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Studies on the effect of application of carbaryl insecticide on Alfalfa (*Medicago sativa* L.)

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Abstract

Alfalfa (*Medicago sativa* L.) is considered as a highly valued legume forage crop. It is infested by various pests particularly the alfalfa weevil. Carbaryl, a carbamate insecticide is used to control these pest infestations. Insecticides are earlier reported to have affected non-target plants but their frequent application to the target plants is also a matter of concern. This study focuses on effect of three different concentrations i.e. Field application dose (FR), 2FR, 10 FR of carbaryl on germination and biochemical parameters of Alfalfa seeds. Additionally, the activity of antioxidative enzymes in leaf tissues was determined. A significant dose dependent decrease ($p<0.01$) in germination percentage and early seedling growth was observed in seeds treated with different concentrations of carbaryl. Interestingly reduction in soluble sugar content, free amino acid content and amylase activity was found in the cotyledons of seeds treated with carbaryl, while the total protein and insoluble sugar content increased significantly. Foliar application of carbaryl showed dose dependent increase in peroxidase (POD) activity in leaf tissues. Leaf protein analysis showed alterations of certain protein bands in carbaryl treated samples. Results obtained indicated carbaryl induced oxidative stress response in Alfalfa.

Keywords:

Alfalfa;
Carbaryl;
Peroxidase;
Germination;
Oxidative Stress.

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1. Introduction

Alfalfa is also known as the "queen of forages" for its high productivity, forage quality, ability of nitrogen fixation, flexible adaptability as well as soil profits [1]. Owing to its nutritional properties, Alfalfa is also effectively incorporated into diets of dairy cattle, sheep, goat and horses norms as recommended by dieticians [1]. Alfalfa is also widely consumed by humans as "alfalfa sprouts" or nutritional supplements prepared from Alfalfa leaves [1]. This crop is often infected by various pests including the alfalfa snout beetle (*Otiorhynchus ligustici* (L).) [2], the parasitic microsporidium *Nosema otiorhynchi* [2] and the infamous Alfalfa Weevil (*Hypera postica*) (Coleoptera: Curculionidae) [3]. Chemical control measures (applied as foliar and seed treatments) are commonly used for such pest control. However, excessive and indiscriminate use of such chemical pesticides often effect nutritional properties of target plants besides causing environmental

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contamination [4]. Long term application of different pesticides also reportedly causes persistent ecological damages [4].

Carbamate are commonly used for preventing and controlling a wide range of pest infestation in different crops [4]. Such pesticides are found to be responsible for suppression of aerobic respiration in plants and interfere with mitotic cell division in both plants and animals [4]. Carbaryl (1-napthyl methyl carbamate) is one such widely used pesticide belonging to the carbamate family and is a carbamic acid derivative [4]. However, carbaryl has been widely reported to be responsible for inhibition of plant growth and cellular respiration [4]. Previous studies have also reported carbaryl to reduce protein mobilization, depletion of nucleic acids, generation of oxidative stress in plant cells and cause changes in nutritional patterns that may exert secondary effects on the ecological components [4].

The present study investigated the effect of carbaryl in field recommended doses both (FR) and that exceeding FR (2FR and 10FR) in the form foliar and seed treatments on germination characteristics, growth, biochemical and enzyme parameters of Alfalfa seeds and seedlings for understanding the impacts of exogenous application of the carbaryl insecticide on target plants.

2. Research Method

2.1. Germination study

Germination studies were conducted according to Goswami *et al.* [4]. Alfalfa *(Medicago sativa L.*) and carbaryl insecticide were obtained commercially. Seeds of uniform size were surface-sterilized in 0.1% *(w/v)* $HgCl_2$ and thoroughly rinsed thrice using sterilized distilled water. Fifty seeds were placed on a petridish (12 cm diameter) containing a sterilized filter paper moistened with 5 mL of respective treatment including control (distilled water) and FR, 2FR and 10FR dilutions of cabraryl insecticide prepared in distilled water (0.125% w/v, FR, 0.25% w/v and 1.25%w/v respectively). The whole experiment was repeated thrice. All petridishes were incubated for seven days in a seed germinator (Yorco Y58765) in dark at 25 ± 2 °C. Emergence of root tips of length 1 mm and higher were accepted as germinated. The germination % of seeds were recorded after each 24 hours upto 120 hours following emergence of radical. Root lengths were determined after 120 hours of onset of germination.

