

Comparative hyperaccumulator of *Azolla pinnata* and *Lemna minor* for livestock wastewater treatment: morpho-physiological and genetic approach

Nor Anis Nadhirah Md Nasir

anisanasir@unimap.edu.my

Universiti Malaysia Perlis <https://orcid.org/0000-0001-9326-4002>

Irnis Azura Zakarya

University Malaysia Perlis: Universiti Malaysia Perlis

Saadi Ahmad Kamaruddin

Universiti Utara Malaysia

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Abstract

The potential of two different aquatic macrophytes, *A. pinnata* and *L. minor* to treat livestock wastewater through phytoremediation were investigated. The physiological includes the removal efficiency of Manganese (Mn) from livestock wastewater by AAS, morphological observation was performed under visual and SEM. RAPD analysis were applied to observe the DNA expression. It was observed that the removal efficiency of Mn, was higher in *L. minor* with 92% removal rate, while in *A.pinnata* RE was at 77% rate. *A.pinnata* exhibits symptoms of cell death by shrinking when exposed to livestock effluent as early as 24 hours but *L.minor* showed no changes. RAPD analysis showed that there are 19% of polymorphism in *L.minor*, in *A.pinnata* there is increase in band intensities. It can be concluded that *L. minor* performed better as a hyperaccumulator in livestock wastewater than *A. pinnata* which exhibits signs of cell death.

Introduction

The problem of environmental pollution caused on by wastewater discharges has spread all over the world. Due to the high levels of nutrients and organic material, untreated agricultural wastewater has been deemed the point source that poses the greatest risk to aquatic ecosystems (Khalid et al. 2018; Bashir et al. 2020). For example, livestock animals require a significant amount of water for direct consumption, upkeep (water services), and the manufacturing of animal feed and return to the environment through animal manure and slurry. Livestock wastewater (LW) can come from a range of sources, including animal waste, newly emerging pollutants like vaccines and antibiotics that are released into the environment through livestock runoff, leaching (Tian et al. 2021) and cage cleaning. Main routes of heavy metals sources in livestock production is by animal feed (Hejna et al. 2019). Important trace elements are commonly used as feed additives in the farming industry to not only meet nutritional needs and prevent nutritional deficiencies, but also to promote health and welfare, optimise efficiency, and improve food safety (Hejna et al. 2021).

Phytoremediation is a biological method of removing heavy metals and other contaminants naturally like in wastewater through the direct use of plants (Ubuza et al. 2020). Metal toxicity in plants can substantially impair growth, nutritional balance, and important metabolic activities including photosynthesis and respiration, which ultimately results in mortality, whether or not the metals are engaged in biological processes (Angulo-Bejarano et al. 2021). The molecular mechanisms enabling metal tolerance and hyper accumulation are mostly controlled by the constitutive high expression of many genes involved in metal absorption and transport, the production of metal ligands, and the responses to oxidative stress (Ingle et al. 2005; Pasricha et al. 2021)

Aquatic plants have been used extensively as phytoremediation in aquatic ecosystems due to their low cost, environmental friendliness, and sustainability (Ansari et al., 2020). In example, *Lemna minor* are used in water quality studies for monitoring heavy metals (Radic et al. 2010) while azolla in wastewater can significantly improve water quality as it is known as bioremediation by regulating oxygen capacity (Sood et al. 2012). Additionally, both of these aquatic plants have significance qualities that make them one of the best alternatives for animal feed applications (Putra and Ritonga, 2018; Md Nasir et al. 2022) biofuel production (Ge et al. 2012; Miranda et al. 2020) due to their higher nutritional content. Therefore, extensive knowledge on the response of these aquatic plants has been investigated to achieve close-loop bio economy employed livestock wastewater treatment. The main objective of this study is to determine the removal efficiency, morphology and genetic of the hyperaccumulator consisting of two different types of aquatic plants which are *L. minor* and *A. pinnata* for livestock wastewater.

Methodology

2.1. Wastewater collections and plants materials

The wastewater used for this experiment was collected from a local livestock production in Chuping located in Perlis, Malaysia. Livestock wastewater samples of 5L was collected during the period of May-July 2022. Samples of *A. pinnata* and *L. minor* was cultivated in container with a dimension of 35cm width, 45cm length, and 12cm depth under greenhouse located at Eco-Pro Training Centre, Kebun Abi, Perlis. 10 grams of samples were applied directly for livestock wastewater treatments and harvested according to specific times of 0, 24, 48 and 72h.

