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Studies on the role of six enzymes in the metabolism of kinetin in mustard aphid, *Lipaphis erysimi* (Kalt.)

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Abstract: *The activity of catalase, glutathione peroxidase, superoxide dismutase, O-demethylase, ATPase and succinate dehydrogenase, belonging to two main classes of detoxification enzymes (i.e. hydrolases and oxido-reductases), mostly involved in metabolism and degradation of xenobiotics in insects, were assessed under the influence of kinetin, a plant growth regulator (PGR). The nymphs (48-52hr old) of Lipaphis erysimi (Kalt.) were permitted to feed on radish plant, Raphanus sativus L. treated with kinetin (400ppm) for 13, 25 and 37hr. It was found that the activity of catalase, glutathione peroxidase and superoxide dismutase increased significantly when compared with the control of the same age group, which indicated that these enzymes might be playing a significant role in the metabolism of kinetin in this insect. The activity of O-demethylase showed an increase upto 25hr of the treatment but it decreased under prolonged treatment whereas the activity of succinate dehydrogenase fluctuated insignificantly. ATPase showed a decrease in the activity with the treatment suggesting kinetin's interference in synthesis of ATPase.*

Key words: *Detoxification enzymes, Mustard aphid, Lipaphis erysimi (Kalt.), Kinetin.*

Introduction

Scientists all over the world are trying to turn all the possible stones in exploring the possible ways and means, on one hand to reduce the load of synthetic organic pesticides and the rate of pollution and yet on the other hand to procure sufficient amount of harvest from crops, in order to meet the ever increasing need of the 6.01 billion hungry mouths.

It is a known fact that phytohormones play an essential role in regulating the life cycle events in plants such as germination, cell division, flowering, fruit ripening, seed and bud dormancy and death. They are also responsible to some extent in establishing seasonal patterns of growth and reproduction in phytophagous insects associated with plants (Scheurer, 1976). These phytohormones along with other secondary plant metabolites are now under the focus of some researchers for their exploration as potential pesticides. As, compared to organic pesticides, they are safer, environment friendly and biodegradable. Among the four major classes of phytohormones, cytokinins influence growth, cell differentiation and cell division in plants. They have also been reported to interfere in the development and reproduction of some insects (Scheurer, 1976). Schaefers and Montgomery (1973) have reported a reduction in the size of strawberry aphid, *Chaetosiphon fragaefolii* (Cockerell) when it was fed on discs of old leaves treated with Kinetin and N^6B. Both kinetin and kinetin riboside adversely affected the growth, development and survival of potato aphid, *Macrosiphum euphorbiae* (Thomas) (Chawla *et al*., 1974). Rup *et al.* (1998) had also studied the influence of fourteen PGRs including kinetin on the population built up of mustard aphid, *Lipaphis erysimi* (Kalt).

The receptiveness and response of an organism to the exogenous application of any compound is dependent upon the development of particular set of enzymatic pathways in its metabolism. In order to understand the mode of action/ influence on development/toxic effect of a compound (xenobiotics/foreign compounds), the studies related to the profile of various enzymes involved in general metabolism, constitute an integral part. Already, Rup *et al.* (2002) have investigated the influence of kinetin on the five enzymes (esterases, gluthathione S-transferases, NADH dehydrogenase, NADH oxidase and gluthathione reductase) and reported a significant increase in the activity of only two of them i. e. esterases and NADH dehydrogenase in *L. erysimi.* Therefore, it was proposed to analyse the profile of six additional enzymes, usually involved in metabolism of xenobiotics, under the influence of kinetin, in mustard aphid *L. erysimi,* a key pest of Brassica crops in India. This aphid has an intimate association with its host plant and is one among the most fecund animals of the world. It has the ability to build up population in a short span of time due to the presence of some unique physiological phenomenon in its life cycle like viviparity, parthenogenesis and polymorphism. In fact it has defied most of the conventional methods of its population control under different agro climatic conditions and the losses caused by it have been reported to vary from 35.4 % to 73.3 % (Bakhetia, 1983). Analysis of these additional enzyme systems involved in metabolism would greatly help in assessment of the possible inclusion of the Kinetin in its management strategies in addition to enhancing the understanding of the basic principles involved in insect plant interactions.

