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Allelopathic response of *Vetiveria zizanioides* (L.) Nash on members of the family Enterobacteriaceae and *Pseudomonas* spp.

Jatin Srivastava · Harish Chandra · Nandita Singh

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Abstract The biological removal of phosphates was carried as a part of treatment strategy. *Vetiveria zizanioides* (L.) Nash belonging to the family Poaceae was used for biological removal of biostimulants. Vetiver reportedly has mycorrhizal association; besides having potential for removal of $PO_4{}^{-3}$ also showed allelopathic affect on the microorganisms present in the water. In fact after a period of 96 h old roots of this plant have been found to have killing effects on the *E. coli, Enterobacter spp. Pseudomonas spp.* belonging to the family Enterobacteriaceae. The paper is opening a new face of study.

Keywords Water treatment · *Vetiveria zizanioides* · $PO_4{}^{-3}$ · Enterobacteriaceae Mycorrhizal association · Allelopathy

1 Introduction

Nutrient removal is essential for aquaculture wastewater treatment to protect receiving waters from eutrophication and for potential reuse of the treated water (Lin Ying-Fen et al., 2002). A number of physical chemical and biological methods used in conventional wastewater treatment have been applied in aquaculture systems. Researchers have found that treatment through wetland systems can remove biostimulants such as nitrates, phosphates, suspended solids, organic matter and microorganisms contained in wastewater (Kadlec and Knight, 1996). The major sources of biostimulants ($PO_4{}^{-3}$, $NO_3{}^{-1}$) in natural and wastewater include drainage from agricultural land, defecation

J. Srivastava (✉) · H. Chandra · N. Singh
Dolphin (PG) Institute of Biomedical & Natural Sciences,
VPO - Manduwala, Dehradun, Uttranchal, Postal Code-248007, India
e-mail: jks_345@rediffmail.com

N. Singh
Eco-Auditing Division, National Botanical Research Institute,
Rana Pratap Marg, Lucknow - 226001, India

from livestock, municipal and industrial effluents, and atmospheric deposition and diffuse urban drainage (Howarth et al., 1996). Wetlands are particularly well-suited for treating non-point sources of pollution, such as urban and agricultural runoff, because they can operate under a wide range of hydraulic loads, have internal water storage capabilities, and can remove or transform a number of contaminants (Kadlec et al., 1997; Kadlec and Knight, 1996; Reed et al., 1995).

Vetiver *Vetiveria zizanioides* (L.) Nash is one of the abundant grasses of wetlands of Indian subcontinent which has potentially been in use for contour protection and essential oil production. Vetiver, owing to its morphological and physiological attributes, and its strong deep penetrating, aerenchymatous roots system has an unusual ability to absorb and capacity to tolerate extreme levels of nutrients; making it an ideal system for wastewater treatment (Lavania, 2004). Vetiver has been reported (Wong, 2003), to contain arbuscular mycorrhizal fungi (AMF). These endo-mycorrhizal fungi could well be producing biotransformations of the vetiver oil (Adams et al., 2004).

In the present study, vetiver grass has been used to treat natural waters for the uptake of biostimulants. The study also revealed the impact of vetiver roots system on the microorganisms especially those belonging to the family Enterobacteriaceae.

2 Materials and methods

2.1 Site conditions

The experimental site was located at National Botanical Research Institute in Lucknow, a city in the northeast of India. The temperature of the site during the experiments was constant around 32°C ± 3°C. The entire study was completed within the months of July to September, the season for the growth of most of grasses. The average rainfall was 112 mm in these three months with a humidity around 78–89%.

2.2 Plant material

Naturally growing vetiver grass was collected from the wetlands of Lucknow city near Banthra Rresearch Center (a sister lab of NBRI). The plants were brought to the laboratory and were cleansed with 1% EDTA to remove elemental impurities that adhere to the roots and the soil particles. The plant material (Vetiver) was then arranged on $30 \times 30\,\mathrm{cm}^2$ of thermocol trays and were allowed to float over a constructed pond containing ground water for the acclimatization. After a period of 15 days, new roots appeared in fluffy bunches. The plant material was harvested and was transferred into 50 liters tubs containing pond water for the phyto-remediation studies.

