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ENHANCEMENT IN PHYTOREMEDIATION OF MUNICIPAL WASTEWATER BY GENETICALLY MODIFYING WETLAND AND NON-WETLAND PLANT SPECIES (NON-EDIBLE)

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ABSTRACT
The enhancement of phytoremediation efficiency of non-edible wetland plant species and non-edible non-wetland plant species using *Agrobacterium rhizogenes* treatment was carried out. *Portulaca oleracea, Chrysopogon zizanioides* and *Alternanthera philoxeroides* showed good transformation results. The transformed *Portulaca oleracea, Chrysopogon zizanioides* and *Alternanthera philoxeroides* showed significant increase in phytoremediation efficiency for the treatment of municipal waste water (MWW) over the non-transformed plants. However, *Eichhornia crassipes* showed no significant difference in its efficiency after the transformation process. As *Portulaca oleracea, Chrysopogon zizanioides* and *Alternanthera philoxeroides* have shown a positive response to transformation and also exhibited an increased efficiency to treat MWW, these plants can be transformed with specific genes for the removal of specific contaminants in waste water and polluted waters.

KEY WORDS: Phytoremediation, Municipal Waste Water, Genetically modified, Wetland, Non-wetland plants, *Agrobacterium rhizogenes*

INTRODUCTION

Phytoremediation is a process which involves the use of plants to carry out treatment of contaminated soil and water. Roots are multifunctional in plants as besides providing anchorage and storage they facilitate absorption of water and minerals. The functional role of the plant roots enables the cleansing capacity of the plants for treatment of contaminated soil and water. The roots of the plants *Arundo donax and Sarcocornia fruticosa* have been used in a horizontal constructed wetland at a tannery waste outlet after its conventional treatment unit. It was found that both plants were able to absorb nutrients from the waste and they grew well (Calheiros *et al*., 2012). Constructed wetlands have been used to treat urban waste water and the efficiency was enhanced by altering the plants used, the bedding material and the flow rate supplied. Aging of wetlands too affected their treatment ability. (Hijosa-Valsero *et al*., 2012). Karami *et al*. (2010) have used various ways for increasing phytoremediation efficiency such as increasing the electrode potential (Eh) and lowering of pH, using chelating agents, micro-organisms (arbuscular mycorrhizal fungi (AMF) and plant growth promoting rhizobacteria (PGPR). The use of *A. rhizogenes* is also of great interest since it can be used as natural vector to transfer new genes to plant cells. Agrobacterim cells can act as a gene vector after genetic manipulation of their plasmids. In practical terms, transformed root may be developed by infecting sterile plant material, leaves or petiole, excised from the explants and transformed to the growth medium (Walton *et al*., 1999). One approach that has raised a lot of interest is the use of *A. rhizogenes*, a soil bacterium able to stimulate the proliferate growth of roots named as 'hairy roots' via plasmid (*Ri* T-DNA) insertion into plant genome. The DNA transforming plasmid of *A. rhizogenes* has ability to insert into the plant DNA thus transforming it. Hairy roots have production rate very similar to those of normal roots and can grow in-vitro without addition of exogenous growth regulators, thus solving the problem of possible inhibitory activity of their phyotohormones. These cultures exhibit a high level of genetic and biochemical stability. One way of increasing the phytoremediation efficiency can be the use of *Agrobacterium rhizogenes* transformation to increase the root biomass and improve secondary metabolite production thus making the plant more efficient. A number of plant species including many medicinal plants have been successfully transformed with the help of *Agrobacterium rhizogenes*. *Agrobacterium rhizogenes* has the capacity of delivering its T-DNA from the *Ti* plasmid into the host cell which modifies the host cell on genetic grounds. This transfer makes the plant cell turn neoplastic producing higher levels of secondary metabolites (Tzfira and Citovsky, 2006). The *vir* genes, *vir* D1 and *vir* D2, and bacterial proteins make it possible for the TDNA to be produced and travel across the plant cell and reach the nucleus. Plant derived organic compounds and sugars are responsible for the activation of *vir* genes. The nuclear targeting of *vir* genes is brought about by importing α protein. Induction of root occurs due to the presence of ***Ri*** plasmids, the T-DNA structure may differ