Materials and Methods

The radish, *Raphanus sativus* (Linn.) plants were grown in clay pots in a glass house for maintaining the culture of the mustard aphid, *L. erysimi* under controlled 10L: 14D

photoperiod regime. One month old (3-4 leaf stage) plants were used for experiments and sprayed with 400ppm (LC_{60}) of kinetin (Rup *et al.,* 1998) by using hand atomizer. The second instar nymphs (48-52hr old) were released on both the treated and control (sprayed with distilled water) plants for 13, 25 and 37hr. The nymphs were collected after specified treatment period and were assayed for six enzymes i.e. catalase, glutathione peroxidase, superoxide dismutase, O-demethylase, ATPase and succinate dehydrogenase.

The activities of these enzymes were estimated by using the various methodologies available in literature after incorporating tissue dependent standardizations. Catalase was estimated with the method given by Bergmeyer (1974). The nymphs were homogenised in 0.05M potassium phosphate buffer (pH 7.0) to prepare 4 ml of 5% w/v homogenate. The supernatant obtained after centrifugation at 10,000 rpm for 20 min at 4°C was used for enzyme estimation. The assay mixture consisted of 2.9ml of H_2O_2 (0.05%) and 0.1ml of the extract to obtain a total volume of 3ml. The decrease in absorbance was recorded at 240nm at intervals of 1min. for five min., at a constant temperature of 25°C.

Glutathione peroxidase activity was measured according to the method standardised by calzyme laboratories, inc. The homogenate (2ml, 5% w/v) was prepared in sodium phosphate buffer (pH 7.0) containing 5.0mM EDTA. It was centrifuged at 12,000rpm for 30min. at 4°C and the supernatant was used for enzyme assay. The assay mixture comprised of phosphate buffer (1.0ml), NADPH (0.2ml), GSSG (0.2ml), NaN_3 (0.2ml), H_2O_2 (0.2ml) and enzyme extract (20µl). The change in absorbance was recorded at 340nm at a constant temperature of 30°C.

The activity of superoxide dismutase was estimated by the methodology of Kono (1978). The homogenate (2ml, 10% w/v) was prepared in sodium carbonate buffer (pH 10.0) and centrifuged at 10,000rpm for 20min. at 4°C. The 2.5ml of assay mixture consisted of 1.3ml of carbonate buffer, 0.5ml of NBT, 0.1ml of TritonX-100, 0.1ml of hydroxylamine hydrochloride and 0.5ml of enzyme extract (the supernatant). The increase in absorbance was recorded at 540nm.

The O-demethylase activity was estimated by following the methodology of Lee and Scott (1989) after standardisation. The homogenate (2ml, 10% w/v) was prepared in tris-HCl buffer (pH 7.7). The supernatant obtained after centrifugation at 12,000rpm for 20min at 0°C was used for enzyme assay. The assay mixture containing tris-HCL buffer (1.6ml), para-Nitroanisol (1ml) and enzyme extract (0.1ml), was incubated at 34°C for 3min. NADPH (0.4ml) was added to this and the reaction was followed for 30min at 34°C, after which the O-demethylated product p-Nitrophenol was measured for its optical density at 400nm. The standard curve was prepared by using serial dilutions of p-Nitrophenol (20-100µM).

The ATPase activity was measured according to the methodology of Kielley (1969) with some modifications. The homogenate (2ml, 5% w/v) prepared in NaCl (0.9%) was centrifuged at 4000rpm for 10min at 4°C. The supernatant was used for ATPase estimation. The assay mixture comprised of enzyme extract (0.05ml), ATP solution (0.25ml), histidine buffer (0.25ml), magnesium chloride (0.1ml) and double distilled water (0.3ml). Incubated the contents at 28°C for 5min, the reaction was stopped by adding perchloric acid (1.0ml) followed by centrifugation at 4000rpm for 5min. The aliquots of the supernatant were analysed for inorganic phosphate. The reaction mixture consisted of supernatant (1.0ml), sulphuric acid (1.0ml), ammonium molybdate (1.0ml), metol (0.1ml) and distilled water (0.9ml). The optical density was read after 10min at 660nm. The standard curve was prepared by using serial dilutions of KH_2PO_4 solution (0.2µm-1µm).

Succinate dehydrogenase activity was measured according to the methodology of King (1967). The homogenate (2ml, 10% w/v) prepared in phosphate buffer (pH 7.8) was centrifuged (12,000rpm) at 4°C for 25min. The assay mixture consisted of 3.0ml. of phosphate buffer, 0.6ml of succinic acid, 0.4ml of BSA, 0.2ml of potassium ferricyanide, 0.7ml of distilled water and 0.1ml of enzyme extract/ supernatant. The decrease in absorbance was recorded at 420nm at constant temperature of 30°C. Six replications were taken in each experiment and comparisons were made by analysing the data with student's't' test.

