAT 45	DAT 60	Total	Mean
T1- Control (No chlorpyrifos)	109.905	148.41	95.88	354.195	118.07
T2- 0.25%(low)	138.21	141.525	66.045	345.78	115.26
T3- 0.75% (medium)	136.68	148.41	84.15	369.24	123.08
T4- 1.25%(High)	96.645	119.85	46.41	262.905	87.64
Mean	120.36	139.55	73.12		
CD(P=0.05)	13.851	19.968	11.778		
CD(P=0.01)	22.36	NS	19.022		
SE(m±)	3.436	4.953	2.921		
C.V.	4.037	5.019	5.65		

DAT (Days after transplanting)

Chlorophyll a content (mg/g fw)

The chlorophyll a content was significantly affected with chlorpyrifos doses. In ashwagandha in general it is increased with passage of time. The maximum value was obtained at 30 DAT 0.100 mg/g and increased with passage of treatment 45 DAT 0.518 mg/g and at 60 DAT 0.555 mg/g. However, the lower doses of pesticides (T_2, 0.25%) and (T_3, 0.75%) have increased chlorpyrifos content 0.402 mg/g and 0.423 mg/g respectively and highest dose of pesticide (1.25%) decreased it 0.352 mg/g (Table no 4.20 fig no. 4.54-4.56).

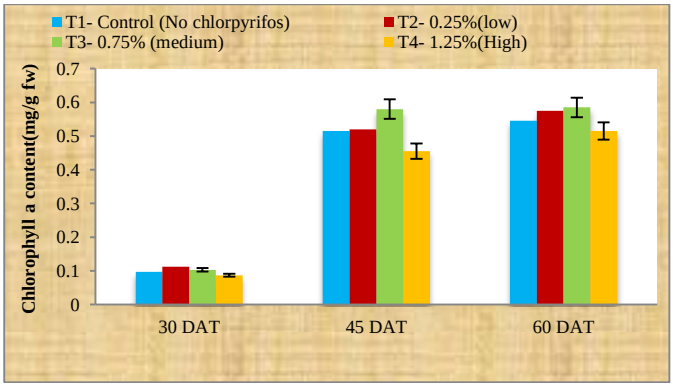

Fig 4: Impact of pesticide on Chlorophyll a content of ashwagandha at various days after treatment 30, 45 and 60 DAT

Table 4: Impact of pesticide on chlorophyll a content (mg/g fw) in in ashwagandha leaves at various growth phases *i.e.*, 30, 45 and 60 DAT.

Chlorophyll a content (mg/g fw) Ashwagandha	DAT 30	DAT 45	DAT 60	Total	Mean
T_1- Control (No chlorpyrifos)	0.097	0.515	0.545	1.157	0.386
T_2- 0.25%(low)	0.112	0.520	0.575	1.207	0.402
T_3- 0.75% (medium)	0.103	0.580	0.585	1.268	0.423
T_4- 1.25%(High)	0.087	0.455	0.515	1.057	0.352
Mean	0.100	0.518	0.555		
CD(P=0.05)	0.014	0.059	0.020		
CD(P=0.01)	NS	NS	0.035		
SE(m±)	0.003	0.015	0.005		
C.V.	4.865	3.984	1.274		

DAT (Days after transplanting**)**

Chlorophyll b content (mg/g fw)

The chlorophyll a content was significantly affected with chlorpyrifos doses. In ashwagandha in general it is increased with passage of time. The maximum value was obtained at T_3 (0.75%) treatment.

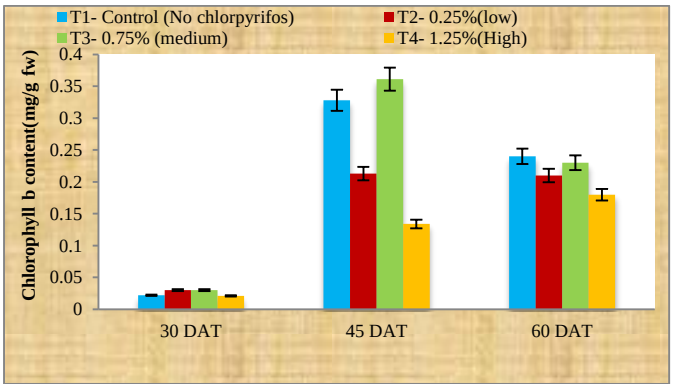

Fig 5: Impact of pesticide on Chlorophyll b content of ashwagandha at various days after treatment 30, 45 and 60 DAT

Table 5: Impact of pesticide on chlorophyll b content (mg/g fw) in ashwagandha leaves at various growth phases *i.e.*, 30, 45 and 60 DAT.

