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HEAVY METAL ACCUMULATION BY *ASTERELLA KHASIANA* (GRIFF) GROLLE AND *CYATHODIUM CAVERNARUM* KUNZE (BRYOPHYTES) FROM NEYYAR WILDLIFE SANCTUARY (KERALA) AND IT'S ADAPTIVE MECHANISM

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ABSTRACT

The habitat diversity, structural simplicity, totipotency, rapid rate of multiplication and high metal accumulation capacity make bryophytes a lead group for pollution studies. *Asterella khasiana* (Griff) Grolle. and *Cyathodium cavernarum* Kunze. the bryophytes growing across different regions of Neyyar wildlife sanctuary have been evaluated as indicators of metal pollution. The estimation of major heavy metals like Cu, Zn, Pb, Ni, Cd and Cr had been carried out in these bryophytes collected from three different micro habitats natural, public and traffic areas using atomic absorption spectroscopy. Liverworts collected from the natural habitats showed low profile of metal accumulation compared to traffic areas. Concentration of Cu and Pb was found to be proportionally high in the species from traffic areas compared. There was negative correlation between heavy metal accumulation and total chlorophyll pigments among the liverworts collected from public and traffic habitats. Chloroplast also showed marginal micro structural variations from natural to traffic area. Antioxidant level and total phenolic content showed positive correlation in the species collected from different habitats to mitigate metal toxicity. Random amplification of polymorphic DNA (RAPD) analysis using 10 primers produced 24 and 22 bands in *C. cavernarum* whereas, in *Asterella khasiana* 24 and 29 bands were observed in the natural and traffic habitat with base pairs ranging between 100-1200 in gel

electrophoresis. The results showed that RAPD analysis could be a useful tool for detection of genotoxic effects of metal toxicity on liverworts.

Keywords: Heavy Metal, Bryophytes, *Asterella khasiana, Cyathodium cavernarum*, Antioxidant

INTRODUCTION

Bryophytes are green terrestrial non vascular plants with simple morphological and anatomical peculiarities. They grow in a variety of habitats especially in moist places on soil, rocks, trunks of trees, fallen log and derive the nutrients directly from the substrate by diffusion. Bryophytes are used as reliable indicators of pollution **[1]**. Bryophytes have high surface area / volume ratio and are able to concentrate heavy metals remarkably than that of higher plants. Pollutants reach the plant tissues from the dry deposition in the form of gases and particles. These substances are readily accumulated and their amount exceeds from those in the surrounding habitat. So bryophytes indicate the presence of element and their concentration gradient in the respective substrata. The unique qualities of bryophytes to accumulate the elements are due to their wide distribution, ability to grow on variety of habitats, large surface area, and lack of cuticle and stomata and evergreen and ectohydric nature of plants **[2]**.

Heavy metals induce diverse cellular stress responses and damage to cellular components including proteins and DNA. DNA-based techniques, like Random Amplified Polymorphic DNA (RAPD), is used to evaluate the variation at the DNA level, and differences can clearly be shown when comparing DNA fingerprints from individuals exposed and/or unexposed to genotoxic agents. The effectiveness of a plant's antioxidant defense may be crucial for elucidating its tolerance mechanisms against heavy metals. The synthesis of diverse non enzymic and enzymic antioxidants may be important for defense mechanisms against a metals action. Low molecular weight antioxidants such as proline, ascorbate or glutathione detoxify oxygen free radicals. Non-protein compounds, rich in –SH groups, are capable of binding metal ions and forming non-toxic complexes with metals. Similarly, antioxidant enzymes such as catalase and peroxidase also mitigate the reactive oxygen species (ROSs) formed as a consequence of oxidative stress.

This study was undertaken to evaluate the impact of heavy metals accumulation among the two liverworts across three microhabitats in terms of pigment composition, ultra

structure of chloroplast, antioxidant status and the genomic DNA.