Results and Discussion

The analysis of six enzymes after the treatment of nymphs of *L. erysimi* with kinetin revealed enzyme specific influence on their activity. The catalase activity during the normal course of development of nymphs showed that it increased by 3 fold from 1.26M/g at the age of 48hr to 3.343M/g at 85hr of age. In the kinetin treated nymphs the activity followed the same trend as that was observed in the control but the values were significantly higher in the treated groups for all the three exposure intervals (Table 1).

The glutathione peroxidase activity showed fluctuations in the nymphs of *L. erysimi* when assessed at different developmental age groups (48, 61, 73 & 85hr). The treatment of nymphs with kinetin induced an increase in the enzyme activity which was highly significant after 13 and 37hr of exposure as compared to the activity in the nymphs of the same age group in control (Table 1).

The estimation of superoxide dismutase (SOD) activity under the normal developmental conditions showed a fluctuating pattern from 48 to 85hr of age. Although in treated nymphs the SOD activity followed the same trend, but the values for treated groups were significantly higher after prolonged exposures at 25 and 37hr as compared to control of the same age group nymphs (Table 1). The O-demethylase activity showed some fluctuations during development from 48 to 85hr of age. The activity of O-demethylase in the kinetin treated nymphs remained at significantly higher levels after 13 and 25hr of exposure but on prolongation of the exposure period to 37hr, it decreased significantly (Table 1).

Table – 1: The influence of kinetin (400ppm) on the activity of six enzymes in *L. erysimi.*

Enymatic activity	Status	Treatment duration (age of nymphs) and " t " values						
		Oh (48-52 hr)	't'	13hr (61-65 hr)	't'	25hr (73-77 hr)	't'	37hr (85-89 hr)
Catalase (M/g)	Control	1.26 ± 0.026	10.14 ** →	2.37±0.106	6.76 ** →	3.26±0.076	1.76 N. S. →	3.43±0.062
	't' value			11.58 ** ↓		4.38 ** ↓		3.28 ** ↓
	Treated	1.26 ± 0.026	73.16 ** →	3.63 ± 0.019	0.04 N. S.	3.63 ± 0.035	1.21 N..S. →	3.71 ± 0.057
Glutathione peroxidase (mM/g)	Control	0.911± 0.112	0.53 N.S. ←	0.845 ± 0.05	1.34 N.S. →	0.931 ± 0.04	2.00 N.S. ←	0.082 ± 0.05
	't' value			5.37 ** ↓		0.27 N.S. ↓		5.49 ** ↓
	Treated	0.911 ± 0.112	3.09 * →	1.325 ± 0.074	5.09 ** ←	0.942 ± 0.012	6.18 ** →	1.107 ± 0.024
Superoxide dismutase (mM/g)	Control	36.28 ± 0.64	11.87 ** ←	27.45 ± 0.38	8.59 ** →	41.88 ± 1.64	4.81 ** ←	33.14 ± 0.79
	't' value			1.74 N.S. ↓		4.31 ** ↓		2.93 * ↓
	Treated	36.28 ± 0.64	37.74 ** ←	28.22 ± 0.22	7.89 ** →	61.24 ± 4.18	3.64 ** ←	42.38 ± 3.05
O-demethylase (mM/g)	Control	5.892 ± 0.018	6.10 ** ←	5.170 ± 0.117	6.71 ** →	5.982 ± 0.031	10.46 ** ←	4.573 ± 0.131
	't' value			3.86 ** ↓		36.47 ** ↓		5.19 ** ↑
	Treated	5.892 ± 0.018	12.77 ** ←	5.623 ± 0.011	52.14 ** →	7.892 ± 0.042	93.94 ** ←	3.893 ± 0.006
ATPase (mM/g)	Control	1.04 ± 0.068	28.70 ** →	4.052 ± 0.080	40.12 ** →	11.4 ± 0.165	17.07 ** →	15.355 ± 0.163
	't' value			10.40 ** ↑		2.61** ↑		29.73 ** ↑
	Treated	1.04 ± 0.068	12.70 ** →	2.658 ± 0.108	38.88 ** →	10.765± 0.179	14.97 ** ←	5.857 ± 0.275
Succinate dehydrogenase (mM/g)	Control	0.728 ± 0.083	1.41 N.S. →	0.865 ± 0.049	4.46 ** →	1.103± 0.022	1.87 N.S. ←	0.948 ± 0.08
	't' value			0.20 N.S. ↑		2.24 N.S. ↑		1.43 N.S. ↓
	Treated	0.728 ± 0.083	1.32 N.S. →	0.852 ± 0.042	0.71 N.S. ←	0.810 ± 0.042	5.37 ** →	1.068 ± 0.024