2.2 Atomic Absorption Spectrophotometer (Aas)

A spectrophotometer (Unicam, Model: 8600, Netherland) mod. 969 was used to conduct the metal tests using Flame Atomic Absorption Spectroscopy (air/acetylene).

2.3 Visual / Scanning Electron Microscope (Sem) Observation

Visual observation of the overall morphology for both aquatic plants were measured and recorded after 0, 24, 48 and 72hours after treatment with livestock wastewater. Several characteristics were observed including leaves size, color and root length. This characteristic have been used by several researchers to observe morphological changes in both aquatic plants as phytoremediation (Brandão et al., 2018; Sood et al., 2012). The sample was prepared dried at a temperature of 30 C and kept in a dry cabinet (MRC, Model: Dycd – 100, UK) before examination under Tabletop SEM (Hitachi Model: TM3000, Japan) observation.

2.4 Dna Extraction / Pcr Amplification

2.4 DNA extraction / PCR Amplification
Prior utilization, all pipetman, tips, and Eppendorf tubes had such a 5-minute UV treatment. Each 100mg of fresh samples were used to extract DNA by using Primeway Plant DNA Extraction Kit (First Base). Total PCR volume reaction consist of 25µL: DNA template (50ng/ml), ultrapure water, Master mix (dNTPS, reaction buffer, MgCl₂) and RAPD Primer. The PCR Thermocycler (BioRad, USA) conditions for *L. minor* were set for an initial denaturation at 95° C for 4 min, followed by 36 cycles of denaturation lasting 60 s at 94° C, annealing lasting 40 s at various temperatures depending on the primer, and extension lasting 60 s at 72° C. The final step was applied for 7 minutes at 72° C for the ultimate extension. For *A. pinnata*, PCR were set for 45 amplification cycles lasting 1 min at 94°C (denaturation step), 1 min at 36°C (annealing step), and 2 min at 72°C (extension step). One extension cycle lasting 7 min at 72°C was then performed. PCR products were analyzed by electrophoresis on a 1.6% agarose gel in 1X TAE buffer (Thermo Fischer Scientific) at 90 V for 90 minutes. The estimated size of DNA molecules ran on gel electrophoresis was calculated using 100 bp plus & 1000bp DNA ladder (Vivantis, MY). Agarose gel was stained with ethidium bromide and observed using a Gel Doc (Biorad, USA)

2.5 Data Analysis

Band profiles were assumed to occur between 200 and 1000 bp for each lane, and software output was also manually confirmed. RAPD polymorphisms and band profiles showed presence, disappearance, and intensity changes.

Table 1 List of the RAPD primers sequences and melting temperatures (Tm) that were utilized in this study.			
Type of aquatic plants	Primer details		
	RAPD Primer	Sequence (5' – 3')	TM (° C)
<i>A. pinnata</i>	C17	TTC CCC CCA G	37. 4
	B06	TGC TCT GCC C	39.8
	C08	TGG ACC GGT G	39.1
	D01	ACC GCG AAG G	40.7
	A13	CAG CAC CCA C	37.7
	B05	TGC GCC CTT C	41.1
<i>L. minor</i>	A12	TCG GCG ATA G	34.0
	B09	TGG GGG ACT	37
	B04	GGA CTG GAG T	32.2
	A04	AAT CGG GCT G	35.1
	B07	GGT GAC GCA G	38.1

Results

1.1 Composition of livestock wastewater

Table 2

The total HMs concentrations in livestock farm effluent and their impact to the human health, and the maximum concentration levels of those HMs in quality standards B.