MATERIALS AND METHODS

Neyyar wildlife sanctuary is spread over the southeast corner of the Western Ghats, Kerala and covers a total area of 128 km^2. It is located between 77°8' to 77°17' East Longitude and 8°29' to 8°37' North Latitude, central location 8°33′N 77°12.5′E. *Asterella khasiana* (Griff) Grolle. and *Cyathodium cavernarum* Kunze. are the liverworts selected for the study. Identification was confirmed by comparing with the voucher specimens from the herbarium of University of Calicut. For the sake of convenience the areas considered were categorized into three microhabitats as Natural, Public and Traffic areas.

Estimation of the heavy metals by Atomic Absorption Spectroscopy

Estimation of the heavy metals in the plant samples was done by atomic absorption spectroscopy according to Allen **[3]**, using AAS (Perkin Elmer, 3030A). The various regions of Neyyar sites were chosen randomly. The estimation of metals was done in the two species.

Antioxidant potential among liverworts by FRAP assay

The total antioxidant power of a freshly prepared, cooled, and filtered infusion (5 g of dry leaves /100 mL of boiling, distilled water) of each sample was measured using the FRAP assay **[4]**.

Total phenolic content

Total phenolic content was determined using Folin-Ciocalteau reagent by measuring the absorbance at 700 nm and was expressed as mg of gallic acid equivalents (GAE) / g of sample **[5]**.

Electron microscopy

Leaf samples were fixed in a mixture of 4% formaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 48 h. The lead citrate processed tissues were examined in a Hitachi 7100 TEM instrument at 75 kV accelerating voltage.

Pigment analysis

Pigments were extracted by grinding 0.2 g fresh sample of leaves in 80% (v/v) acetone at room temperature for 24 h in the dark according to Wellburn **[6]** with some modifications.

Quantification of H_2O_2

Hydrogen peroxide (H_2O_2) concentration was determined according to Loreto and Velikova **[7]**.

Estimation of lipid peroxidation

The content of total 2-thiobarbituric acid reactive substances (TBARS) was estimated by the method of Cakmak and Horst **[8]**. The absorbance of the supernatant was recorded at

532 nm in a spectrophotometer and corrected for non-specific turbidity by subtracting the absorbance at 600 nm.

Genomic DNA Isolation and RAPD-PCR analysis

DNA was isolated from fresh frozen samples as per the CTAB method **[9]**. The PCR amplification was carried out with ten 10-base pair random primers. RAPD-PCR protocols were done according to Williams and Kubelik **[10]**. Primers with random nucleotide sequence were chosen from Operon Technologies. The PCR amplification products were separated in 1% agarose gel using Tris-Borate- EDTA (TBE) buffer and Gene Ruler 100bp DNA ladder (Fermentas, Germany). All the PCR examinations were carried out by Bioer XP thermal.

Statistical analysis

The SPSS (version 17.0) program was used for analysis of variance and comparison of the means was performed by Duncan's method at $P < 0.05$.

RESULTS

Quantification of heavy metals in the liverworts

Results of Pb, Cd, Ni, Zn, Cu and Cr assessment in the liverworts collected from different microhabitats of Neyyar Wild life sanctuaries were reflections of soil contamination. Maximum uptake capacity of Cu was found in mature thallus of *C. cavernarum* in traffic areas than the *A. khasiana*. Maximum values of accumulation of Zn were recorded in mature thallus of *A. khasiana* than the *C. cavernarum* at traffic areas of Neyyar. Similarly, analysis of thallus for Pb and Ni were also increasing from natural areas to traffic areas. Maximum accumulation of Ni has been recorded in mature thalli of *A. khasiana* than *C. cavernarum* in traffic areas of Neyyar. The thalli analysis of Pb showed that the *C. cavernarum* accumulated more in mature thalli from traffic areas of Neyyar and less in thalli of *A. khasiana* from natural areas. Maximum accumulation of Cr and Cd were observed in the mature thalli of *C. cavernarum* of the traffic areas while minimum in the thalli of *A. khasiana* in natural areas **(Table 1)**.

