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INTERACTION OF ANTHROPOGENIC POLLUTANT WITH DISEASE INCIDENCE IN A HIGH ALTITUDE AMARYLLIDES AND ITS CONTROL.

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ABSTRACT

The plant *Hippeastrum vittatum* of Amaryllidaceae family, growing exclusively at a high altitude, near Mansar Lake, Jammu(North India), showed a characteristic infective lesions. Here, the mechanism of disease initiation in leaves, its stimulating cause and correlated metal interaction was studied, with the aim of suggesting an effective control. Infection is a natural phenomenon: which may be due to air-borne fungal spore and increased anthropogenicity, including significant bloom in Tourism in the recent years, thus affecting the vegetation cover in the Lake vicinity. The total disease incidence on the leaf was found to be 20%-30% and fungal pathogen *Alternaria* spp. was isolated from the leaf. Analysis of the phyllosphere pollutant revealed detectable concentrations of Copper and Zinc. Metal tolerance of the same fungi was performed and percentage germination was studied. Verification of Koch's postulate, showed a 3.34 mm^2 increase in disease lesion area on the leaf and the least reduction in spore frequency in case of Copper but 77% reduction in spore frequency in case of Zinc. This showed that Copper accelerated the growth of the fungus, while zinc massively regressed it. Hence, an unique antagonistic metal-spore interaction was studied. To suggest for a control, test fungicides Blitox and Bavistin were used. Fungicide bioassay demonstrated that Blitox at 1500ppm was effective in controlling spore germination. Since, Zinc also showed reduction in fungal infectivity, it can be a potent control. Precisely, our studies suggest, that effective concentrations of fungicide and zinc can protect the plant from anthropogenicity caused disease incidence.

KEYWORDS: *Alternaria* spp, anthropogenic, fungicide, *Hippeastrum vittatum*, Mansar Lake, metals.

INTRODUCTION

The Mansar Lake(Fig 1(a)) ,60 Km east of Jammu, at an altitude of 666m above mean sea level, is the largest freshwater lake of Jammu and Kashmir(North India). In the recent years, the flourishing tourism industry has increased human involvement leading to general increase in the pollutant levels of the lake environment. As shown in Fig 1(b),anthropogenic activities like disposal of sewage waste and faecal matter, overuse of pesticides etc, have all contributed to the cultural eutrophication of the lake (Kotwal, Sahi 2013)[1]. Typical red necrotic lesions with well defined margin (Mamgain, Roychowdhury 2013),[2] which is the characteristic feature of pathogenicity caused by Alternaria genus, was observed on the leaves of *Hippeastrum vittatum* of Amaryllidaceae family growing in the Mansar lake vicinity. This flowering plant was earlier observed to be damaged by fungal pathogens like *Stagonospora curtisii* (Tarabeih, Michail, 1980).[3] The characterization of the fungal pathogen was done by routinely used spore morphology study. The interaction between the fungal pathogen and heavy metals like Copper and Zinc were studied. Using the biomass reduction assay and metal uptake assays, the antagonistic effect of copper and zinc on the growth of the fungus was detected. Effective concentration of fungicide Blitox and zinc successfully reduced the growth of the fungal pathogen acting as a potent control of the diseases. The aim of this work is to report the nature of the disease in the high altitude Amaryllis, to characterize the nature of the pathogen and elucidate means of control of this disease along with possible correlation of the disease with increase in anthropogenic activity.

Fig 1(a)

Fig 1(a) and (b) showing Mansar lake being polluted due to Anthropogenic activities.

Fig 1(b)

MATERIAL AND METHOD

(a) Phyllosphere Characteristics

The infected leaf of the plant was washed with 50 ml sterile water and the pH, electrical conductivity and Total Dissolved Solid (TDS) was calculated. The water sample was

acidulated with 33% Nitric Acid and probable metal detection was done. Zinc and Copper were detected by the method of Inductively Coupled Plasma Mass Spectrometry (ICPMS). The main principle of ICPMS is ionization of the sample with inductively coupled plasma source which converts the atoms of the elements in the sample to ions. These ions are separated, detected and qualified in the mass spectrometer.

