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Topsin-M application affected nucleic acid, amino acid and phenol contents of *Cicer arietinum* L.

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Abstract

The effect of elevated concentrations of fungicide Topsin-M on nucleic acid, amino acid and phenols of *Cicer arietinum* L. was studied. Topsin-M was applied to *Cicer arietinum* L. foliage at seedling and at fruiting stage. It was observed that nucleic acid (DNA and RNA) contents of the crop decreased, while amino acid and phenolic contents increased as a result of such foliar application of Topsin-M. We found that amino acids such as glycine, asparagines, alanine, methionine, phenyl alanine were present both in control and treated plants, while cystein, proline, tryptophane and valine contents were found in higher quantities in treated samples as compared to control ones. Reduction in nucleic acid, changes in amino acids and an increase in total phenolic contents were significantly higher when higher concentration of fungicide was applied to the crop.

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Introduction

Agricultural practices, observed through the physiological aspect, aim at maximizing the photosynthetic efficiency of cultures and directing photo-assimilates toward the formation of grains and other yield factors, instead of other unproductive energy consumptions. Cultivated plants are often subject to a variety of toxic substances leading to important yield reductions (Ezzahiri, 2001).The infections caused by fungi impair the efficiency of the cultures, reducing the tissue area for photosynthetic activity and inhibiting the translocation of assimilates, from their source of production up to the areas of growth and deposition of yield material (grains, fruits, etc.). The diseases also deviates the photo-assimilates toward unproductive consumptions of fungicidal growth and metabolism, plant defense reactions and respiration of lesioned tissue, which can be considerably greater consumers of resources than respiration in unaffected tissues. The attack by phytopathogens thus presents a strong impact on several of the plant's physiological processes, all of which are relevant for production and quality, being that efficiency with fungicide prevents these functional disturbances in the plant. Therefore, the most important contribution provided by the pyraclostrobin molecule to agriculture is derived from its wide range fungicidal activity (Ammermann, *et al.,* 2000). Several have been developed for studying genetic diversity in plant and animal species. The most widely used DNA marker technology among plant species includes restriction fragment length polymorphism (RFLP) (Williams *et al.* 1990). Genetic diversity is a key to breeding new chickpea cultivars. Some studies using cultivated chickpea and its wild relatives have estimated the genetic variation with isoenzyme analysis and seed storage protein analysis (Ahmad and Slinkard 1992).

Fungicides are those chemicals which control the infection of the pathogens. Topsin-M [1, 2 di –{3-methyl carbonyl 2- thioureido} benzene] is a systemic fungicide in which the active ingredient is thiophanate – methyl. It belongs to the chemical family benzimidazole effective against wide range of foliar disease. It is used against the control of diseases affecting legumes, cucurbits, malvaceous and solonaceous crops (Sobti, 1993). Although these fungicides are used against diseases but some time their use also disturbs the metabolic processes (Siddiqui and Ahmed, 1996) and indiscriminate use of the systemic fungicides could causes direct and indirect problems and causes phyto toxic effect on the host (Mukerjee and Gopal, 1996; Singh, 1991). It is presumed that the metabolic changes induced by systemic fungicides effects the host metabolism (Berger and Cwick, 1990). It has been suggested that plants sprayed with chemical pesticide suffer from chemical stress and phenolic compound produced as a result of stress may act as a protective compounds against pest and diseases (Friend, 1977, Siddiqui, *et al.,* 1997). Phyto toxin in the form of phenolic compounds are responsible for limiting cell division, nodulation and respiration, photosynthesis, disruption of cell membrane and reduction in total protein and carbohydrate content of the various plant species (Siddiqui, *et al.,* 1997).

The present investigation was held on Chick pea (*Cicer arietinum* L.) plant to examine the effect of systemic fungicide Topsin-M, on amino acid, nucleic acid (DNA and RNA) and phenolic contents.