The percentage of inhibitory effect on germination to control was calculated by using the following formula [5]:

$I = 100 - (E_2 \text{ X } 100/E_1)$ …….. (1)

Whereby, I, E_1 and E_2 denoted the % inhibition, response in control and response in treatment respectively.

The percentage of phytotoxic effect on germination and growth parameters to control was calculated as follows [6]:

PI = [(radical length in control- radical length in test] x 100)/radical length in control] ...…….. (2)

Whereby, PI is the percentage phytotoxicity.

Fresh weight and dry weight of Alfalfa seeds were determined before and after oven drying seed samples at 80 ± 2 °C for 12 h [7].

2.2 Analysis of biochemical parameters of treated seeds

Analysis of biochemical parameters of treated seeds were conducted according to Goswami *et al.* [4] and Dkhil and Denden [8]. Biochemical parameters of treated seeds including soluble and insoluble carbohydrate, protein and free amino acid content were analysed according to Trevelyan & Harrison [9], Lowry *et al.* [10] and Rosen [11] respectively.

2.3 Foliar application of pesticide

The four-week-old Alfalfa seedlings were grown in flower pots in greenhouse. The seedlings were sprayed with different treatments including control (water) and different dilutions of carbaryl (0.125% w/v, 0.25% w/v and 1.25% w/v) prepared in water corresponding to FR, 2FR and 10 FR dose of carbaryl. Leaf samples were collected from each treatment after 24 h of insecticide application for analysis of protein profile and activity of antioxidant enzymes.

2.4 Leaf protein profiling

Leaf protein was extracted by homogenizing 100mg of leaf tissue in 1.0 ml (10% w/v) of phosphate buffer pH 7.0. The homogenate were centrifuged at 12,000g at 4°C and supernatant were used for protein assays.

Estimation of total protein in leaves of seedlings was done according to the method proposed by Lowry *et al.* [10] using bovine serum albumin (BSA; Sigma Chemical, St. Louis, USA) as standard protein.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of the protein samples was carried out according to Laemmli *et al.* [12]. Immediately after electrophoresis, the gels were subjected to Coomassie blue staining overnight on a gel rocker and further destained using distilled water and scanned using HP Jet scanner.

2.5 *Assay of antioxidant enzymes*

l00 mg leaf tissue was collected from plants subjected to different treatments, washed thoroughly using distilled water, homogenized with chilled phosphate buffer (0.1M, pH 7.0) and centrifuged at 14,000g for 15min at 4 °C. The resulting supernatants stored at -20 °C for further studies. The protein concentrations of leaf crude extract were determined according to the Lowry *et al*. [10]. Alterations in catalase (CAT) and soluble peroxidase (POD) activities of the leaf tissues were determined according to Chandlee and Scandalios [13] and Cippolani [14] respectively with some modifications [4]. Changes in enzyme activities were determined spectrophotometrically using a UV Spectophotometer (Shimadzu UV -1601).

2.6 Analysis of peroxidase in gels

Native PAGE was carried out according to Goswami *et al.* [4]. Immediately after electrophoresis, the gel was immersed in phosphate buffer (100 mM, pH 6.0) for 15 minutes and repeated twice. The bands of peroxidase enzyme were developed by immersing the gel in a solution prepared with 0.25% guiacol and 0.125 % H_2O_2 in darkness for 30 min with gentle rocking.

2.7 Statistical analysis

The data were represented as Mean ± S.D. One-way analysis of variance (ANOVA) was determined using GraphPad InStat 3 (San Diego, CA, USA) in order to compare means of different treatments taking $p \leq 0.01$as significance level.

3. Results and Analysis

3.1 *Effect of carbaryl concentrations on germination and growth of Alfalfa seeds*

The treatment of Alfalfa seeds with different concentrations of carbaryl showed a significant decrease ($p \leq 0.01$) in germination percentage when compared with control. (Table 1, Fig 1).