2.3 Mycorrhizal roots

To study Vetiver that was not infected with mycorrhizal fungi, plants were also grown from the seeds on the sterilized soil. The plants grown from seeds, however, took twice as long as untreated plants to grow sufficiently to be harvested for essential oil content. The association of mycorrhizal fungi was detected by the staining of the roots with Trypan blue in lactophenol after the treatment of 10% KOH and 30% H_2O_2 as bleaching agent.

2.4 Pond water sampling

Pond water was collected from sites earmarked for the studies located inside the urban area of Lucknow city. Water was collected in iodide washed jerry cans and was analyzed within 24 h after sampling.

2.5 Sampling and analyses

Water sampling was done every after 48 h from the day the experiment was setup. The microbial populations around the roots were sampled in pre-sterilized glass vials provided by Borosil Inc. Water samples for nutrient analysis were analyzed within 24 h of sampling to avoid physico-chemical changes. The water samples were analyzed for the nitrogen (NO_3^{-1}–N), (NH_4^{+1}–N) and Ortho-phosphate (PO_4^{-3}–P) according to the (Standard methods 1996). Plate count method was performed for the enumeration of the bacteria in water.

2.6 Microbial assay

Microbes were first isolated on the nutrient agar plates as bacterial isolates from the pond water. *Escherichia coli, Entrobacter spp. Pseudomonas spp*. belonging to the family Enterobacteriaceae were cultured on selective media provided by Hi Media India and were characterized by biochemical assays. Individual isolates were identified by biochemical assays using the Enterobacter identifying kit KB 001 Hi Media. The essential oil of vetiver roots was obtained from the Biomass Biology Division NBRI Lucknow, for the antimicrobial studies 10 mg oil was dissolved in 5% sterilized di-methyl sulfoxide (Qualigens Inc.), and a disc—diffusion assay was performed to ascertain the response of vetiver oil. The zone of inhibition was measured with the help of vernier calipers.

3 Result

3.1 Untreated pond water

A comparative study was performed on the pond water with or without the treatment of vetiver grass. Table 1 shows the changes that occurred in the untreated pond water. Chemical analysis revealed pH values rose over the 10 d trial period i.e., 7.9 ± 0 to an alkaline i.e. 8.5 ± 1.0. Species of nitrogen showed a conventional pattern of changes whereby the NO_3^{-1}–N ions were reduced throughout the experiment and reached a minimum value of 38.9 ± 7.2 from a value of 73.3 ± 3.13. On the other hand, (NH_4^{+}−N) ammonium level increased achieving a maximum value of 37.0 ± 0.23 from a minimum value of 14.4 ± 0.38 on the day one. Phosphate (PO_4^{-3}–P) content in the untreated water was found to vary and remained the same or near to the original value of 7.6 ± 0.26. The microbial population showed an increase, the total number

Table 1 Microbial and nutrient status in untreated water sample

			Untreated pond water			
S. no.	Parameters (mg/l)	Pond water	48 h	96 h	144 h	240 h
1.	pH	7.9 ± 0	8.1 ± 0.1	8.3 ± 1.3	8.3 ± 0.7	8.5 ± 1.0
2.	PO_4^{-3}– P	7.6 ± 0.27	7.01 ± 0.8	6.91 ± 1.25	7.41 ± 0.43	7.23 ± 1.8
3.	NO_3^{-1}–N	73.3 ± 3.13	70.1 ± 4.7	54.7 ± 6.3	43.0 ± 0.81	38.9 ± 7.2
4.	NH_4^+–N	14.4 ± 0.38	16.8 ± 1.76	28.1 ± 0.28	33.7 ± 1.21	37.0 ± 0.23
5.	T. CFUs	$177 \times 10^3 \pm 84.13$	–	–	–	$98^a \times 10^4 \pm 11.4$
6.	*Pseudomonas ssp.*	$44.3 \times 10^3 \pm 13.1$	–	–	–	$56^a \times 10^3 \pm 19.0$

[a]The counts were taken only in the final samples.