Materials and methods

The present investigation was conducted in the field on *Cicer arietinum L.* at Department of Botany, University of Karachi. Experiment was designed as completely randomized block. In the field 12 rows each of 6m² were prepared for study. Three replicates for each treatment including control were used. Seeds were surface sterilized with 1% $HgCl_2$ and sown in rows with distance of 30 cm. plants were irrigated twice a week. Different solutions of Pesticide (Topsin-M) i.e. 1000ppm, 1500ppm and 2000ppm were prepared and sprayed on plant twice.

First Spray= 15 days after emergence of seedlings
Second Spray= At fruiting stage.

Leaf samples were collected after each spray for the

analysis of nucleic acid, amino acid and phenols as:

Extraction and Estimation of Nucleic Acid

RNA and DNA and DNA were determined by the method of (Schmidt and Thannhauser, 1945). For the extraction, 0.5 gm plant material was crushed in mortar with 0.5 PCA (chilled) and kept for 30 minutes at room temperature and then centrifuged at 1000 rpm for 5 minutes. The supernatant was discarded. The residue was again centrifuged in 1 ml of 0.2N PCA (chilled) at 1000 rpm for 5 minutes. The supernatant was again discarded and residue was again centrifuged in 1 ml of ethanol: ether (1:1) at 1000 rpm for 5 minutes. Supernatant was again discarded.

Removal of RNA

1 ml of 0.3N KOH was added to the residue and kept for 1 hour at 37°C. After incubation 2 drops of 70% PCA was added and then 1 ml of 1N PCA (chilled) was added and allowed to stand for 10 minutes and centrifuged at 1000 rpm for 10 minutes. Supernatant was pooled for the estimation of RNA. To the residue 0.5ml of 0.5 N PCA (chilled) was added and centrifuged for 3 minutes at 1000 rpm. Supernatant was pooled with previous one for RNA estimation.

Removal of DNA

To the remaining residue 1 ml of 0.5N PCA (chilled) was added and kept in boiling water bath for 10 minutes and the cooled. Centrifuged it for 5 minutes at 1000 rpm and supernatant was cooled. To the residue 0.5 ml of 0.5N PCA was added and centrifuged for 5 minutes. The supernatant was pooled with the previous one and treated for DNA estimation.

Reagents

1. Orcinol reagent: 1 gm $FeCl_3$ dissolved and made upto 1 L with extra pure HCl. Just before use dissolve orcinol in $FeCl_3$ solution) 1 mg/ml of $FeCl_3$ solution).

2. Diphenylamine Reagent: 27.5 ml of concentrated H_2SO_4 made up to 1 L withglacial acetic acid. Just before use add DPA in the acid solution in the ratio of 10ml/ml.

Estimation

RNA

For the estimation of RNA to 1 ml of supernatant 4 ml of orcinol reagent was added. The resultant solution was heated in boiling water bath for 20 minutes and then cooled. For reagent blank 1 ml of 0.5N PCA was taken and treated in the same way. Optical density was recorded at 600nm against reagent blank.

DNA

To 1.5 ml 4.5 ml of DPA reagent was added and heated in boiling water bath for 20 minutes. The solution was cooled. Blank was prepared by taking 1.5 ml of 0.5N PCA treated in the same way. Optical density was recorded at 600nm against reagent blank.

Amino Acid Estimation by Harbon (1987)

I gm of plant material was cut into small pieces and placed in a conical flask. 10 ml of 80% ethanol was added and boiled on water bath for 10 minutes. Then the material was kept overnight. It was centrifuged twice at 4000 rpm. Supernatant was taken in another tube and the volume of the supernatant was reduced upto 1 ml at 40 °C. After that 1 ml of 50% ethanol was added to the reduced extract to make the volume upto 2 ml.

Plant material was loaded on a chromatogram and dried at room temperature. The chromatographic tank was saturated by the solvent which was used for the estimation of amino acids. The chromatogram was put into the tank carrying the solvent n-butanol, acetic acid and water at ration of 120:30:50 for 7 hours (Harbor, 1973). After that chromatogram was dried at room temperature the spraying was done on the chromatogram with 0.2% ninhydrin in acetone. After spraying the chromatogram was immediately put into heating chamber at 80 °C for about 10 minutes. Amino acids appeared as colored spots. All the spots were marked and examined under ultraviolet light. Rf values were calculated by taking the distance traveled by solute and the distance

traveled by solvent. For the identification Rf values of unknown amino acids were compared with the standard map of known amino acids.