Metal accumulation in bryophytes was determined by soil chemistry, physical properties of the metal containing particles, space variability (vertical and horizontal) of samples, nature of the source of pollution, metal state, relative concentrations, alkalinity or pH. In addition to the amount, quality and temporal development of the emission many edaphic and biological factors also regulate pollutant accumulation in the vegetation **[11, 12]**. The solubility of heavy metals usually

increases with decreasing pH. Some metals can also be substituted for others by ion exchange. The relative accumulation of different metals in certain species may also vary with the total metal load. Major source of heavy metals in the study areas are metallurgical processes, automobile exhaust emission, oil combustion and processing of crustal material. The presence of Cu, Zn, Pb, Ni Cd and Cr elements may seriously retard the potential colonization of polluted sites by sensitive herbals. Meanwhile, liverworts can accumulate particulate matter effectively with varied accumulation due to the elemental composition and thallus tissues nature. Ni, Cu, and Cr were found to be highest range in both the liverworts as compared to Pb and Cd. The lowest concentration of heavy metals was found in the natural places of the study region due to prominence of conserved area. The traffic region experienced high concentration of metals due to heavy traffic of vehicles amounting to high air pollution. Cu and Pb are responsible for the heavy metal pollution and accumulation in both the species among the sites studied. The uptake of heavy metals in the liverworts may certainly be influenced by climate, especially humidity and wind velocity. Different plant species show varying resistance to air borne and soil accumulated toxic elements, which is reflected in their growth survival and occurrence along the pollution gradient. However, the actual degree of exposure to toxic elements is not the same for all the plant species growing at the same distance from an emission source because of difference in the elemental uptake mechanisms **[13]**. The genetic make-up of the plant greatly influences its metal uptake potential. Huang *et al*. **[14]** found that Pb accumulation varied significantly in different species grown in similar environments. It is concluded that Cu and Pb are responsible for causing pollution in the studied sites and higher accumulation of heavy metal concentration was shown by *A. khasiana* due to larger leaf surface area and more tolerance capacity as compared to *C. cavernarum.*

Pigment composition

Chl a, chl b and total chlorophyll were found maximum in the liverworts collected from natural habitats compared to public and traffic. The leafy thallus of *C. cavernarum* from natural area had higher total chlorophyll content than those of *A. khasiana*. Decrease in Chl b content was more marked than that of chl a in the tested liverworts ($P < 0.05$). Meanwhile, the carotenoid content was conspicuously greater in the liverworts of traffic habitat than in natural sites **(Table 2)**. The decline in chlorophyll content in plants exposed to metal stress is believed to be due

to inhibition of δ-aminolevulinic acid dehydratase **[15]** and protochlorophyllide reductase **[16]** associated with chlorophyll biosynthesis or impairment in the supply of Fe^{2+}, Zn^{2+} and Mg^{2+} **[16, 17]**. Similar decrease in chlorophyll content under heavy metal stress was reported earlier in cyanobacteria, unicellular chlorophytes (*Chlorella*), gymnosperms (*Picea abies*) and angiosperms, such as sunflower **[18]** and in almond **[19]**.

Ultra structure of chloroplast

C. cavernarum from natural habitat displayed chloroplasts with a lenticular shape, dense stroma with intact thylakoid membrane and packed grana. Ferritin aggregates were also noticed. However, species from traffic area showed marginal alterations in the chloroplasts such as swollen size but with loosely organized thylakoids in the stroma. Compact and electrodense precipitates were often visible in the cells. Similarly, the plant showed some enlarged plastoglobuli in the chloroplast **(Figures 1a & b)**.

A. khasiana from natural habitat showed elongated chloroplast with dilated stroma. They were probably built up from ferritin-like compounds. The thylakoid system with well developed grana composed of 10-20 thylakoids. Small electron dense plastoglobuli were present in lower number and starch was absent in all sections. *A. khasiana* collected from traffic area showed vacuolation in the chloroplast with marginal membrane injuries. Similarly, the crystalloid bodies displayed a disturbed shape, wavy appearance of granal and stromal thylakoids with swollen intrathylakoidal region. In addition, electron-dense plastoglobuli and faint cytoplasmic lipid droplets were noticed. Some chloroplasts presented dark plastoglobuli and crystalline deposits. Similarly, rounded plastids with inclusions also appeared in the plants **(Figures 2a & b)**. The microstructural analysis in the liverworts from traffic habitat suggested the marginal dislocation of thylakoids, stroma, thylakoid membrane and these changes probably, lowered the affinity between the chlorophyll and the chloroplast protein, and decreased the activity of the chlorophyll enzyme, which in turn, promoted chlorophyll break down. Pigment compositions in the species corroborate this statement. The stability of the plant chloroplast ultrastructure is closely related to metal stress resistance **[20]**. However, morphologically the liverworts not showed any serious disorders suggesting that the plants are tolerant to heavy metal accumulation.