Heavy metals (HMs)	Concentrations (mg/L)	Maximum concentration levels (MCL) of heavy metals Standard B (mg/L)
Cr	ND	0.05
Pb	ND	0.5
Mn	2.2	1.0
Ni	ND	1.0
Cu	0.121	1.0
Zn	0.677	2.0
As	ND	0.1
Al	0.699	15.0
TP	97.7	ND
TN	164.6	ND

1.2 Atomic Absorption of spectrophotometer (AAS) for manganese concentration in *L. minor* and *A. pinnata*

1.2.1 Removal efficiency of aquatic plants in livestock wastewater (%)

$$\text{Removal of efficiency (\%)} = \frac{C_i - C_e}{C_i} \times 100$$

where,

C_i = mean concentration amount of heavy metal initial

C_e = mean concentration amount of heavy metal at specific time

Table 3

The removal efficiency (RE) of Mn in aquatic plant species, including *L. minor* and *A. pinnata*, at various time exposure

Treatments	Types of aquatic plants	Exposure time						
		0 hours	24 hours		48 hours		72 hours	
		(concentration mg/L)	(concentration mg/L)	Removal efficiency (%)	(concentration mg/L)	Removal efficiency (%)	(concentration mg/L)	Removal efficiency (%)
Livestock Wastewater (LW)	<i>Lemna minor</i>	16.6 ± 0.1	27.6 ± 0.1	66	53.1 ± 0.1	92	74.7 ± 0.1	41
	<i>Azolla pinnata</i>	21.1 ± 0.1	29.4 ± 0.1	39	31.4 ± 0.4	6.8	55.6 ± 0.5	77

Table 4
The correlation between the accumulation rate of *L.minor* and *A.pinnata* with exposure time

		Time	LemnaMinor
Time	Pearson Correlation	1	.989*
	Sig. (2-tailed)		.011
	N	4	4
LemnaMinor	Pearson Correlation	.989*	1
	Sig. (2-tailed)	.011	
	N	4	4
*. Correlation is significant at the 0.05 level (2-tailed).			

		Time	AzollaPinnata
Time	Pearson Correlation	1	.918
	Sig. (2-tailed)		.082
	N	4	4
AzollaPinnata	Pearson Correlation	.918	1
	Sig. (2-tailed)	.082	
	N	4	4

Table 5
Morphology description of *L. minor* and *A.pinnata* according to different exposure time

Types of aquatic plants	Morphological characteristics	Exposure time			
		T0	T1	T2	T3
<i>L.minor</i>	Leaf shape	Normal	Normal	Normal	Normal
	Root	Long	Long	Long	Long
	Color	Pale green	Light green	Light green	More light green than pale green
<i>A.pinnata</i>	Leaf shape	Looks fresh, normal shape	Shrink	Shrink	Shrink
	Root	Long	Short	Short	Disappeared
	Color	Light green	Black + green	Black + green	Black + green

1.4 Sem Observation

1.5 Genetic profile of *A. pinnata* and *L. minor* using RAPD Marker

Result And Discussion

The composition of livestock wastewater collected from sampling site showed excessive levels of the heavy metal manganese (Mn), as listed in Table 2. Manganese is one of the elements that has previously been reported to be present in cow dung (Gupta et al. 2016). In livestock production this element is an essential trace mineral known as manganese performs as both an enzyme component and an enzyme activator (Spears 2019). However, manganese toxicity can result from over consumption of this mineral which affects the central nervous system, (Sidoryk-Wegrzynowicz and Aschner 2013).

The plants were revealed to accumulate large levels of Mn in a concentration and time dependent way (Fig. 1). The elimination of the abovementioned heavy metals was equivalent to their net sorption in plants due to natural precipitation. *L. minor* sp. showed greater sorption capability than *A. pinnata*. *L. minor* was highly efficient in absorbing heavy metals (Mn) in livestock wastewater with a RE of 92% at 48 hours treatment while *A. pinnata* shows RE of 77% at 72 hours exposure as recorded in Table 3. A significant strong positive relationship between retention time and heavy metal showed significance value $p \leq 0.05$ (Table 4). According to Paolacci et al. (2021), the duckweed removal system showed larger plant densities resulted in a higher removal rate per square metre of water, increasing the number of plant's capacity to absorb nutrients. This species has long thin root which has known to be main characteristics of greater removal hyperaccumulator in livestock wastewater. Our findings is similar to (Bokhari et al. 2016; Al-Khafaji et al. 2018), for the removal of heavy metals, with RE of > 80% which showed *L. minor* are an excellent candidate.