Determination of Phenolic Compounds

Estimation of phenolic content is based on Folin-Ciocalteu method (1927) as modified by (Swain and Hillis, 1959).

Reagent A

Folin-Ciocalteu reagent 1:9 ratio (1ml Folin-Ciocalteu reagent + 9 ml of distilled water).

Reagent B

Saturated solution of sodium bicarbonate

1 gm sample was plunged into 1N hot HCl with the result that the tissues were killed immediately. After that tissues were crushed in a mortar using 10 ml of 1N HCl. The crushed material was taken in a tube and boiled for about half an hour on water bath. Then it was filtered and the filtered was left out in vacuum over CaCl2 in a desiccator's room temperature until drying. 0.5 ml of pure ethanol was added to the dried extract. After 5 minutes 0.1 ml of extract was taken in another tube and 0.5 ml Folin-Ciocalteu reagent (1:9) and 5 ml of distilled water was added to the extract. Tube was shaken for 3 minutes and then 1 ml of saturated NaHCO3 solution was added. Tube was shaken again and then incubated about half an hour at 26 ^{o}C. Optical density was recorded at 660nm against reagent blank. For reagent blank, 0.1 ml of distilled water was taken as treated in the same manner as the phenol solution. Amount of total phenolic content (ug/ml) was calculated by using standard curve.

Results and discussion

Nucleic acid (DNA and RNA), amino acids and phenolic contents are affected by fungicide Topsin-M in the treated plant at high concentration as compare to control. It has been reported that stress condition causes abnormalities in the biochemical pathway due to which toxic phenolic compounds like flavones are formed (Reid *et al.,* 1992).

Table 1. Effect of elevated concentrations of fungicide Topsin-M on different amino acids of *Cicer arietinum* L. when applied at seedling stage.

Amino Acids	Control	1000 ppm	1500 ppm	2000 ppm
Cystein	-	-	+	++
Aspartate	-	-	-	-
Threonine	-	-	+	-
Glycine	+	-	+	+
Glutamate	-	-	-	-
Arginine	-	-	-	-
Tyrosine	-	-	-	-
Proline	-	+	+	+
Asparagine	+	+	++	+
Tryptophane	-	++	+	++
Alanine	++	+	-	+
Methionine	-	+	++	++
Phenylalanine	+++	+	++	+
Leucine	+	+	-	+
Valine	-	+	+	+

Phenolic content are significantly ($P<0.001$) increased in the treated plants with respect to concentration as compare to control (Fig. 1) that indicated the stress condition developed by the use of agrochemicals (Siddiqui and Ahmed, 2006). It has been suggested that the plants treated with agrochmicals such as systemic fungicides suffer from the chemical stress (Siddiqui*et al.,* 1997) and these phenolic content which produced due to stress act as a protective compounds against pathogens and other pests (Friend, 1977).

Table 2. Effect of elevated concentrations of fungicide Topsin-M on different amino acids of *Cicer arietinum* L. when applied at fruiting stage.

Amino Acids	Control	1000 ppm	1500 ppm	2000 ppm
Cystein	+	-	-	+++
Aspartate	+	-	++	+
Threonine	+	-	++	+
Glycine	-	-	+	+++
Glutamate	++	-	-	++
Arginine	+	++	++	+
Tyrosine	+	-	-	+
Proline	-	+	++	-
Asparagine	+	-	+	++
Tryptophane	+	+	++	++
Alanine	+	+	++	+++
Methionine	+	-	+	++
Phenylalanine	+	+	-	++
Leucine	+	-	-	++
Valine	-	+	+	-

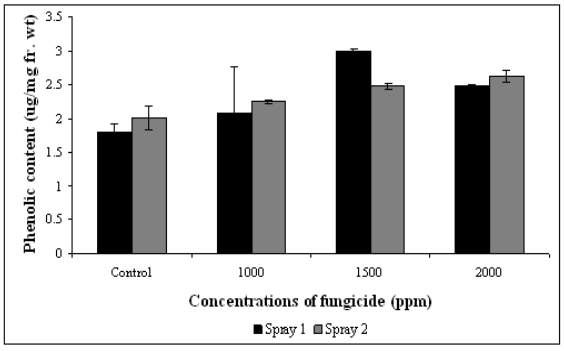

Fig. 1. Effect of different concentrations of Topsin-M on phenolic contents of *Cicer arietinum* L.