Hydrogen peroxide (H_2O_2) content and lipid peroxidaion (LPX)

The H_2O_2 concentration of leafy thallus of the liverworts was significantly increased with traffic samples, suggesting that the most H_2O_2 accumulated in the polluted site as a consequence of metal toxicity. Lipid peroxidation profile was at par with habitat influenced by the accumulated metals ($P \leq 0.05$) **(Table 3)**. MDA was higher under traffic conditions compared with natural habitats in both the liverworts. *A. khasiana* at traffic site caused 3.5 ± 0.02 MDA content compared to 1.3 ± 0.06 at natural habitat (Table 3). Meanwhile, in *C. cavernarum* the values are 2.6 ± 0.04 and 1.2 ± 0.08 respectively. The MDA accumulation was more pronounced in *A. khasiana* than in *C.* cavernarum. Malondialdehyde (MDA) is a secondary end product of polyunsaturated fatty acid oxidation, is widely employed to measure the extent of lipid peroxidation as indicator of oxidative stress. Peroxidation of lipids in plant cells appears to be initiated by a number of ROSs. The result showed that lipid peroxidation to an extent was influenced by heavy metal stress in both the species. However, the values were less compared with other angiosperms treated with metals **[21]**. H_2O_2 concentration leaf tissue was enhanced with increasing metal toxicity **(Table 3)**. Probably it was connected with the decrease in water potential, which might have limited H_2O_2 diffusion from the place of its generation. Together with higher hydration of tissues, H_2O_2 migrates more easily within a cell and reacts with some cell compounds resulting in lipid peroxides formation.

Antioxidant potentiality and total phenol content

The phenol content significantly influenced by metal accumulation levels in both liverworts (significant at $P \leq 0.05$) **(Table 3)**. An enhancement of phenolic compounds was reported under different environmental and stress conditions. An increase of phenolics may be correlated to the increase in activity of enzymes involved in phenolics metabolism **[22]**, suggesting synthesis of phenolics under heavy metal stress. The phenolics are generally thought to prevent oxidative damage by scavenging ROS and by breaking the radical chain reactions during lipid per oxidation, these antioxidative effects require the reduced form of phenolics, in the oxidized form act as prooxidants. The antioxidant activity of phenolics is mainly due to their redox properties, which allow them to act as reducing agents, hydrogen donors, singlet oxygen quenchers and metal chelators **[23]**.

The antioxidant potentiality by FRAP assay displayed an increased profile in the liverworts collected from traffic habitats, compared with natural. The higher the FRAP

value the greater is the antioxidant activity. In *C. cavernarum*, the antioxidant potentiality increased from 90.6 ± 0.09 (natural) to 180 ± 0.05 in traffic samples. Similarly, in *A. khasiana* the respective values are 70.6 ± 0.06 and 138 ± 1.6. Under metal stress conditions, a constitutively high antioxidant capacity can prevent cellular damages due to ROS formation including H_2O_2 formation in chloroplasts. Hydroxyl and carboxyl groups in phenolics can bind particularly to iron and copper and chelate metal toxicity **[24]**.