In addition to physiology capability, the morphology of the plants should be considered in order to determine their potential for RE. Visible observation of the plants indicated that healthy plants appeared to be in great health and to have a good adaptation of their surroundings. The morphology description of *L. minor* and *A. pinnata* has been recorded in Table 5. At the end of exposure period, leaves of *L. minor* turned pale green to yellowish color whereas those *A. pinnata* leaves turned into brown and black (Fig. 2). It is known that the plants leaves are more sensitive to environmental changes than its other organs (Brandão et al. 2018). The greenish colour of the plants has been turned to pale green and almost white in colour due to mechanism of chlorophyll occurred in the aquatic plants. This is similar to finding by Wang et al. (2014), the fronds of *L. minor* exposed to high nitrogen ammonia concentrations during the cultivation process, had a chlorotic and white colour that was gradually extending from the edge to the centre of each frond. The decrease in chlorophyll content may be caused by chloroplast membrane peroxidation or by Pb ions substituting for magnesium in chlorophyll molecules (Peng et al. 2019). Based on this study, *A. pinnata* could not be further applied as phytoaccumulator as this plants show sign of cell death as early as 24 hours exposure. According to Sudiarto et al. (2019), the wastewater probably possessed characteristics that prevented the plants from growing and absorbing nutrients. These results demonstrate the potential of using appropriate floating plant species in phytoremediation, as different plant species resulted in diverse removal efficiencies of Mn from livestock wastewater. *A. pinnata* exposed to livestock wastewater had leaf surfaces that were obviously different from the untreated control (Fig. 3). The leaf surface of *A. pinnata* was decreased by the livestock wastewater treatment and it may be that an excess of Manganese (Mn) resulted in shrinking. The key features of Program Cell Death (PCD) are shrinking of the protoplast and nucleus, condensation of chromatin, breaking of DNA, and vacuolization. According to Temmink et al. (2018), when maximum fixation rates by diazotrophic symbionts were reached for high P levels, the plants turned chlorotic. Evidently, the diazotrophs were unable to fix extra N that might have been used to boost biomass growth. This is in line with a study by Muradov et al. (2014), observed that *Azolla* plants were more sensitive to anaerobically digested swine wastewater (ADSW), becoming brown from the centre of the fronds and ultimately dying after 5 to 7 days at concentrations of 50 to 10%. Excessive nutrient in livestock wastewater lead to stressing in nutrient stoichiometry which may give death effect to the *A. pinnata*. According to Temmink et al. (2018), at greater P concentrations ($> 50 \mu\text{mol}^{-1}$) chlorosis appears to be brought on by iron (Fe-) rather than a lack of nitrogen (N-). A study by Sangwijit et al. (2021), observed that another aquatic macrophytes *H. verticillata* was unable to grow in university canteen wastewater (UCW) as a result of its photosynthesis being inhibited by high nutrients, turbidity, and organic substances. The morphology of the aquatic plants' roots has also been examined in this study for any alterations that might occur when exposed to livestock wastewater. Based on SEM observation root surface of the treated samples in both aquatic plants seen to shrink and shorten. Heavy metals are adsorb in cationic form with negative cell walls on the surface of the roots due to the existence of cellulose, pectins, and glycoproteins, which function as particular ion exchangers (Arif et al. 2016).

Using the RAPD technique, rearrangements, point mutations, insertions and deletions of DNA, and ploidy alterations in genomic DNA caused by genotoxic substances found in excessive levels in the environment (Erturk et al. 2013). RAPD profile in this study confirmed genotoxic effect of livestock wastewater which alter *L. minor* and *A. pinnata* genome. DNA profiles have shown increased/decreased band intensities, the emergence of new bands, and the removal of typical bands. In this study, overall seven (or 58%) of these primers produced significant band, but another 5 primers were not functional for amplification. From functional primers C17, amplified > 10000bp bands and produced four reproducible band profiles that might be used to determine treated treatments from controls (Fig. 4A). No new bands appeared or disappeared, however there was a significant increase in band intensities in *A. pinnata* with control and different exposure time in livestock wastewater. The band intensities was increased at T1-T3 compared to the control. The increment in band intensities has been reported to occur in DNA profiles of *Urtica pilufera* when exposed in different concentration of Cadmium (Cd) (Dogan et al. 2016). In another study, various band intensities was observed in DNA profile of *Sphagnum palustre*, suggesting similar genotoxic effect related to heavy metals (Sorrentino et al. 2017). From another functional primers, bands obtained using B07 ranged from 200 bp to 1000bp (Fig. 4B). Primer B07 produced 21 reproducible band profiles and also demonstrated that DNA fingerprints between livestock wastewater exposed and control group were clearly distinguished (Fig. 4A). The variation in band intensities were observed as decreased in 800bp T1 and T2 and increase in T3 in treated group. Moreover, new band appeared between 800bp to 1000bp in T3 was observed in *L.*