Nucleic acids (DNA and RNA) contents were significantly (P< 0.001) reduced with the higher concentration (2000 ppm) of the Topsin-M (Fig. 2). It has been suggested that reduction observed in nuclear DNA content in maiz stem tissues after fungicide treatment and this reduction of DNA in maize seedling due to fungicide treatment has been observed to be concentration dependent (Murphy *et al.,* 1996). Certain metabolites and natural hormones which effected with the pesticides inhibited protein synthesis in various ways. These compounds enter the nucleus, precipitate or remove the histone shields and uncover some portion of the DNA code for transcription due to that the RNA content was decreased in the higher doses of fungicides. It has been suggested that 2,4-D decreased number of histone suggesting that the herbicides influence protein synthesis by acting at the histone DNA level (Cherry, 1976). Topsin-M at higher concentration induced greater inhibitory effect in RNA and DNA levels of the *Cicer arietinum* L. plant.

Amino acids which were observed in treated and control plants neutral amino acids i.e. Glycine, Aspargine, Alanine, methionine and phenyl alanine were found in both treated and control plants (Table 1). While cystein, proline, tryptophane and valine were found only in appreciable amount in treated samples as compare to control after first and second spray. The accumulation of amino acids might be the consequent of protein hydrolysis which might enter the tricarboxylic acid cycle either pyruvate by diamination or by transamination with alpha ketogluteric acid or oxalo acetic acid [Siddiqui and Ahmed, 2000]. Activation of enzymes involved in amino acid and amides biosynthesis in plants have been observed in certain cases resulting in the increase level of various amino acids and amides [Ahmed *et al.,* 1985]. Resistance to the simple

protectants fungicides such as copper and sulphur compounds is rare and unimportant [Ashidu, 1965]. In general the development of resistant strain is not a major problem but resistance to the systemic fungicide is much more important. [Schroeder and Provvidenti. 1969] reported the occurrence of strain of the cucumber mildew pathogen *Sphaerotheca fuliginea* which was highly resistant to benomyl. Since then the development of resistance to benomyl in various pathogens has been reported [Bent, 1978]. There is evidence that resistant strains lack vigour and are unable to compete with sensitive ones on absence of fungicide (Hollomon, 1978).

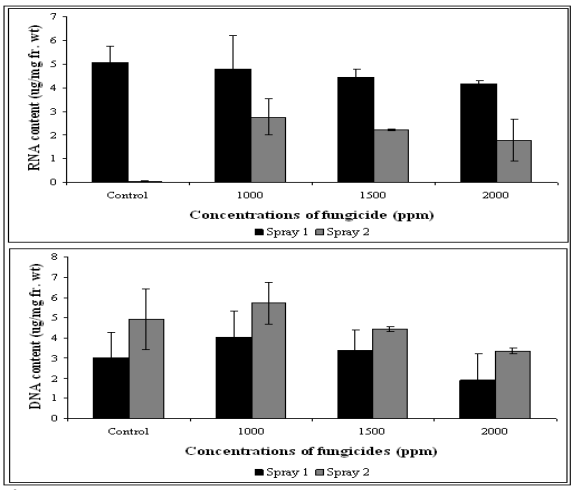

Fig. 2. Effect of different concentrations of Topsin-M on nucleic acid contents of *Cicer arietinum* L.

The present investigation suggested that repeated application of systemic fungicides and their high doses are injurious for host plant and results in decrease on nucleic acid and change in metabolism. The basis of present finding suggested that criteria regarding for the use of systemic fungicides against disease should be followed strictly with regards to their concentration, time of application and the stage of host in order to reduce phytotoxicity.

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