but the *rol* genes remain the same for TDNA. The roots here are transgenic roots and the shoots remain non-transformed. (Tzfira and Citovsky, 2006; Anand *et al*., 2007; Chabaud *et al*., 2006; Gelvin, 2012; Mohajjel-Shoja, 2011). The agrobacterium roots produced differ from each other in their genetic nature, phenotype, metabolite content and growth due to random pairing of T DNA copies in plant genome. (Foyer and Noctor, 2009). Studies on enhancement of secondary metabolite production in various plants by *Agrobacterium rhizogenes* has been carried by many workers. The exogenous activity of conversion of hydroquinone to arbutin (pharmaceutically important compound) and secondary metabolite production was enhanced in *Physalis ixocarpa* (Bergier *et al*., 2012). Similarly, *Bacopa monnieri* a medicinally important plant showed increased secretion and growth on *Agrobacterium rhizogenes* infection (Majumdar *et al*., 2011).Pepper plant gained resistance against *Phytophthora capsici* post an *Agrobacterium rhizogenes* transformation. *Agrobacterium rhizogenes* transformation was used to carry out transformation of *Eucalyptus camaldulensis.* The plant has a good wood quality, good growth rate and good adaptability. The regeneration of plant using *Agrobacterium* strain *A4RS* proved to give good results and the confirmation of modification was carried out by using *GFP* cloning. (Balasubramanian *et al*., 2011). Resveratrol production in *Arachis hypogaea* was studied, where an *Agrobacterium rhizogenes* transformation increased the secondary metabolite production. (Kim *et al*., 2008). In a similar study, metal treatment was improved using strains of *Agrobacterium* carrying out transformation of the plant *Arabidopsis thaliana*. The bacterial gene *Mer* A was inserted into the plants which resulted in increased conversion of mercury (Hg^{2+}) to less toxic form mercury (Hg^{o}) (Karenlampi *et al*., 2000). Therefore, in this study an attempt has been made to enhance phytoremediation of municipal waste water by increasing the root biomass through transformation of wetland (non-edible) and non- wetland (non-edible) plant species using *Agrobacterium rhizogenes*.

MATERIALS & METHODS

Plant Species Used

Four plant species were used for the study. The wetland plant species were Alternanthera *philoxeroides* (Alligator weed, Family: Amaranthaceae), *Eichhornia crassipes* (Water hyacinth, Family: Pontideriaceae) and non-wetland plant species were *Chrysopogon zizanioides* (Vetiver Grass, Family: Poaceae); *Portulaca oleracea.(Purslane). Eichhornia crassipes* and *Alternanthera philoxeroides* were obtained from Powai Lake, Mumbai and the *Chrysopogon zizanioides* and *Portulaca oleracea* was obtained from the nearby nurseries.

Adaptation of land plants to the water environment

Plants like *Eichhornia crassipes* and *Alternanthera philoxeroides* are adapted to the water environment. Hence, they could be directly employed for the treatment process but plants like *Portulaca oleracea* and *Chrysopogon zizanioides* being terrestrial plants needed to be adjusted to the water environment. Initially, the non-wetland plants were kept in soil with water for 7 days. Then soil was partially cleaned, with water, and plants were reintroduced to fresh water and maintained for another 7 days. Then the plant roots were cleaned properly and were made soil free and reintroduced to fresh water. They were maintained in fresh water for 15 days. The water was changed at an interval of 5 days. Additional support was provided by using gravels.

Support media used

The support media used in the setup of the mesocosms for the treatment of municipal wastewater (MWW) were gravels and balls made up of waste plastic carry bags. These support media were non-biodegradable. The support media were obtained from the local dealers in the city and collected as a waste material.