Table 2 Microbial and nutrient status in treated water with *Vetiveria zizanioides*

			Treated pond water			
S. no.	Parameters (mg/l)	Pond water	48 h	96 h	144 h	240 h
1.	pH	7.9 ± 0	7.2 ± 0.29	7.1 ± 0.1	7.0 ± 0.3	7.0 ± 0
2.	PO_4^{-3}– P	7.6 ± 0.27	6.92 ± 0.42	4.39 ± 0.3	3.9 ± 0.1	1.08 ± 0.1
3.	NO_3^{-1}–N	73.3 ± 3.13	44.2 ± 4.67	49.4 ± 1.3	58.9 ± 2.4	62.7 ± 1.8
4.	NH_4^+–N	14.4 ± 0.38	14.8 ± 0.16	6.9 ± 1.4	2.8 ± 0.4	0.97 ± 0.1
5.	T. CFUs	$177 \times 10^3 \pm 84.13$	–	–	–	$308.5^a \pm 97$
6.	*Pseudomonas ssp.*	$44.3 \times 10^3 \pm 13.1$	–	–	–	$377^a \pm 23$

[a]The counts were taken only in the final samples.

of colony forming units (CFUs) and *Pseudomonas* spp. reached to $98 \times 10^4 \pm 11.4$ & $56 \times 10^3 \pm 19.0$ from a value of $177.0 \times 10^3 \pm 84.13$ & $44.3 \times 10^3 \pm 13.1$ respectively.

3.2 Treated pond water with *Vetiveria zizanioides* (L.) Nash

The results of water quality of treated pond water with the vetiver grass has been shown in (Table 2). A consistent reduction in each parameter except in pH and NO_3^{-1} was observed. The reduction in the ortho-phosphate (PO_4^{-3}–P) reached a value 1.08 ± 0.13 achieving 85% removal efficiency. The depletion in $NH_4^+ - N$ from a value of 14.4 ± 0.38 to a level of 0.97 ± 0.1 was recorded at 240 h. The microbial count in the treated water was found reduced quite significantly. The total CFUs and *Pseudomonas* spp. were found on the 240th h 308.5 ± 96.9 & 377.0 ± 23.0 from a value of $177.0 \times 10^3 \pm 84.13$ & $44.3 \times 10^3 \pm 13.1$ respectively.

3.3 The allelopathic response of Vetiver oil

The natural Vetiver grass when grown in $30 \times 30\,cm^2$ of trays produced a root biomass <3.2 kg in 30 days, while those were grown from seeds (seedlings) yielded <3.86 kg in almost 60 days (Table 3). The infected (i.e. mycorrhizae positive) and non-infected (i.e. mycorrhizae negative) plants yielded essential oils from their roots 0.256 ± 0.21 & 0.219 ± 0.1 respectively. The activity of the essential oil recovered from the roots of both types on the microbes (Table 4). Essential oil extracted from the

Table 3 Biomass and oil production by the *Vetiveria zizanioides* grown in the mesocosm

Mesocosm	Surface area (cm^2)	Root Biomass (kg)	Root Biomass (kg)	% Essential oil in (infected)	% Essential oil (non-infected)
Vetiveria zizanioides	30 × 30	3.2 ± 1.87	3.86 ± 2.10	0.256 ± 0.21	0.219 ± 0.1

Table 4 Anti microbial activity of Vetiver oil on the members of Enterobacteriaceae

S. no.	Microbes	Zone of inhibition (cm) Essential oil (infected)[a]	Zone of inhibition (cm) Essential oil (non-infected)
1.	*Enterobacter spp.*	1.935 ± 1.35	0.155 ± 0.07
2.	*Escherichia coli*	0.750 ± 0.06	0.810 ± 0.13
3.	*Pseudomonas fluorescence*	NIL	NIL
4.	*Pseudomonas spp.*	1.025 ± 0.89	NIL

[a]Roots are infected with mycorrhiza.

mycorrhizae infected roots shows an inhibition zone on *Enterobacter* spp.*, Escherichia coli, Pseudomonas fluorescence, Pseudomonas* spp. 1.935 ± 1.31, 0.750 ± 0.06, Nil & 1.025 ± 0.89 respectively. The essential oil from the non-infected roots of vetiver grass showed, however, the activity on the same in order of 0.155 ± 0.07, 0.810 ± 0.13 Nil & Nil respectively.