Chlorophyll b content (mg/g fw) Ashwagandha	DAT 30	DAT 45	DAT 60	Total	Mean
T_1- Control (No chlorpyrifos)	0.022	0.328	0.240	0.590	0.197
T_2- 0.25%(low)	0.030	0.213	0.210	0.453	0.151
T_3- 0.75% (medium)	0.030	0.361	0.230	0.621	0.207
T_4- 1.25%(High)	0.021	0.134	0.180	0.335	0.112
Mean	0.026	0.259	0.215		
CD(P=0.05)	NS	0.070	NS		
CD(P=0.01)	NS	0.115	NS		
SE(m±)	0.005	0.017	0.012		
C.V.	27.766	9.537	8.056		

DAS (Days after sowing**), DAT (**Days after transplanting**)**

Total Chlorophyll Content

The total chlorophyll content affected with chlorpyrifos doses. In ashwagandha increased concentration of pesticide decreased the total chlorophyll content gradually (Table no 6 fig no. 6)

Ito *et al.* (1996) also reported the increased chlorpyrifos concentration. This increase in chlorophyll- b may be due to the inter conversion of chlorophyll-a to chlorophyll-b. The loss of chlorophyll content in treatment may be due to the interference in fat metabolism inhibiting root and shoot growth, photosynthesis, nutrient uptake, leaf area, biomass etc. recorded by Pandolfini *et al.* (1992). Reduction in Chlorophyll contents at high concentrations of chlorpyrifos may be due to the inhibition of their biosynthesis or breakdown of pigments or their precursors and decrease in leaf area with increasing concentration of pesticides, which is in agreement with the study on inhibitory action on photosynthetic apparatus by Sresen *et al.* (2000) on Chlorophyll of spinach. Similar results have been obtained by Ashrafi *et al.*

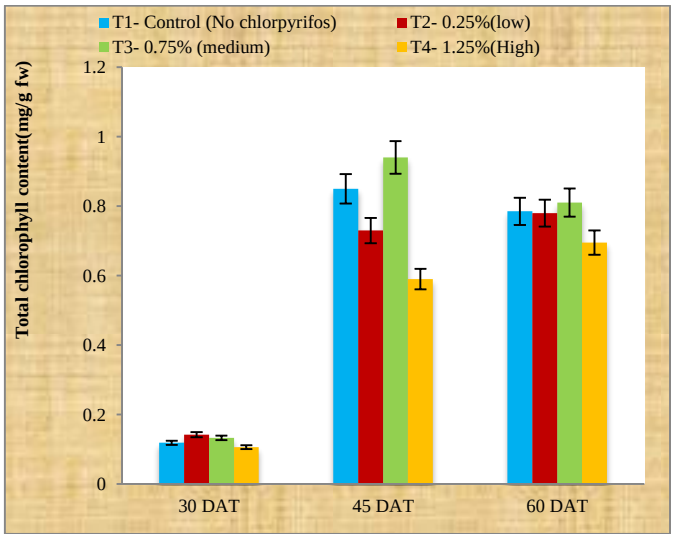

Fig 6: Impact of pesticide on Total chlorophyll content of ashwagandha at various days after treatment 30, 45 and 60 DAT

Table 6: Impact of pesticide on total chlorophyll content (mg/g fw) in ashwagandha leaves at various growth phases *i.e.*, 30, 45 and 60 DAT.

Total chlorophyll content (mg/g fw) Ashwagandha	DAT 30	DAT 45	DAT 60	Total	Mean
T_1- Control (No chlorpyrifos)	0.119	0.850	0.785	1.754	0.585
T_2- 0.25%(low)	0.142	0.730	0.780	1.652	0.551
T_3- 0.75% (medium)	0.133	0.940	0.810	1.883	0.628
T_4- 1.25%(High)	0.106	0.590	0.695	1.391	0.464
Mean	0.125	0.778	0.768		
CD(P=0.05)	0.017	0.105	0.038		
CD(P=0.01)	NS	0.161	0.069		
SE(m±)	0.004	0.026	0.009		
C.V.	4.912	4.726	1.724		

DAT (Days after transplanting**)**

Conclusions

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 *ie.*, 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. The ashwagandh was initially affected but later it increased its morphophysiological traits.

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