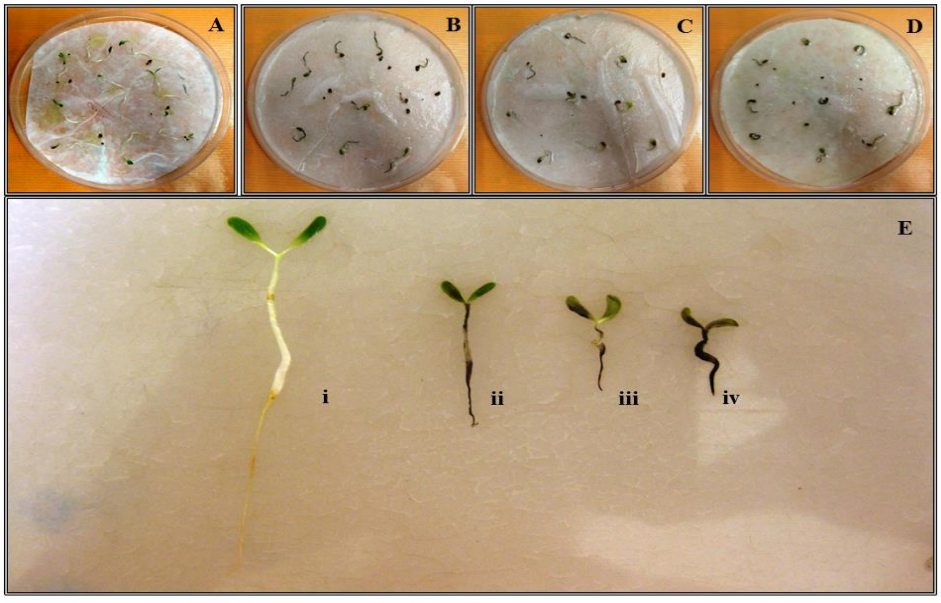

Figure 1. Effect of carbaryl exposure on Alfalfa seeds. Figures A-D showing seeds treated with control, FR, 2FR and 10FR doses of carbaryl respectively. Figure E (i-iv) showing morphological alterations in single individuals from treatments A-D respectively.

Table 1. Mean root and shoot length, % germination, T_{50}, Vigor index, % phytotoxicity, % inhibition of carbaryl treated Alfalfa seeds

Treatments	Mean root length (in mm)	Mean shoot length (in mm)	% germination after 120hrs	T_{50} (hrs)	Vigor index	% phyto-toxicity	% Inhib-ition
Control	3.05 ± 0.04	0.76 ± 0.01	100.00 ± 3.46	8.8 ± 0.69	654.12 ± 3.46	4.54 ± 0.38	-
Carbaryl FR	0.4 ± 0.05	0.32 ± 0.009	85.00 ± 2.78	11.2 ± 4.26	323.33 ± 3.21	49.99 ± 17.27	20.83 ± 5.21
Carbaryl 2FR	0.25 ± 0.04	0.1 ± 0.02	72.00 ± 10.55	14.5 ± 3.12	223.33 ± 4.93	49.99 ± 17.27	25.83 ± 7.22
Carbaryl 10FR	0.23 ± 0.03	0.09 ± 0.02	64.00 ± 10.43	15.2 ± 1.22	204.47 ± 2.02	88.11 ± 3.69	8.33 ± 11.55

Results indicated that root lengths of all seeds treated with different concentrations of carbaryl were found to be significantly reduced ($p \leq 0.01$) in comparison to seeds treated as control. Both % inhibition and % phytotoxicity calculated for seeds treated with carbaryl was significantly higher ($p \leq 0.01$) than those treated as control.

Germination of seeds is a complex process guided by various metabolic pathways. It starts with the uptake of water and is completed with the appearance of the embryo, in the form of radicle in most species of plant [15]. Alteration in germination of seeds could be linked up with impairment of certain biochemical processes during seed germination [4]. Results obtained in this study are similar to those reported previously [4, 16-20].

3.2 Effect of carbaryl concentrations on biochemical parameters of carbaryl treated Alfalfa seeds

The amount of protein, free amino acid, total soluble and insoluble carbohydrate and amylase activity in both control and treated seeds has been shown in Table 2.