(b) Isolation and Identification of the Causal Organism

The infected leaf fragments were surface sterilized in mercuric chloride and sterile water and placed aseptically in Potato Dextrose Agar (PDA) slants and incubated for four days at room temperature (37˚C). Fungal colonies were seen on the PDA slants. Microscopic observations of spore suspension at 45X indicated the causal organism to be *Altenaria* spp.

Potato Dextrose Agar Composition

Potato-200g

Dextrose - 200g

Agar - 20g

Distilled Water- 1000ml

pH- 5.6

Streptomycin(5 mg/ml) – 6ml added to 1 litre medium to obtain final concentration of 30µg/ml (it is added to prevent growth of bacteria).

(c) Slide Bioassay

20µl of spore suspension on a clean glass slides was treated with different parameters like 5% sugar solution, 30ppm copper solution, 200pm zinc solution and 30 ppm blitox and bavistin fungicide solution were added in different combinations. These slides were placed on Petri dishes lined with Whatman no.2 filter paper and sufficient moisture was provided and incubated at room temperature for one day. The percentage germination and germ tube length were observed under microscope at 45X at these parameters.

(d) Koch`s Postulates

To verify Koch`s Postulates, healthy leaves of the same family were surface sterilized and placed on petri dishes lined with Whatman no.2 filter paper. The leaves were inoculated with 20µl of fungal spore solution along with the same parameters as in slide bioassay and incubated at room temperature for 4 days. Similar red disease lesions were observed on leaves and their cross- section under microscope showed presence of the spores of *Alternaria* spp. as denoted in the slide bioassay.

(e) Biomass Reduction Assay

In sterile 250 ml flasks, 50ml of sterile Potato Dextrose Agar media containing different concentrations of Copper(10ppm, 30ppm, 50ppm) and Zinc(100ppm, 150ppm, 200ppm) solutions, blitox(1500ppm) and bavistin (1500ppm)fungicides were inoculated with circular fungal colony discs of 1cm diameter and incubated at room temperature for 10 days. The black cottony fungal mesh formed was filtered and dried at 90˚C for 1 hour. The reduction of the fungal biomass was noted in the presence of fungicide and the change in the fungal biomass was noted in the presence of metal solutions with respect to control solution. The biomass from metal solution flasks that showed LD 50 were acidulated with 33% Nitric acid and the acidulated solutions were sent for metal uptake detection. The method of ICPMS was used to quantify the metal uptake by the fungi at LD50.

RESULTS

The project was initiated with the objective of characterizing and identifying the infecting organism causing a typical disease lesion in the high altitude *Hippeastrum vittatum* and to detect whether the anthropogenic events in that area has caused the same.

(a) Phyllosphere Analysis

The analysis of the sterile water in which the infected leaf was washed, exhibited the results as indicated in table 1.

Table 1: Analysis of water sample after washing the *Hippeastrum vittatum* leaf.

PHYLLOSPHERE CHARECTERISTICS	VALUES
pH	7.8
Electrical Conductivity	1.46µS
Total Dissolved Salt (TDS)	0.67ppm
Metals detected	• Zinc- 2mg/l Copper- 0.03mg/l

The causal organism identified by microscopic examination of spore suspension as indicated in Fig.2, obtained from inoculating the infected leaf in potato dextrose agar medium was *Alternaria* spp . The same has a growth optimum at pH 6.5-8 (Curran, 1980).[4] Comparing with the pH value of the water as mentioned in table 1, it can be inferred that the high altitude environment in which