Agrobacterium rhizogenes proliferation

The virulent *Agrobacterium rhizogenes* strain was obtained from NCIM, National Chemical Laboratory, Pune, India. The media YEMA and NA media used in performing the study were acquired from Hi-Media Laboratories Pvt. Ltd. and Merck Qualigen, Mumbai, India. *Agrobacterium rhizogenes* strain was isolated using different media to select an appropriate growth medium. YEP media was made using yeast extract, sodium chloride and peptone (10 g, 5 g and 10 g, respectively for 1 litre YEP medium). Alternately, agar was added (15g/L) for preparing YEP agar plates for isolation. (S. Murugesan *et al*., 2010; Ali *et al*., 2010). Murashige and Skoog media was prepared by preparing stock solutions (A, B, C, and D). Solution A (NH_4NO_3, KNO_3, $CaCl_2$, $MgSO_4$, KH_2PO_4), Solution B (H_3PO_4, $MnSO_4.H_2O$, $ZnSO_4.7H_2O$, KI, $NaMo_4.2H_2O$, $CuSO_4.5H_2O$, $CaCl_2.6H_2O$), Solution C (Na_2.EDTA, $FeSO_4.7H_2O$) and Solution D (Nicotinic acid, Pyridoxal HCL, Thiamine HCL, Glycine, Myoinositol). 200 ml media was prepared by adding stock solution A (10 ml) stock solution B (1 ml) stock solution C (1 ml) stock solution D (1 ml). Additionally, 6 gm sucrose was added followed by 1.6 gm agar. The final pH was adjusted to 5.8. The *Agrobacterium* strain was inoculated into the media to check for appropriate media supporting optimum growth. All inoculated media were inoculated at 28ºC for 3-5 days and were observed. After five days, strains from YEMA and YEP agar were re-cultured on YEMA plates and were incubated for 5 days at 28 ºC. Colonies from YEMA were selected and inoculated in distilled water at 28 ºC for 24 hrs. They were then subjected to centrifugation at 1500 rpm for ten minutes and density adjustment 1.0 at 600 nm in saline was done.

Infection to the plant with Agrobacterium rhizogenes

Two parallel sets were run, laboratory setup (controlled conditions) and field setup (uncontrolled conditions). In the laboratory saline suspension of *Agrobacterium rhizogenes* was taken in syringe and was then injected into plant nodes and roots, alternately wounds were made on the roots using surgical blade. Suspensions were added to the containers of plants. All plants (*Alternanthera philoxeroides, Eichhornia crassipes, Chrysopogon zizanioides, Portulaca oleracea)* were exposed to *Agrobacterium rhizogenes* suspension and subjected to 28 ºC for three days. The plants were washed and *Agrobacterium rhizogenes* containing water was replaced with normal distilled water after three days exposure time. The plants were removed from the incubator and kept on tissue culture rack under controlled light conditions for 3-4

weeks. Further, the plants were exposed to direct sunlight and maintained in the open environment. In the field setup, the plants after infection were directly exposed to the sunlight and no special conditions like temperature or pre-adjusted photoperiod were provided. For confirming that *Agrobacterium rhizogenes* was promoting rooting *Alternanthera philoxeroides* and *Portulaca oleracea* were cut at the nodal segments and were exposed to 0.1% $HgCl_2$ for 3 minutes, followed by distilled water exposure for 3 minutes. Then injuries were made to all the segments and they were introduced to culture density adjusted *Agrobacterium rhizogenes* suspension, maintaining them at 28°C for 90 minutes. Antibiotic washing was followed by washing with sterilized distilled water. They were then placed in sterilized MS Media tubes and exposed to pre-adjusted photoperiods in the tissue culture rack.

Testing efficiency of transformed plant species in each support media

Infected wetland plants were weighed. Gravels and plastic bag balls were added to tanks separately. 160 number of gravels of 2.0cm size and 15 number of plastic bag balls were added to individual tanks, followed by the addition of plant species. Equal amount of MWW was added to all the tanks. The MWW analysis at HRT 3 and 5 days was carried for efficiency calculation.

Analysis of MWW

The parameters of MWW analyzed were BOD, Nitrate N, Phosphate P. APHA, 20th edition was followed for the protocols for analysis.