RAPD analysis

Total genomic DNA of *C. cavernarum* and *A. khasiana* grown in natural and traffic area were isolated and RAPD-PCR was carried to evaluate the effects of traffic pollution in terms of environmental risk connected with its potential mutagenic effects in the liverworts**.** Total 10 primers were tested in RAPD-PCR experiments. The RAPD fingerprints showed sound differences between natural and traffic polluted liverworts with apparent changes in number, size and the intensity of amplified DNA fragments **(Figures 3a and b & 4a and b)**. Different polymorphic bands were detected with all the primers tested, except primer 9 and 10 with *C. cavernarum* and primer 5 with *A. khasiana*. In each case polymorphisms were due to the loss and/or formation of new bands in the traffic samples compared with the natural habitat. Similarly, differences were noticed with the change in the intensity of the bands **(Table 4)**. A total of 24 RAPD bands were observed in *C. cavernarum* from natural habitat ranging between 100-1200 bp. Meanwhile, bands were restricted to 22 in traffic samples (10 new bands and 12 shared with natural samples). The most obvious band losses of *C. cavernarum* was with primer 1, (1200, 870,700 bp bands), primer 2, (1200 bp band), primer 3 (560, 900 bp band), primer 4, (450,800, 900 bp bands), primer 7, (500 bp band) and primer 8 (320, 640 bp bands). In contrast, traffic species had some different bands i.e., with primer 1 (900, 800 bp), primer 2 (600 kb), primer 3 (520 bp), primer 4 (650, 760 bp), primer 7 (320, 540, 620, 760 bp). A total of 24 RAPD bands were detected in *A. khasiana* by using nine random primers. The obvious band losses were with primer 1 (400 bp band), primer 2 (700, 1200 bp), primer 3 (500 bp), primer 4 (680, 700 and 800 bp) and primer 10 (400 bp). The appearance of new PCR products from the species of natural habitat are primer 1 (620, 720 bp), primer 2 (740 bp), primer 3 (820 bp), primer 4 (1031, 760 bp), primer 6 (1500 bp), primer 8 (660, 800, 1031 bp), primer 9 (320 bp) and primer 10 (500, 600 bp).

Modifications of band intensity and loss of bands are likely to be due to one or a combination of the following events such as changes in oligonucleotide priming sites, mainly due to genomic rearrangements and less likely to point mutations; DNA damage in the primer binding sites; and interactions of DNA polymerase in test organisms with damaged DNA. On the other hand, the appearance of a new DNA band could occur because some oligonucleotide priming sites could become accessible to oligonucleotide primers after structural change or because some changes in DNA sequence have occurred due to mutations, large deletions, and/or homologous recombination. Appearance of new bands may also be the result of genomic template instability related to the level of DNA damage and the efficiency of DNA repair and replication **[25, 26]**. Similarly, when Taq DNA polymerase encounters a DNA adduct, there are a number of possible outcomes including blockage, bypass, and the possible dissociation of the enzyme/adduct complex, which will cause the loss of bands **[27]**. The present results support this claim that DNA polymorphisms detected by RAPD can be considered as a powerful biomarker assay for detection of the ecogenotoxic effects of environmental pollutants like heavy metals. As a tool in risk assessment, the RAPD assay can be used in characterization of metal hazard in soil. The RAPD-PCR based assay is fast, reliable and easy to conduct for assessment of environmental hazardous metals on plants **[28]**.

CONCLUSION

In conclusion, the two liverworts showed varied response of heavy metal accumulation sampled from the three microhabitats such as natural, public and traffic. The ROS H_2O_2, lipid peroxidation, pigmental composition and ultra structure of chloroplast substantiate the metal accumulation in the thallus i.e., high in traffic samples than natural habitat. However, the liverworts mitigate the oxidative stress effectively by antioxidant machinery. The varied RAPD profiles (disappearance and appearance of DNA bands) presented here also support the view that RAPD analysis was novel for hazard identification in risk assessment of ecogenotoxicological studies. Further studies are warranted at molecular and biochemical level to know the mechanism of metal tolerance in this lower group.

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Table 1: Heavy metal accumulation in *C. Cavernarum* and *A. khasiana* (mg/100g dry weight) collected from different habitats of Neyyar wild life sanctuary

Heavy metal	*C. cavernarum*			*A. khasiana*		
	Natural	Public	Traffic	Natural	Public	Traffic
Copper	3±0.98	9±0.88	15±1.3	2±0.01	7±0.46	8±0.54
Zinc	2±0.4	5±0.32	7.2±0.76	1±0.02	4±0.55	10.5±0.22
Lead	2±0.45	9±0.43	18.3±2.2	3±0.01	7±0.27	11.5±0.56
Nickel	2±0.12	4±0.25	15.4±1.4	4±0.03	10±0.82	18.5±0.49
Cadmium	0.2±0.002	1.3±0.03	3.5±0.87	0.2±0.001	1±0.002	2±0.03
Chromium	0.2±0.001	1.3±0.01	3.5±0.32	0.2±0.001	1±0.004	2±0.11