** = Significant at 1 % , * = Significant at 5 % , N.S. = Non significant

↑ = upper reading higher, ↓ = lower reading higher, → = reading on right higher, ← = reading on left higher

The ATPase activity showed a continuous increase as the nymphs matured from the age of 48hr to 85hr. The treatment of nymphs with kinetin significantly suppressed the ATPase activity after all the time exposures and the suppression was highly significant after 37hr by being approximately one third of what it was in control at the same age of the nymphs (Table 1).

Estimation for the succinate dehydrogenase (SDH) activity during the normal course of development from 48 to 85hr showed a fluctuating trend. The treatment with kinetin resulted in a decline in the activity of SDH except after 37hr of treatment where the activity was higher than in the control of the same age group of nymphs (Table 1).

Among the six enzymes investigated for their activity under the influence of kinetin, a significant increase was perceived in the activity of catalase, glutathione peroxidase and superoxide dismutase. The O-demethylase activity, which showed an initial increase after treatment, got significantly suppressed with prolonged treatment, whereas the activity of succinate dehydrogenase fluctuated insignificantly. On the other

hand, ATPase activity got significantly suppressed with kinetin treatment.

The enzyme catalase is found in all aerobic micro organisms, plant and animal cells where it decomposes hydrogen peroxide. Its involvement in the detoxification of xenobiotics has been reported in some cases. It has been shown as a positive longevity determinant in the two different strains of the house fly *Musca domestica* (L.) (Sohal *et al.*, 1987). Sharma *et al.* (1997) reported more than 57% increase in catalase activity as compared to control in the larvae of vinegar fruit fly, *Zaprionus indianus* (Gupta) under the influence of 25ppm concentration of kinetin whereas it was 120% in the pupal stage. A corroboratory increase in the catalase activity was observed by Keywanlee and Berenbaum (1990) in the fruit fly, *Drosophila melanogaster* (Meigen) when fed on plants rich in furanocoumarins and they suggested the involvement of catalase in the detoxification of oxygen radicals generated from furanocoumarins of the host plants. Later on, Figueroa *et al.* (1999) also reported a 2 fold increase in catalase activity in grain aphid, *Sitobion avenae* (Fab.) fed on 2mM DIMBOA (2,4-Dihydroxy-7-methoxy-1, 4-benzoxazin-3-one). Loayza *et al.* (2000) hypothesized that catalases played an important role in the detoxification of plant secondary metabolites such as hydroxamic acid in *S. avenae.* Different enzymes have been reported to be more active in reducing hydrogen peroxide to water in different cell lines of *Spodoptera frugiperda* (Smith) (Wang *et al.*, 2001). Catalase was more active in TN-SBI-4 cell line compared to other cell lines. Paes *et al.* (2001) concluded that in *Rhodnius prolixus* (Stal.) chemicals which resulted in increase in free radicals were metabolized by catalases. The increase observed in the catalase activity in the present findings in *L. erysimi* could be related to its significant role in the detoxification of free radicals generated by kinetin treatment.

The enzyme glutathione peroxidase (G.Px.) belongs to the family of selenoproteins and plays an important role in defence mechanisms of animals against oxidative damage by catalyzing the reduction of a variety of hydroperoxides, using glutathione as a reducing substrate. Lee (1991) noticed an inhibition in G.Px. activity under the influence of two plant phytotoxins (xanthotoxins and harmine) in cabbage looper, *Trichoplusia ni* (Hubner) and black swallow tail, *Papilio polyxenes* (Fab.). Similarly, Leszczynski *et al.* (1993) reported a decrease in the G.Px. activity in grain aphid, *S. avenae* when fed on wheat cultivar containing high concentration of cereal allelochemicals (phenolic compounds and hydroxamic acid). Wang *et al*. (2001) observed that glutathione peroxidase which is ubiquitously active in mammalian cells under oxidative stress was not active in any of the *S. frugiperda* or *T. ni* cell lines. However, induction of glutathione peroxidase reported in *Apis mellifera* Spibola with exposure to flumethrin was correlated to elevated oxidative stresses by Neilson *et al*. (2000a). In the same year Neilson *et al.* (2000b) related the activated glutathione peroxidase activity in *Coccinella septempunctata* L. to elevated oxidative stress caused due to low quality food. A significant increase in the G.Px. activity in the present findings could be associated with its involvement in reducing the oxidative stress caused with kinetin application in the mustard aphid.