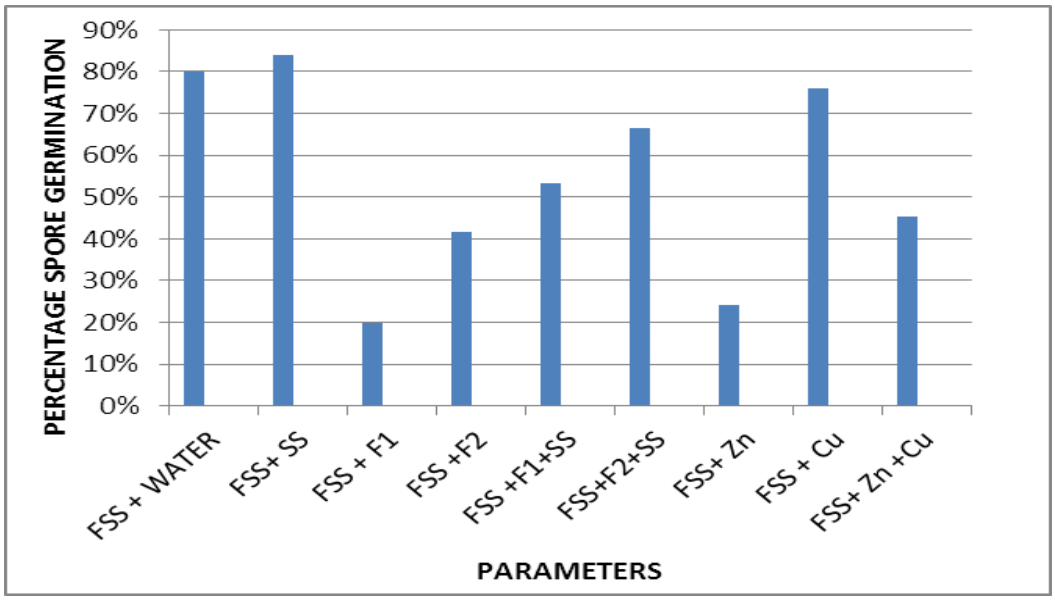
Fig 2.- *Alternaria* spp. spores in spore solution

the plant flourishes has a prevalent optimum pH, favoring the growth and germination of the infecting organism.

The detected metals from the water sample are Copper and Zinc (table 1). As studied from references (Cunnane, 1981),[5] there might be an antagonistic interaction of copper and Zinc with the causal organism. Hence the purpose of our forthcoming experimental protocols was to verify a similar interaction.

(b) Slide Bioassay

Effect on percentage spore germination of the isolated fungus due to various parameters like Sugar, Metals: Zinc (50ppm) and Copper (10ppm), fungicides Blitox and Bavistin (both 30 ppm), was observed through a Slide Bioassay as demonstrated in Table 2. The percentage spore germination of the control and the respective decrease in percentage spore germination in case of Zinc, Blitox, Bavistin and Copper with respect to the control was studied. As observed from Graph 1, Copper exhibited the minimal decrease whereas Blitox and Zinc showed the maximal, respectively. An increase in percentage spore germination in case of 5% Sugar solution assures the fact that the infecting organism *Aternaria* spp. utilizes sugar for its growth and germination.



Graph 1- Showing variation in percentage spore germination under different parameters.

Table 2: Slide Bioassay.

Parameters	Percentage Spore Germination	Increase or Decrease in Percentage	Germ Tube Length (µm)	Standard Error
a)FSS + Water (control)	80%		24.15	2.19
b)FSS+ SS	83.87%	4.8% increase	12.92	3.34
c)FSS+ F1	20%	75% decrease	9	3
d)FSS +F2	41.67%	47.91% decrease	8.4	2.19
e)FSS+F1+SS	53.3%	33.37% decrease	6	0.89
f)FSS+F2+SS	66.6%	16.75% decrease	16.8	3.34
g)FSS+Zn	24%	70% decrease	6.25	0.89
h)FSS+ Cu	76%	5% decrease	8.5	1.78
i)FSS+Zn+Cu	45.4%	43.25% decrease	12.81	2.19

NOTE: *FSS- Fungal Spore Suspension, SS- Sugar Solution, F1- Fungicide Blitox, F2- Fungicide Bavistin, Zn- Metal Zinc and Cu- Metal Copper.*

(c) Verification of Koch's postulates

Koch's postulate was performed with the same parameters as in slide bioassay, to verify the infectivity of the causal organism as in Fig. 3 and to observe the corresponding change in lesion diameter on the leaf surface and spore frequency with respect to the control (Table 3). Copper demonstrated the maximum disease lesion area with a high spore frequency whereas fungicide Blitox and metal Zinc showed a decrease in area with reduced spore frequency as reflected in Table 3. The second fungicide Bavistin influenced a lesser decrease in lesion area than that of the former.