Table 2. Biochemical parameters of carbaryl treated Alfalfa seeds

Treatments	Fresh Wt. (mg)	Dry Wt. (mg)	Protein content (mg/mg seed tissue)	Free amino acid content (mg/100 mg seed tissue)	Soluble carbohy-drate content (mg/mg seed tissue)	Insoluble carbohy-drate content (mg/mg seed tissue)	Amylase activity (mg of maltose produced/mg protein/hour)
Control	0.05 ± 0.002	0.01 ± 0.0007	0.10 ± 0.004	59.8 ± 4.16	0.33 ± 0.03	0.03 ± 0.001	1.06 ± 0.10
Carbaryl FR	0.05 ± 0.002	0.02 ± 0.0001	0.27 ± 0.01	47.8 ± 2.49	0.30 ± 0.03	0.10 ± 0.007	0.38 ± 0.03
Carbaryl 2FR	0.06 ± 0.004	0.01 ± 0.0007	0.39 ± 0.17	36.2 ± 1.00	0.11 ± 0.01	0.24 ± 0.024	0.29 ± 0.22
Carbaryl 10FR	0.05 ± 0.002	0.01 ± 0.0007	0.17 ± 0.003	17.3 ± 0.68	0.05 ± 0.004	0.35 ± 0.03	0.15 ± 0.01

A significant increase ($p<0.01$) in the protein content, insoluble sugar content and fresh weight and dry weight of seeds and a significant reduction ($p<0.01$) in the free amino acid, soluble sugar content and amylase activity was observed in seeds treated with different concentrations of carbaryl in comparison to those treated as control. Similar results were previously reported by Chopra and Nandra [21]. According to the results obtained in this study, the reduced formation of soluble sugars in carbaryl treated seeds may be attributed to the the suppressed amylase activity observed in the same as amylase is reportedly responsible for degradation of storage polysaccharide pool during seed germination [22]. Similar results were also reported previously [19]

3.3 Effect of foliar application of carbaryl on peroxidase and catalase activity in leaf samples

The effect of foliar application of carbaryl treatment on peroxidase (POD) and catalase (CAT) activity in leaf samples of four week old Alfalfa plants were studied in a dose dependent manner and the results have been shown in figure 2. Spectrophometric analysis of POD activity showed a significant ($p<0.01$) dose dependant increase in carbaryl treated leaf samples in comparison to those treated as control. However, plants treated with lowest (FR) concentrations of carbaryl the change is not significant in comparison to control, but the activity increased significantly in plants exposed to higher concentrations (2FR and 10FR respectively) of carbaryl. Analysis of the representative bands of POD obtained in native gels (figure 2A inset) were also in accordance with results obtained spectrophotometrically. Oxidoreductase enzymes like POD are considered responsible for metabolism of pesticides in plants [23]. The dose dependant increase of POD activity in leaf samples of Alfalfa observed in this study could have been related to detoxification mechanisms of H_2O_2 by POD [24].

However, CAT activity was found to be significantly suppressed ($p<0.01$) in plants treated with different concentrations of carbaryl in a dose dependent manner in comparison to those treated as control. This suppression of CAT activity may have had occurred due to inhibition of synthesis of enzyme and its subunits or due to a flux of superoxide radicals that are known to suppress CAT activity [4,25].