minor. This finding is similar with Ozyigit et al. (2021), whereby a new band was found in the 200 µM Cd-Ni treatment level. A total of 19% polymorphism were detected in *L.minor*. The presence of polymorphism in RAPD profiles demonstrates that these plants have evolved to withstand the stress caused by the presence of heavy metals in livestock wastewater. In other study by (Swaileh et al. 2006), observed the RAPD profile of raw wastewater whereas raw wastewater is much more genotoxic than treated wastewater (TWW) and the treatment process in this treatment plant removes some, but not all, genotoxic substances from wastewater. Another study by (Yigider et al. 2016) revealed that Mn stressors caused *Z. mays* seeds to develop several primers known as Stowaway, Sukkula, BARE 1(0), N-57(Nikita), and Nikita-E2647 LTR retrotransposon polymorphism. In contrast to *A.pinnata*, although this plant did not induce any polymorphism, all treated samples at all exposure times showed increased in band intensities as compared to untreated samples. There are several studies reported that heavy metals induced plant stresses which cause retrotransposons' transcriptional levels to increase (Cong et al. 2019; Gallo-Franco et al. 2020). According to Ozyigit et al. (2019), by analyzing differences in DNA banding profiles between collected sample species using these molecular markers, it is possible to determine the damaging effects of heavy metals on the integrity of genetic material. This is due to the genetic instability of plants due to heavy metals exposure to the cells (Morales et al.2016). A few studies investigated the genotoxicity by DNA molecular markers in aquatic plants so far (Tanee et al. 2016; Ozyigit et al. 2021), to our knowledge, this paper is the first to contribute to livestock wastewater contained excessive of Mn.

Conclusion

The concrete results of this study revealed that the hyperaccumulator consisting of aquatic plants, namely, *L.minor* for the livestock wastewater treatment is a dependable and environmentally preferable choice for treating livestock wastewater. *L.minor* demonstrated a higher RE of 92% and less toxicity on the plant cell as early as 24 hours after exposure that can be observed visually and under SEM as compared to *A. pinnata*. Future research on the underlying mechanisms the toxic effects of livestock wastewater that result in plant cell death should be done. *A. pinnata* had a lower accumulation capacity of Mn (55.6mg/L) than *L.minor* (74.7mg/L). *L. minor* samples treated with livestock wastewater showed a total of 19% polymorphism, and *A. pinnata* samples displayed an increase in band intensities, indicating that the DNA expression changes except for *A. pinnata*, which exhibits signs of cell death that make this plant fragile in comparison to *L. minor*. This environmentally friendly technology could be used in industry to reduce costs associated with heavy metal removal in livestock wastewater.

Declarations

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Author's contribution:

Conceptualization: Nor Anis Nadhirah Md Nasir. Data curation: Saadi Ahmad Kamaruddin. Methodology: Nor Anis Nadhirah Md Nasir. Supervision: Irnis Azura Zakarya. Validation: Irnis Azura Zakarya. Writing – original draft: Nor Anis Nadhirah Md Nasir. Writing – review & editing: Irnis Azura Zakarya