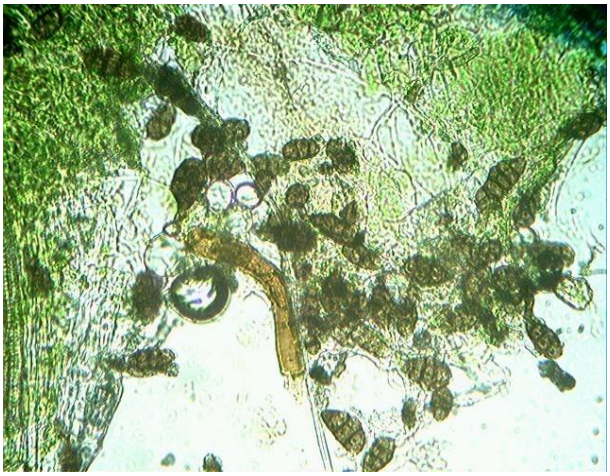
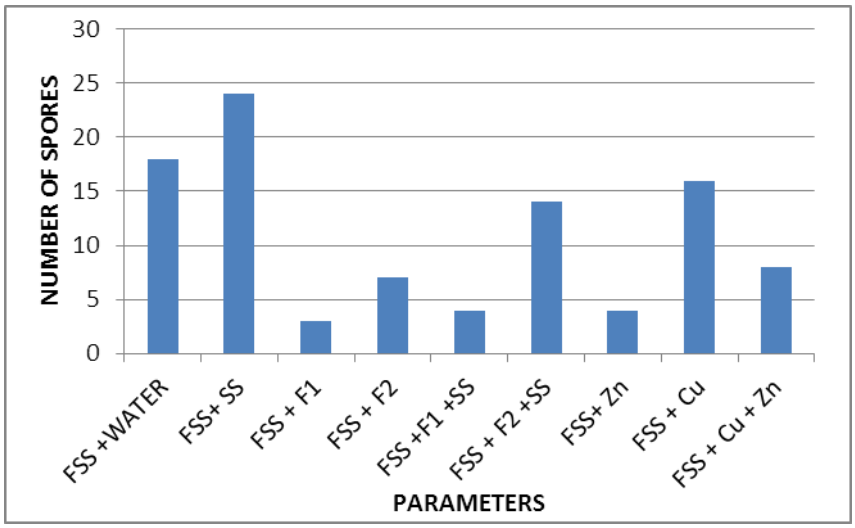


Fig 3.-*Alternaria* spp. spores in the leaf to verify Koch's Postulate

Table 3: To verify Koch's Postulate

Parameters	Total Inoculum (µL)	Total Area of Inoculation (mm^2)	Total Area of Infection (mm^2)	Number of Spores	Area Under Microscope (mm^2)
a)FSS+Water (Control)	40	201.6	12.56	18	13.93
b)FSS+SS	40	201.6	12.56	24	54.35
c)FSS+F1	40	201.6	3.14	3	0.87
d)FSS+F2	40	201.6	5.30	7	8.91
e)FSS+F1+SS	60	314.15	3.14	4	3.48
f)FSS+F2+SS	60	314.15	7.068	14	50.30
g)FSS+Zn	40	201.6	4.52	4	4.21
h)FSS+Cu	40	201.6	15.90	16	10.66
i)FSS+Zn+Cu	60	314.15	12.56	8	7.83

NOTE: FSS- Fungal Spore Suspension, SS- Sugar Solution, F1- Fungicide Blitox, F2- Fungicide Bavistin, Zn- Metal Zinc and Cu- Metal Copper.



Graph 2-Showing variation in number of spores of *Alternaria* spp. under different parameters.

So, from the results of the above experiments as indicated in Graph 2, it can be inferred that the two metals Copper and Zinc, illustrate an antagonistic interaction with the infecting organism *Alternaria* spp, as proposed earlier.