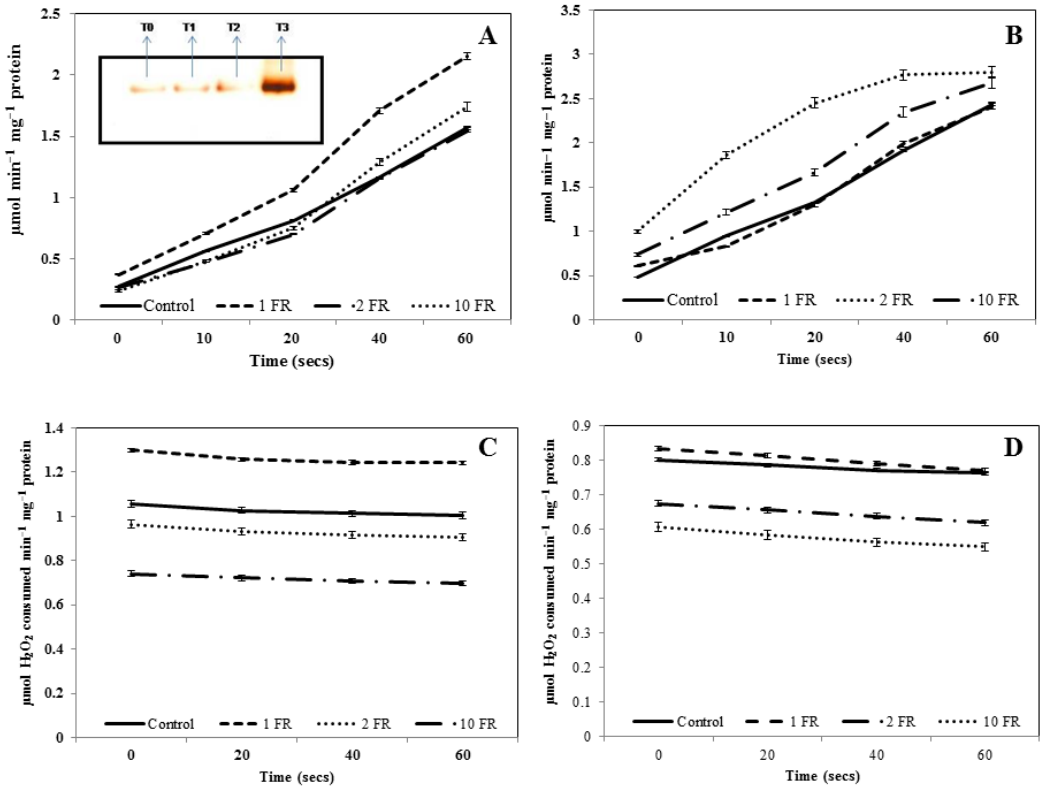

Figure 2. Effect of different treatments on peroxidase and catalase activities of Alfalfa after foliar application. Figures A and B show changes in POD activity recorded after 24 h and 72 h of exposure respectively; inset shows the substrate gel where T0-T3 represented plants treated with foliar application of control, FR, 2FR and 10FR doses of carbayl respectively. Figures C and D show changes in CAT activity recorded after 24 h and 72 h of exposure respectively.

3.4 Protein profile of Alfalfa leaves

The different protein banding patterns in the leaf extracts of control and carbaryl treated Alfalfa plants have been shown in Figure 3.

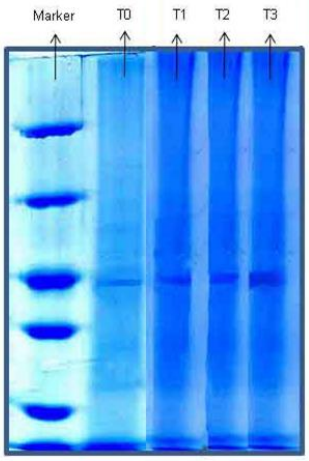

Figure 3. SDS–PAGE of samples from each dose of insecticides-treated leaves of Alfalfa (*Medicago sativa*) after 24hours of treatment. Lane Marker represents Genei molecular marker (116.3−14.4 kDa); Lane T0 represents Control; Lanes T1-T3 represent treatments of with Carbaryl (FR, 2FR and 10FR respectively).

The overall protein content was found to have increased in leaf tissues as a result of carbaryl treatment. SDS PAGE analysis of protein samples extracted from leaves of carbaryl treated Alfalfa plants exhibited an amplification of a high molecular weight band (~ 45 kDa) in samples treated with higher concentration (10FR) of carbaryl. Besides, additional bands of low molecular weight (~ 18 kDa) were also observed with increasing concentrations of carbaryl. Such amplification of protein in plants treated with different concentrations of protein may be attributed to plant response against stress induced by the foliar application of pesticide. Similar results were also previously reported by Ganguly *et al.* [26] and Goswami *et al.* [4].

4. Conclusion

The carbaryl treated Alfalfa plant showed a significant dose dependent alterations in growth, germination, biochemical and enzyme parameters in comparison to plants treated as control. Excessive exogenous application of carbamate insecticide was found to induce oxidative stress in exposed Alfalfa plants marked by significant increase in activity of peroxidase enzyme with dose. Hence results reported in this study indicates that carbamate insecticide application should be carried out judiciously and cautiously for avoiding negative impacts on target as well as nontarget plants.

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