Table 2: Pigment composition (mg/g) in *C. Cavernarum* and *A. Khasiana* collected from different habitats of Neyyar wild life sanctuary

	Natural				Public				Traffic			
	Chl a	Chl b	Total Chl	Caro	Chl a	Chl b	Total Chl	Caro	Chl a	Chl b	Total Chl	Caro
C.cavernarum	0.54± 0.09	0.29± 0.22	0.83± 0.31	0.6± 0.03	0.4± 0.03	0.20± 0.05	0.8± 0.31	0.71± 0.03	0.38± 0.04	0.18± 0.08	0.56± 0.31	0.7± 0.01
A. khasiana	0.48± 0.11	0.25± 0.02	0.73± 0.01	0.6± 0.09	0.5± 0.03	0.19± 0.02	0.83± 0.31	0.64± 0.07	0.30± 0.06	0.17± 0.04	0.47± 0.31	0.6± 0.02

Table 3: H_2O_2, Lipid peroxidation, Antioxidant potentiality, Total phenol content in *C. Cavernarum* and *A. Khasiana* collected from different habitats of Neyyar wild life sanctuary

	C. cavernarum			*A. khasiana*		
	Natural	Public	Traffic	Natural	Public	Traffic
H_2O_2 (µmol/ gDW)	1.2±0.03	2.9±0.04	4.5±0.03	1.2±0.05	3.3±0.04	5.3±0.05
Lipid peroxidation (µmol/ gDW)	1.2±0.08	2±0.02	2.6±0.04	1.3±0.06	2.7±0.01	3.5±0.02
Antioxidant potentiality(µm/g)	90.6±0.09	150.5±0.01	180±0.05	70.6±0.06	85.7±0.02	138±1.6
Total phenol (mg/g)	1.1±0.04	1.6±0.88	2.2±0.56	0.98±0.59	1.3±0.27	1.87±0.99

Table 4: Changes in RAPD bands in the liverworts from natural and traffic habitats

Primers	*C. cavernarum*			*A. khasiana*		
	Natural	Traffic		Natural	Traffic	
	Total no. of bands	Total no. of bands	New bands	Total no. of bands	Total no. of bands	New bands
1	3	2	3	3	2	1
2	3	1	1	4	1	2
3	4	1	2	2	1	1
4	4	2	3	4	2	3
5	1	-	-	-	-	-
6	2	-	-	1	1	-
7	1	4	1	1	-	-
8	6	-	2	3	3	-
9	-	-	-	5	1	-
10	-	-	-	1	2	1

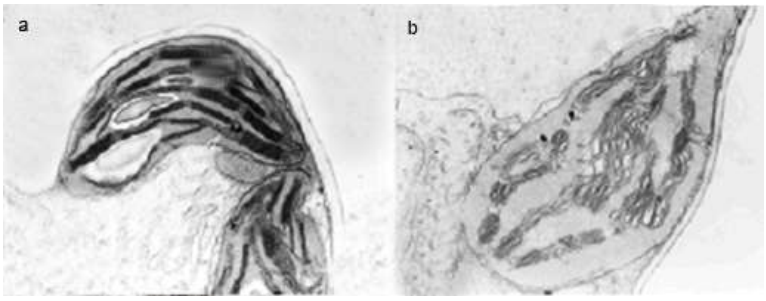

Figure 1a & b: Ultrastructure of chloroplast of *C. cavernarum* a- natural habitat b- traffic habitat

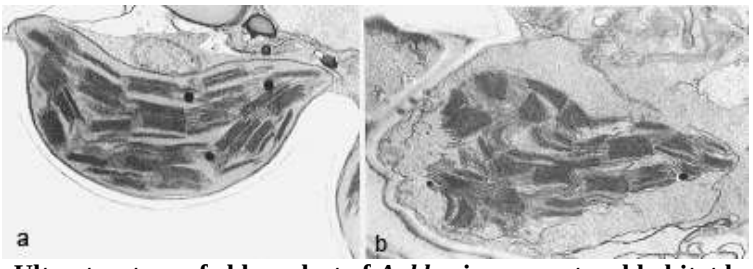

Figure 2a & b: Ultrastructure of chloroplast of *A. khasiana* a- natural habitat b- traffic habitat