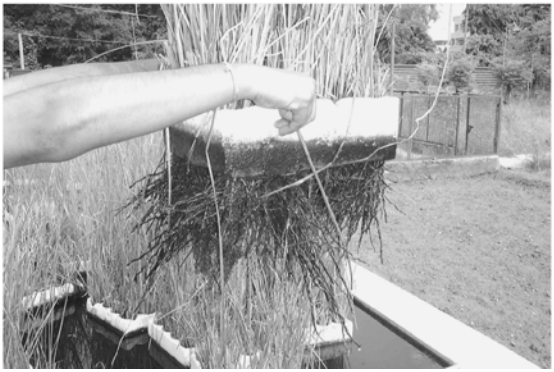
4 Discussion

The carbonate ions get reduced because of the algal growth causing an increase in pH of water (Tchobanoglous and Schroeder, 1985, p. 137). The reduction in PO_4^{-3} content may be attributed to the growth of algae and the accumulation by microbes in their outer cell wall (Vassilev et al., 1999). Nitrates and ammonia are associated with each other as the anoxic conditions favors the conversion of nitrate into ammonia (Tchobanoglous and Schroeder, 1985).

The treated experimental set-up where the water was in contact with the roots of grass that grows vigorously in water (see plate) and because of its expanded surface area, an increased uptake of nutrients from water has been recorded following the direct assimilation of water column P by the roots (Dierberg et al., 2002). As far as the ammonia removal is concerned, earlier workers (Lin Ying-Fen et al., 2002) have already demonstrated the same results where nitrogen removal was achieved with 86%–98% efficiency for ammonium nitrogen (NH_4^{+1}–N) with the increase of nitrate content. The reduction in microbial population specifically belonging to the family Enterobacteriaceae (as observed in the experiments) are attributed to the allelopathic effect of essential oil that is sparingly water soluble. The phenomenon of AMF for protecting plants from root pathogens is known from studies of Dodd (2000), the root branching also get affected as the AMF determines plant dependence on the fungal symbiosis. The presence of AMF in freshwater wetlands (Miller, 2000), suggests that the ecology of this association must play an important role in the wetland vegetation. Vetiver grass has been reported (Wong, 2003), to contain mycorrhizal association that

Mesocosms of *Vetiveria zizanioides* floating in an experimental tank containing water

Profusely grown root of Vetiver grass in the mesocosm



transforms the essential oil. In some studies intracellular bacteria are reported to be associated with essential oil cells in vetiver roots (glands) and bio-transformations of the oil. In a study (Adams et al., 2004), unidentified biotic factors (apparently bacteria or fungi) appear to enhance the oil production in normal vetiver by both increasing yield and by the generation of signature oil compounds. This may be the reason for the lower recovery of essential oil from non-infected roots of vetiver.

Because the surface area of the roots was sufficient enough in the experimental tubs to cover most of the water, hence exposed to most of the microbial clusters. The anti-microbial assay with both types of (infected roots with mycorrhiza and non-infected roots with mycorrhiza) exhibit different affects on microbial population. The member of Enterobacteriacae are responsible for phosphate solubilizing in media and in the case of scarcity of phosphate in media, these bacteria accumulate PO_4^{-3} in their outer layer of cell wall. Reports (Vassilev et al., 1999) suggest that in media (water) the mycorrhization along with enterobacters enhance phosphate uptake. The killing of the members of Enterobacteriaceae may be attributed to the conditions of reduced phosphate content that led the bacteria to accumulate more phosphate that switches on the competition between the fungal association and bacterial cells, where fungal association with roots transform the oil making it to have a killing effect on the bacteria to control the phosphate assimilation in microbes. The oil transformation however has been well documented in reports (Adams et al., 2004).