RESULTS & DISCUSSION

Agrobacterium rhizogenes growth was observed for choosing the appropriate media for its proliferation. Based on the above obtained results (Table 1), YEMA was chosen as the proliferation media. No contamination was observed in the YEP medium and it was chosen as the maintenance medium for the strain. Infection was carried out using the bacterium growing on YEMA plates. New root initiation and elongation under uncontrolled conditions were similar to those under controlled conditions in the laboratory. Therefore, for root initiation and for further growth, controlled environment is not needed. Upon infection, rooting was seen after 15-20 days in *Alternanthera philoxeroides*, *Chrysopogon zizanioides and Portulaca oleracea* (Plate 1, 2 and 3). As random injuries were made with surgical blade, a branching root system was observed on existing roots.

Before transformation

After transformation

PLATE 1: Roots of *Alternanthera philoxeroides*

Before transformation

After transformation

PLATE 2: Roots of *Portulaca oleracea*

Before transformation

After transformation

PLATE 3: Roots of *Chrysopogon zizanioides*

TABLE 1: Growth pattern

Media used	Day 1	Day 2	Day 3	Day 4	Day 5
Nutrient agar	-	-	+	+	++
YEP	-	-	+	+	++
YEMA	-	-	++	+++	+++++
YEP with sucrose and agar	-	-	+	+	++
YEP broth	-	-	+	++	++
MS Media	-	-	+	++	+++

The plants were showing a well defined hairy root system. According to Eapen and D'Souza (2005), it is essential to have plants with highly branched root systems with large surface area for efficient uptake of toxic metals and other pollutants. Experiments had shown that *Agrobacterium rhizogenes* could enhance the root biomass in some hyperaccumulator plants. The hairy roots induced in some of the hyperaccumulators were shown to have high efficiency for rhizofiltration of radionuclide (Eapen *et al*., 2003) and heavy metals (Nedelkoska and Doran, 2000).

Transformed plants were tested for their efficiency in MWW treatment at HRT of 3 and 5 days. On obtaining the percentage reduction for the transformed and non-transformed plant species for the two support materials statistical analysis (SPSS 13) was performed to check for any significant difference between the transformed and non transformed plant species with respect to the efficiency exerted by them in the respective support materials. With gravel as a support material, transformed *Portulaca oleracea* was found to be significantly different in terms of BOD and phosphate removal in comparison to non-transformed plants. The transformed *Alternanthera philoxeroides* showed significant difference in Phosphate removal in comparison to its non-transformed plants, while *Chrysopogon zizanioides* was found to be significantly different in terms of BOD and Phosphate removal between transformed and non-transformed plants. *Eichhornia crassipes* showed no significant difference in any of the parameters tested which explains that the *Agrobacterium rhizogenes* infection treatment was of no effect (Table 2,3).

TABLE 2: Removal % of various parameters in gravels using transformed and non-transformed plant species

mg/L	BOD		Nitrate N		Phosphate P	
HRT (days)	3	5	3	5	3	5
Alternanthera philoxeroides						
TP	68 ± 3	73 ±6	63 ± 17	82±9	63 ± 2	94±3
NTP	58 ± 6	61±11	51 ± 5	56 ±13	47 ± 1	51±1
Chrysopogon zizanioides						
TP	69 ± 0.25	75 ± 7	71 ± 5	80±13	84 ± 5	92 ± 1
NTP	60 ± 1	60 ± 4	54 ± 3	64±10	67 ± 5	75±1
Eichhornia crassipes						
TP	71 ± 3	73±6	62 ± 2	77±5	82 ± 5	89±3
NTP	69±0.3	77±10	62±1	74±9	80±4	89±1
Portulaca oleracea						
TP	68±3	75±4	59±12	64±6	79±5	85±1
NTP	58±4	66±4	41±9	56±13	57±2	60±6

TP: Transformed Plants, NTP: Non-Transformed Plants

TABLE 3: Anova table showing significant difference in BOD, Nitrate, Phosphate between transformed and non-transformed plant species in gravel