(d) Biomass Reduction Assay

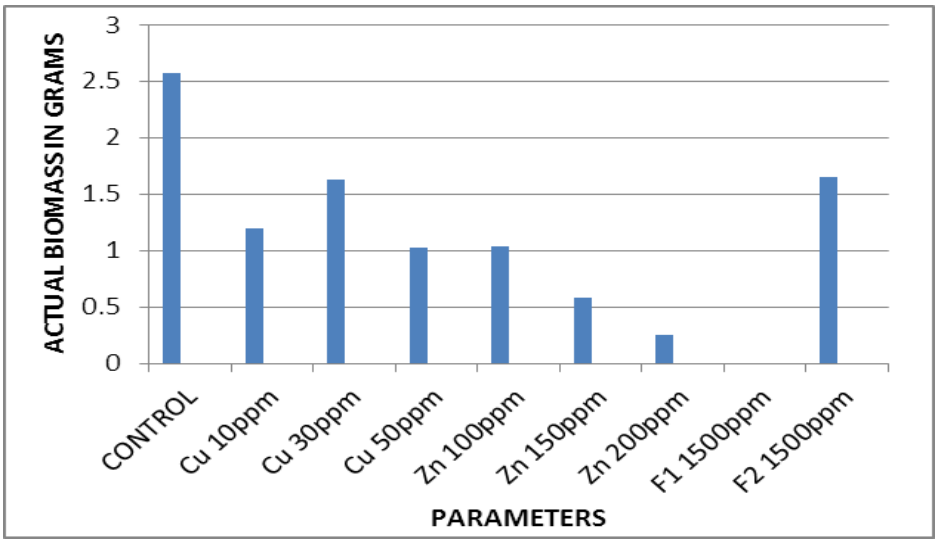
Biomass reduction assay with fungicides: Blitox and Bavistin represented that Blitox was most effective at 1500 ppm, completely inhibiting the growth of the disease inducing *Alternaria* spp, whereas Bavistin at the same concentration with respect to the control, showed a reduction in fungal biomass but did not completely inhibit it (Table 4). Similarly, in case of metals Copper and Zinc, at different concentrations, Bioassay was performed as

shown in Table 4. As shown in Graph 3, there was a prominent decrease in biomass on increasing Zinc concentration, but for Copper at the lowest concentration, the biomass reduction with respect to the control, was the least, showing a further reduction at the highest concentration. The Metal uptake ability of the causal organism was checked.

Table 4: Biomass reduction assay.

PARAMETERS	WEIGHT OF ALUMINIUM FOIL(gm)	DRY WEIGHT OF FUNGAL MESH(gm)	ACTUAL BIOMASS(gm)
a)Control	0.215	2.792	2.577
b)Cu-10 ppm	0.197	2.13	1.196
c)Cu- 30ppm	0.190	1.822	1.632
d)Cu-50ppm	0.166	1.186	1.020
e)Zn-100ppm	0.173	1.212	1.039
f)Zn-150ppm	0.217	0.794	0.577
g)Zn-200ppm	0.173	0.419	0.246
h)F1-1500ppm	0.230	No growth	No growth
i)F2-1500ppm	0.241	1.895	1.654

NOTE: *FSS- Fungal Spore Suspension, SS- Sugar Solution, F1- Fungicide Blitox, F2- Fungicide* Bavistin *Zn- Metal Zinc and Cu- Metal Copper.*



Graph 3:- Showing the actual biomass obtained at different concentrations of different parameters in biomass reduction assay.

(e) Metal Uptake

Table 5 clearly shows and suggests that *Alternaria* spp, infecting the leaf of *Hippeastrum vittatum*, had up taken considerable amount of Zinc, which thus reduced its growth, imparting a toxic effect on the same. On the other hand, Copper uptake ability was extremely less, with no proper toxicity observed in the fungi.

Table 5: Metal uptake.

PARAMETERS	METAL	CONCENTRATIONS(mg/l)
a)Control	Zn Cu	1.06 0.09
b)Cu-50ppm	Cu	>1
c)Zn-100ppm	Zn	58.80

DISCUSSION

Mansar Lake, one of the two biggest jewel lakes in Jammu and Kashmir, is the only source of fresh water to support the livelihood and biodiversity of the surrounding habitation. The littoral zone around the lake supports the wide variety of micro and macro vegetation including Acacia, *Bahunia variegate*, *Pinus gerardinia*, Barbados lily (*Hippeastrum vittatum*). Surprisingly a typical disease lesion or leaf spot was noted on a Hippeastrum plant of the pristine environment. The leaf spot was caused by *Alternaria spp*. Similar leaf spot disease by *Alternaria alternata* has been reported in *Withania somnifera* (Ashwagandha) (Pati et al., Jan 2008)[6]; *Avicenna marina* (Forski) in Saudi Arabia (Hashem, ABD allah et al.,2014)[7] and Sun flower in Greece (Anastasia, Costas).[8] It was also widely reported by other *Alternaria* spp. on Carnation (*Dianthus caryophyllus*) in seven different locations of high altitude Kashmir valley (Nissar, Khurshid, Vaseem et al., Aug 2006)[9] and in Poplar plants of Himachal Pradesh (Sharma and Sharma, 2000).[10]

Fig 4(a)

Fig 4(b)

Fig 4(a) is the *Hippeastrum vittatum* plant near mansar lake with red lesions as seen in fig4(b), from which the experiment is performed.