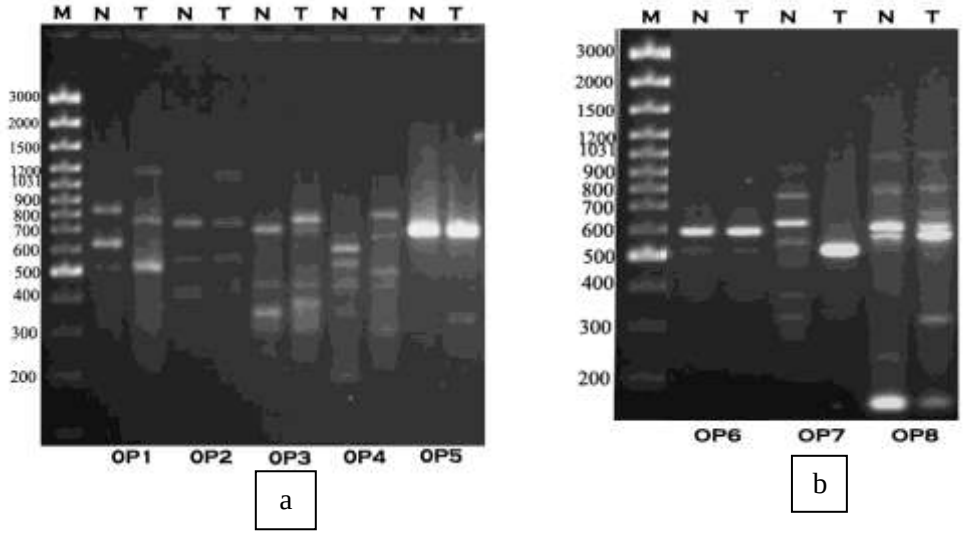

Figure 3a and b: RAPD profiles of genomic DNA from *C. cavernarum* using primers from OP 1 -5 and OP 6 - 8, M – marker, N- natural habitat, T – traffic habitat

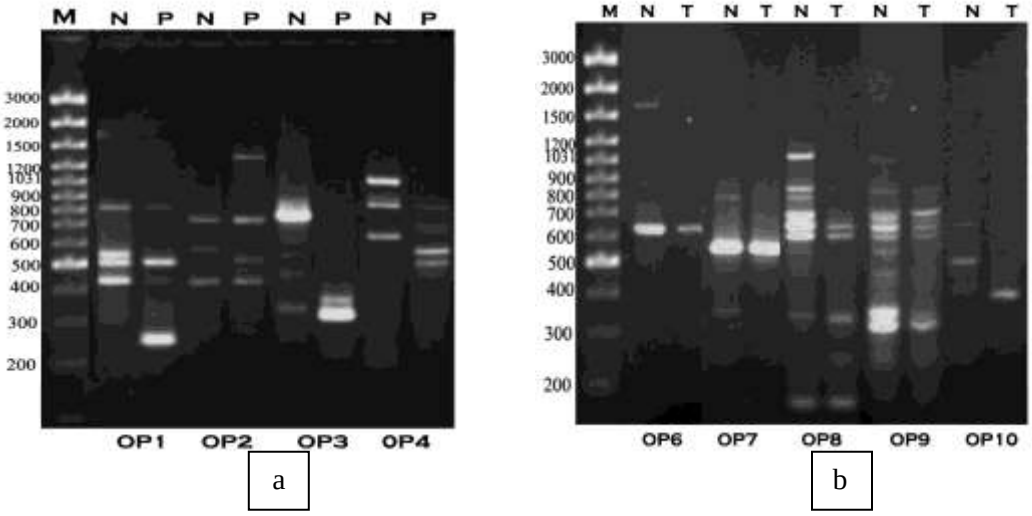

Figure 4a and b: RAPD profiles of genomic DNA from *A. khasiana* using primers from OP 1 -4 and OP 6-10, M – marker, N- natural habitat, T – traffic habitat