The superoxide dismutase acts on superoxide radical (O^{2-}) and provides the first line of defence against toxicity from free radicals generated during metabolism and helps in development of resistance (Paes *et al*., 2001; Wang *et al*., 2001). Nivsarkar *et al*. (1991) reported that alpha –terthienyl, an insecticide and phototoxic compound inhibits SOD activity completely in all the four instars of mosquito larvae in the presence of ultraviolet light. Jiang *et al.* (1999) detected the activity of SOD in fourth instar larvae of cutworm, *Spodoptera litura* (Fab.) and compared the effects of the two photosensitive compounds, α-terthienyl and 1-phenyl-4- (3,4-methylene dioxy) phenyldiaethylene on superoxide dismutase activity under the specified conditions. Three types of superoxide dismutase were identified; Cu, Zn-SOD in mitochondrial fraction and Fe-SOD in post mitochondrial fraction in a dipteran, C*hironomus riparius* Mg. (Choi *et al*., 1999). The role played by superoxide dismutase in first line of defence system against toxicity of kinetin could be responsible for observed increase in its activity in mustard aphid.

O-demethylase is an important enzyme of cytochrome P_{450} dependent monooxygenase system. It can act on a number of substrates like p-nitroanisole, methoxychlor, p-methoxyphenol and 7-methoxy-4-methyl coumarin (Hodgson 1985). Rose *et al.* (1989) reported that the midgut homogenate from last instar of soybean looper, *Pseudoplusia includens* (Walkers) reared on leaves of resistant soybean cultivator (PI 227687) through out life had 2 fold greater O-demethylase activity than those reared on leaves of susceptible soybean cultivator (Bragg). The relationship among O-demethylase (MFO) and phoxin resistance was studied in lepidopteran, *Helicoverpa armigera* (Hubner) by Tang *et al.* (2000). The activities of pNA –O-demethylase in the resistant strains were 3.4 –fold higher than those in the susceptible strain, when enzyme activity was expressed in units of µ mol. / mg protein/ 30 min. They concluded that O-demethylase might play an important role in phoxin resistance in *H. armigera.*. In 1983, Yu studied the effects of various plant substances and host plants on the O-demethylase activity in the fall armyworm, *S. frugiperda*, maintained on a meridic diet. He found that the O-demethylase activity of larvae after feeding on these plants was as follows; Corn >Potato >Sweet potato >Soybeans. The ratio in the activity of O-demethylase for soybean and corn fed larvae was 5.7- fold. The initial increase and than suppression in the activity of O-demethylase activity in the present findings suggest that this enzyme plays a significant role in the defence of mustard aphid only initially and then only catalases perform this function.

The ATPase is an energy generating enzyme complex of respiratory chain which catalyses the synthesis as well as hydrolysis of ATP, depending upon the requirement of the body. The influence of any exogenous compound on the synthesis of

ATPase automatically affects the well being of the organism. A corroboratory report to the present findings has been cited by Luo and Bodnaryk (1988), where inhibition in ATPase activity by an insecticide, Allethein in brain of lepidopterans was observed. Another insecticide, DDT has been found to be a potential inhibitor of ATPase acitivity in *Spodoptera littoralis* (Boisd) and *A. mellifera* (Younis *et al*., 2002).

Succinate dehydrogenase belongs to complex-II of respiratory chain and is present in inner mitochondrial membrane. A decline in the succinate dehydrogenase activity with dimilin, baytex and K-othrine was observed in the bug, *Diplonychus indicus* Venk. and Rao (Raja and Venkatesan, 2001). In the present observations kinetin treatment does not seem to interfere in this enzyme system as its activity is not affected significantly.

The present investigation strengthens the hypothesis regarding the vital role played by various enzyme systems in the metabolism of xenobiotics and in conferring resistance in insects. It may be concluded that in addition to esterases and NADH dehydrogenase (Rup *et al.,* 2002) the catalase, glutathione peroxidase and superoxide dismutase might also be playing an important role in metabolism of kinetin in this aphid as their activity levels increased with treatment. They might be helping to provide an important defence mechanism against allelochemicals to the mustard aphid, *L. erysimi*. Moreover, the suppression of ATPase activity suggests the interference of kinetin in the metabolic pathway of this aphid.

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