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Figures

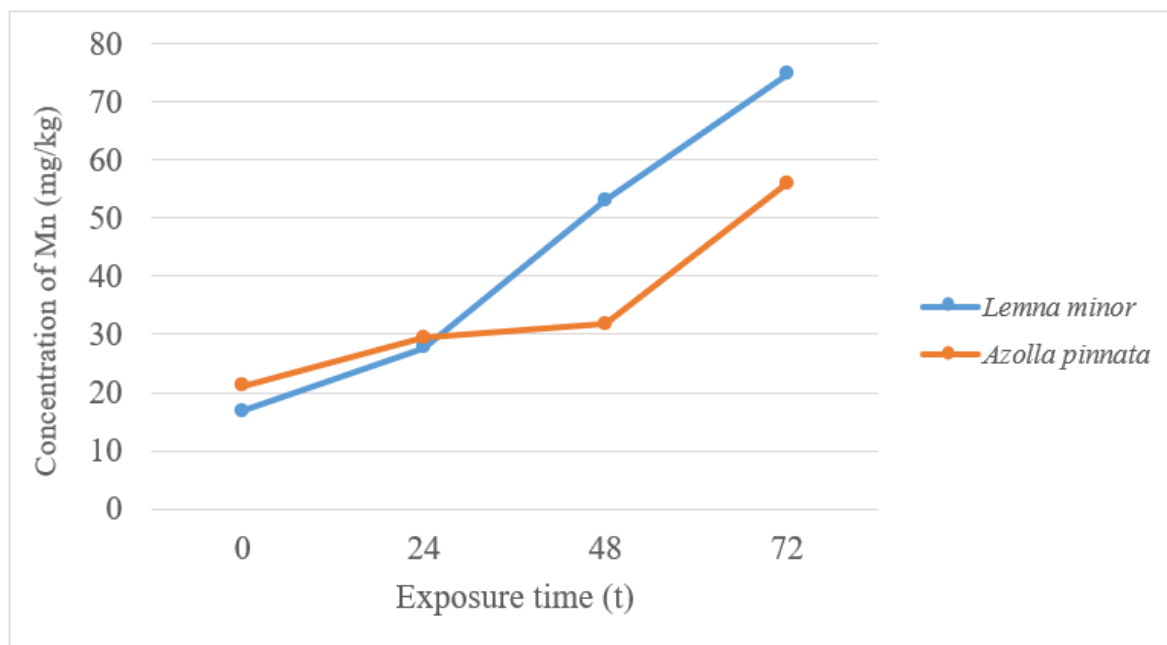


Figure 1

Different concentration of Manganese (Mn) in two types of aquatic plants, *L. minor* and *A. pinnata*



Figure 2

Visual observation of *L. minor* (A-D) and *A. pinnata* (E-H) treated in livestock wastewater at different exposure time