5 Conclusion

The pond water containing biostimulants led to the growth of microflora as well as macrofauna. The frequent disposal of municipal waste in natural water systems such as ponds, pools, & lakes along with the leached nutrients provide suitable growing conditions for microbes especially the members of family Enterobacteriaceae. *Vetiveria zizanioides* (L.) Nash grass, which was used for the treatment of the natural waters, the results revealed that there exist allelopathic responses of vetiver roots on certain bacterial members especially belonging to the family Enterobacteriaceae. Vetiver oil quality differs depending upon the infection with mycorrhizal fungi, as these fungi reportedly are able to transform the composition of oil. The oil from both types of plant material one that was having mycorrhizal association with the roots and the other that was not having such association showed quite different response on the bacteria isolated from the experimental pond water systems (tubs) confirmed that the allelopathic response of vetiver is more than a fact however, more research work is needed on the bacterial population belonging to other families.

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References

Adams, R.P., Habte, M., Park, S., and Dafforn, M.R.: 2004, 'Preliminary Comparision of Vetiver Root Essential Oils from Cleansed (Bacteria and Fungus—Free) Versus Non-Cleansed (Normal) Vetiver Plants,' *Biochemical Systematics and Ecology* **32**, 1137–1144.

American Public Health Association: 1996, *Standard Methods for the Examination of Water and Wastewater*. APHA, AWWA, and WPCF, Washington, D.C.

Dierberg, F.E., DeBusk, T.A., Jackson, S.D., Chimney, M.J., and Pietro, K.: 2002, 'Submerged Aquatic Vegetation—Based Treatment Wetlands for Removing Phosphorous from Agricultural Runoff: Response to Hydraulic and Nutrient Loading,' *Water Resource* **36**, 1409–1422.

Dodd, J.C.: 2000, 'The Role of Arbuscular Mycorrhizal Fungi in Agro and Natural Ecosystems,' *Outlook on Agriculture* **29**(1), 55–62.

Howarth. R.W., Billen, G., Swaney, D., Townsend, A., Joworski, N., Lajtha, K., Downing, J.A., Elmgren, R., Craco, N., Jordan, T., Berendse, F., Freney, J., Kudeyarov, V., Murdoch, P., and Zhao-Liang, Z.: 1996, 'Regional Nitrogen Budgets and Riverine N & P Fluxes for the Drainages of the North-Atlantic Ocean: Natural and Human Influences,' *Biogeochemistry* **35**, 39–75.

Kadlec, R.H., Burgoon, P.S., and Henderson, M.E.: 1997, 'Integrated Natural System for Treating Potato Processing Waste Water,' *Water Science Technology* **35**, 263–270.

Kadlec, R.H. and Knight, R.L.: 1996, *Treatment Wetlands*, CRC Press, Boca Raton FL, USA, p. 893.

Lavania, U.C.: 2004, 'Vetiver System Ecotechnology for Water Quality Improvement and Environmental Enhancement,' *Current Science* **86**(1), 11–14.

Lin, Y.-F., Jing, S.-R., Lee, D.-Y., and Wang, T.-W.: 2002, 'Nutrient Removal from Aquaculture Wastewater Using a Constructed, Wetlands System,' *Aquaculture* **209**, 169–184.

Miller, S.P.: 2000, 'Arbuscular Mycorrhizal Colonization of Semi-Aquatic Grasses Along a Wide Hydrologic Gradient,' *New Phytologists* **145**, 143–155.

Reed, S.C., Crites, R.W., and Middlebrooks, E.J.: 1995, 'Natural Systems for Water Management and Treatment,' McGraw Hill, New York NY.

Tchobanoglous, G., Schroeder, E.D.: 1985, *Water Quality*, Wesley Publishing Company, USA.

Vassilev, M., Azcon, R., Barea, J., and Vassilev, N.: 1999, 'Effect of Encapsulated Cells of Enterobacter Spp. On Plant Growth and Phosphate Uptake,' *Bioresource Technology* **67**, 229–232.

Wong, C.C.: 2003, 'The Role of Mycorrhizal Associated with *Vetiveria zizanioides* and *Cyperus polystachyos* in the Remediation of Metals (Lead & Zinc) Contaminated Soils'. M.Phil thesis, Hong Kong Baptist University, Hong Kong.