This leaf spot disease of *H. vittatum* is due to pervasive and increasing anthropogenecity around the lake area and a relationship between two, percentage of disease plant and human intervention has been observed. Investigations reported a serious stretch of anthropogenic activities in the lake vicinity: Cattle Bathing, Discharge of fertilizer, Insecticides, Animal excreta, Cremation Remnants and most importantly Tourism and Boating and thus

construction of building and shops for the purpose (Kotwal and Sahi, Aug 2013)[1] thus polluting the lake. The heavy metal content of Copper, Zinc, Manganese, Lead, and Cadmium has been reported (Gupta and Dogra, 1999)[11] in the lake waters, thus creating perfect conditions for growth of fungal pathogens, creating the disease lesion.

Our data indicated severe leaf infection (Increased spore frequency) due to biosorption of heavy metal Copper and reduced spore frequency of *Alternaria* spp. on uptake of Zinc; thereby establishing an antagonistic relationship of the interacting metals with the fungal species. Similar antipathetic interaction was noted in *Alternaria* spp. isolated from contaminated sites when their metal uptake ability was tested: Fungi was able to grow at Copper concentrations of 7.5-10 mM or higher, whereas high concentration of zinc was toxic, thus controlling the germination frequency (Srivastava and Sharma, Apr 2013).[12] However, some reports also demonstrated contradicting manifestations on fungal growth due to the effect of these heavy metals: Copper and Zinc; whereby *Alternaria* spp. was seen to tolerate substantial amounts of both metals (Hashem, 1997).[13]

The optimum growth and sporulation of *Alternaria* spp. was recorded on PDA medium. Our results corroborated with the findings of Ranjegathesh (2003). The optimum pH range for growth of the same was 6.5-8 which confirms with the results of Philomena et al. and Azmi et al. (June, 1997),[14] who reported that a slight neutral pH favours the growth of certain species of Alternaria.

The use of control has become an inevitable requirement for the survival of this ornamental *Hippeastrum vittatum*. From our study, Blitox was reported as the effective fungicide (1500 ppm) as compared to fungicide Bavistin for checking the disease incidence on this plant. In contrast to this, in Carnation, Blitox (Copper Oxychloride) was reported to show an increase in disease intensity whereas use of systemic fungi toxicant like Penconazole 10 EC (0.05%) was effective in controlling Alternaria leaf spot of the same during 2002-2005 (Qazi et al., Aug 2006).[9]Zinc, as per our data, reduces the disease incidence on *H.vittatum* and can be reported to be an important fungicidal agent. So to preserve the ornamental plant, spraying with Zinc.

CONCLUSION

On a concluding note, the executed protocols identified the phyllosphere metal pollutant Copper to be the probable influential cause of the disease incidence in the high altitude

Hippeastrum vittatum of Amaryllideceae family. Anthropogenicity induced pollution in the vicinity of the pristine Mansar Lake is the contributing factor for such a destruction. Another phyllosphere component, Zinc, was able to contradictarily reduce the germination of *Alternaria* spp. spores along with Blitox as an effective fungicide. So, an unique antagonism was noted between the interactions of the two metals with the causal organism.

This disease lesion on the leaf surface of *Hippeastrum vittatum* is very rare in such a high altitude and has been recently aggravated due to ever increasing consequences of human intervention. In the present scenario, the beauty of the heavenly Mansar Lake is getting diminished, thus inflicting repercussions, such as this unconventional Leaf spot. It is believed that controlled release of pollutants in such a serene spotless environment, and efficient use of fungicides such as Blitox and Zinc compounds, can only reduce the incidence of such diseases and thereby preserve the rich floral beauty.

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