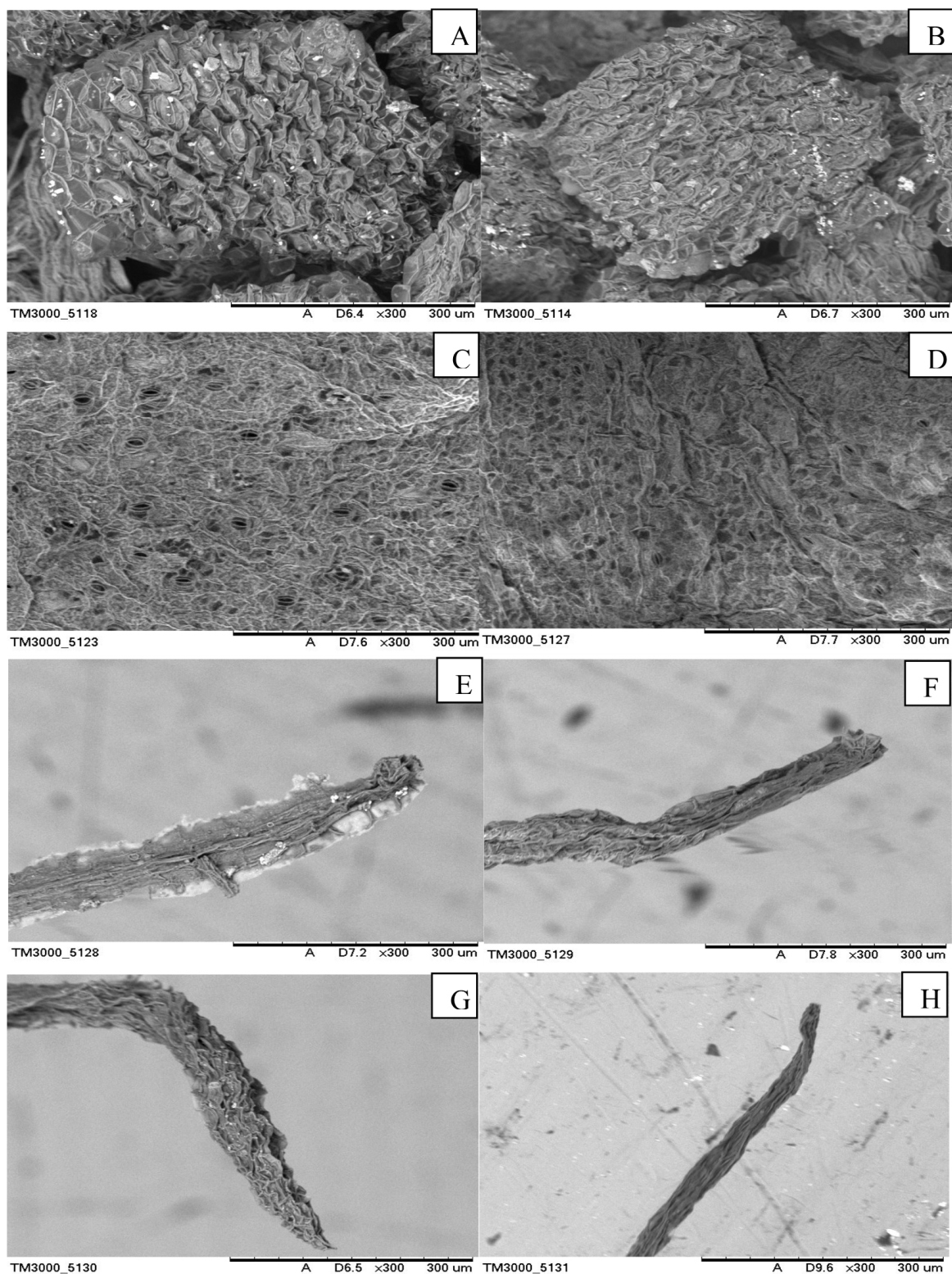


Figure 3

Leaf surface of the *A. pinnata* (A, B) and *L. minor* (C, D) under SEM observation respectively. (A, C) SEM images for the untreated samples. (B, D) showed the root surface exposed with livestock wastewater after 24 hours. Root surface of the *A. pinnata* (E, F) and *L. minor* (G, H) under SEM observation respectively. (E, H) SEM images for the untreated samples. (F, H) showed the root surface with livestock wastewater after 24 hours exposure

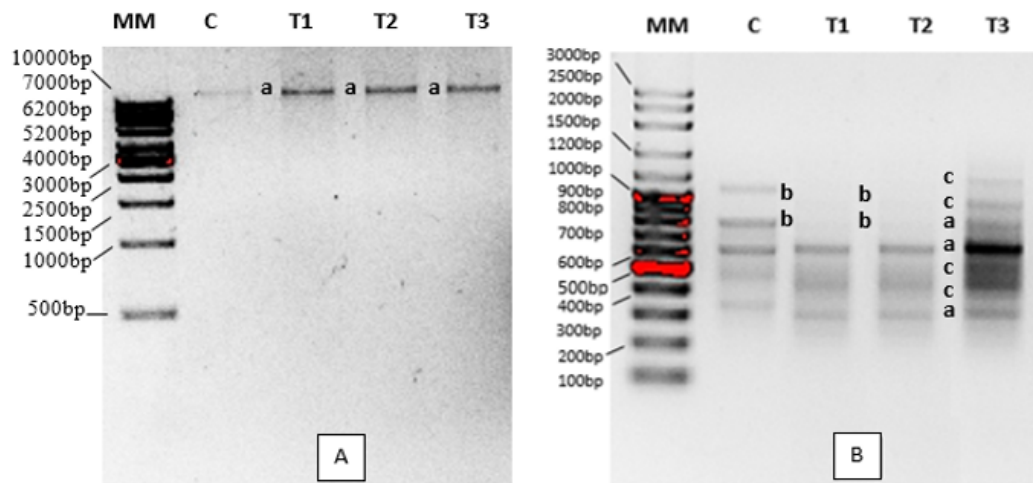


Figure 4

Representative of RAPD profiles of genomic DNA of *A. pinna* exposed in livestock wastewater at T1, T2 and T3, obtained using Primer C17 (A), DNA of *L. minor* exposed in livestock wastewater at T1, T2 and T3. Obtained using Primer B07 (B). Note (a) indicates increase in band intensities (b) decrease in band intensities (c) appearance of new bands (d) disappearance of normal band