Gravel	BOD				NITRATE N				PHOSPHATE P		
	df	SS	F	SIG	SS	SS	F	SIG	SS	F	SIG
1	5	598	3.35	0.14	538	1598	6.9	0.05	2833	676	0.00
2	5	576	13.76	0.02	547	974	2.9	0.15	424	227	0.00
3	5	299	0.33	0.59	233	234	0.24	0.64	27.5	0.71	0.446
4	5	206	13.42	0.02	571	584	0.86	0.4	1057	40.6	0.003

1 -Alternanthera philoxeroides (Alligator weed); 2- *Chrysopogon zizanioides* (Vetiver Grass); 3- *Eichhornia crassipes* (Water hyacinth); 4- *Portulaca oleracea (purslane).*

With waste plastic as a support material, the transformed *Alternanthera philoxeroides* showed significant difference in all parameters in comparison to its non-transformed plants. *Eichhornia crassipes* showed no significant difference in any of the parameters tested which explains that the *Agrobacterium rhizogenes* infection treatment was of no effect. *Portulaca oleracea* was found to be significantly different in terms of BOD and nitrate removal in comparison to non-transformed plants (Table 4, 5). On completion of the treatment process, it was observed that the transformation process increased the efficiency of municipal waste water treatment by these plants. This is attributed to the increase in root biomass due to the formation of additional roots and hairy roots

and also it could be due to increase in secondary metabolite production. Several workers have reported an increase in secondary metabolie production on *Agrobacterium rhizogenes* infection (Bergier *et al*., 2012 and Majumdar *et al*., 2011). *Eichhornia crassipes* showed no significant difference between the transformed and non transformed plants treatment efficiency and hence, it was concluded that the transformation process had no effect on this plant in terms of increasing the efficiency.

TABLE 4: Removal % of various parameters in waste plastic using transformed and non-transformed plant species

mg/L	BOD		Nitrate N		Phosphate P	
HRT↦	3	5	3	5	3	5
Alternanthera philoxeroides	63±6	79±6	58±4	79±9	67±4	85±3
TP	55±4	58±6	42±5	58±4	46±4	71±2
NTP						
Chrysopogon zizanioides	65±3	74±13	71±3	75±1	69±3	86±4
TP	60±7	67±6	60±4	66±14	54±3	62±2
NTP						
Eichhornia crassipes	73±3	75±3	65±12	68±2	81±3	86±3
TP	71±3	81±4	64±12	66±11	83±4	83±3
NTP						
Portulaca oleracea	63±6	68±0.2	53±5	62±9	67±5	70±3
TP	57±0.3	64±0.2	42±3	59±1	55±4	68±3

TP: Transformed Plants, NTP: Non-Transformed Plants

TABLE 5: Anova table showing significant difference in BOD, COD, Nitrate, Phosphate between Transformed and non -transformed plant species in waste plastic

Waste Plastic		Bod			Nitrate			Phosphate		
	df	SS	F	SIG	SS	F	SIG	SS	F	SIG
1	5	1094	23.83	0.008	855	11.9	0.02	324	39	0.03
2	5	489	0.631	0.471	110	0.024	0.008	1756	3.8	0.12
3	5	117	4.24	0.108	250	0.043	0.846	48	1.14	0.34
4	5	81.3	0.167	0.00	800	0.054	0.02	110	1.6	0.2

1 -Alternanthera philoxeroides (Alligator weed), 2- *Chrysopogon zizanioides* (Vetiver Grass), 3- *Eichhornia crassipes* (Water hyacinth), 4- *Portulaca oleracea (purslane).*

CONCLUSION

Portulaca oleracea, Chrysopogon zizanioides and *Alternanthera philoxeroides* showed good transformation results with *Agrobacterium rhizogenes*. The transformed *Portulaca oleracea, Chrysopogon zizanioides* and *Alternanthera philoxeroides* showed significant increase in phytoremediation efficiency as compared to non-transformed plants in both gravel media and waste plastic media. As these plants have shown a positive response to transformation and also an increased efficiency to treat MWW, these plants can be transformed with specific genes for the removal of specific contaminants in waste water and polluted water. As *Agrobacterium rhizogenes* was able carry out transformation successfully in the absence of controlled environmental conditions, it can be used to enhance the efficiency of any existing constructed wetland system.

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