Advance Research in Agriculture and Veterinary Science
ISSN 2348-5353
Adv. Res. Agri. Vet. Sci. Vol. 1, No. 2, 2014: 65-71
Academy for Environment and Life Sciences, India
Available at www.aravs.org

ORIGINAL ARTICLE

ANALYSIS OF SOIL MICROBIAL HEALTH AND PYOLUTEORIN GENE ANALYSIS OF THE PADDY RHIZOSPHERE SOIL OF THE SALINE COASTAL AGRICULTURE SYSTEM OF KANCHEPURAM DISTRICT, TAMILNADU, INDIA

VASANTHABHARATHI V AND JAYALAKSHMI S
Faculty of *Marine Sciences, Annamalai University, Paragipettai Tamilnadu, India*

KEYWORDS
Paddy,
Rhizosphere,
Pyoluteorin,
Salinity.

Article History:
Received on
29 April 2014

Accepted on
23 May 2014

Published on
10 June 2014

ABSTRACT: Paddy rhizosphere Soil samples were collected from different sites along the coastal agriculture belt of Kanchepuram district, Tamil Nadu,India.. The intact root systems from all host plants were collected and soils closely adhering to the roots were used for our studies. The rhisozphere soil samples are named as D1 to D22 and their salinity were recorded. High leval of saliniy was recorded with D17. Minimum was observed with D1.Bacterial population was high in D14, low in D18.Fungal population was high in D16 low in D2.Actinomycete population was high count with D12, minimum in D11. Our results demonstrate clearly that expression of the pyoluteorin biosynthetic genes at different level of salinity in the rhizosphere soil was not affected. It can also be inferred that those Pseudomonas sp which produce pyoluteorin were also not affected by the different levels of salinity.

INTRODUCTION

Soil is a dynamic, living matrix that is an essential part of the terrestrial ecosystem microbes are the main contributors for the dynamicism of soil. Microbes are very important as almost every chemical transformation; taking place in soil involves their active contributions such as carbon, nitrogen cycles, soil fertility which are required for crop productivity (Glick, 1995). They are responsible for recycling of nutrients in the soil (Van loon *et al.*, 1998). Similarly, most of the soil microbes have been found to produce compounds such as vitamins, plant hormones which can improve plant health and crop yield. Certain native microbes present in the soil are antagonistic to plant and can prevent the infections of the crop plants (Weller, 1998). Microbial processes are important for the management of farming system and improvement of soil quality. Microbial respiration of soil has received considerable attention because it can be used as a soil quality indicator (Brendecke *et al.*, 1993) and it is one important variable to quantify soil microbial activity (Alef, 1995)

The study of rice rhizosphere is of vital importance as they are directly involved in increasing crop productivity. The rhizosphere is a zone of great microbial activity with population density 10-200 times greater than the adjacent bulk soil. Microbial diversity is the backbone of agriculture ecosystems and considered the most valuable resource for mankind (Miller and Wood, 1996).

On the other hand abiotic stress like soil salinity, drought and temperature cause serious loss to crop productivity all over the world. Higher salt levels negatively affect plant and soil micro flora (Velusamy, 2003).

It has been estimated that 23% of agricultural soils are affected by problems related to high salinity (Anonymous, 1987). Most crops are sensitive even to relatively low levels of salinity, and in the case of legumes, there is an additional problem because not only the plant but also the symbiotic bacteria (members of the family *Rhizobiaceae*) are sensitive to salinity (Chien *et al.*, 1992) both at the free living stage and during the symbiotic process (Zahran and Sprent, 1986).

Corresponding Author: VASANTHABHARATHI V, Faculty of Marine Sciences, Annamalai University, Paragipettai Tamilnadu, India. **Email:** bharathigene@rediffmail.com

A diverse group of bio-control agents such as Bacteria, Fungi and Actinomycetes exist in nature such as *Bacillus sp, Pseudomonas sp, Lactobacillus sp* and some of the fungi like *Trichoderma sp, peniciilu citrinium and sterptomyces sp.* Pyoluteorin (Plt) is an antibiotic substance produced by certain strains of *Pseudomonas* species, such as *Pseudomonas fluorescens*. It is composed of a bichlorinated pyrrole linked to a resorcinol moiety it can inhibit phytopathogen fungi, including the plant pathogen *Pythium ultimum* effectively and suppress plant disease caused by this fungus .In some instances it contributes to the ecological competence of the producing strain within the rhizosphere. The present study assesses the microbial health of rhizosphere soil, salinity and pyoluteorin gene analysis of the rhizosphere soil.

MATERIALS AND METHODS

2.1. Rhizosphere Soil Sample Collection

Paddy rhizosphere Soil samples were collected from different sites along the coastal agriculture belt of Kanchepuram district, Tamil Nadu. The intact root systems from all host plants were collected and soils closely adhering to the roots were used for our studies. Appropriate sterile materials were used and sterile conditions were maintained during the study. Totally 22 rhizosphere soil samples were collected. All samples were taken at a depth of 0 – 30 cm and they were packed in plastic bags after labeling.

2.2. Texture of the soil sample

The texture of the soil samples varied from sandy, clay to loamy. The general packages of practice followed by the farmers were recorded.

Table 1: General package of practice followed by the farmers in paddy field

Days and events	Practice
	Seed treatment
	Seed at the rate of 24 kg per acre Asozpirillum and phosphobacteria at 2g/kg seed. Wet germination in 2 days taken place.
10 Days after sowing	Nursery preparation at 8 cents for every acre of land cultivated.2kg DAP/cent+cowdunk.1kg pudrone-sand mixture applied half cm water maintained in the nursery
25 Days	Mainland management prior to transplanting rice plough,land leveling FYM (2bags/acre)and DAP complex (1/acre)
	Transplanting of rice takes plac 25-30 days in nursery with 6 ft maintained between the hills
15 Days	Weeding
16 Days	bag urea /acre,1/2 bag potash ,5-10 kg/acre neem cake
20 Days	Pesticide application
40 Days	Weeding, Urea bag urea /acre potash1/2 bag.
45 Days	Pesticide application
85-90 Days after transplant	Harvest operations

2.3. Determination of soil salinity

Salinity was recorded by using Refractometer.

2.4. Assessment of Microbial population

2.4.1. Enumeration of bacteria

Serially diluted 0.1 ml of soil samples were spread in to Nutrient agar media plate (HI-MEDIA).Then plates were kept in an incubator at 35◦c for 24 hours. After the incubation, colonies were counted and calculated.

2.4.2. Enumeration of Fungi

Serially diluted 0.1 ml of soil samples were spread in to Potato dextrose media plate (HI-MEDIA).Then plates were kept in an incubator at 35◦c for 2 to 3 days. After the incubation colonies were counted and calculated.

2.4.3. Enumeration of Actinomycetes

Serially diluted 0.1 ml of soil samples were spread in to Actinomycete isolation agar (HI-MEDIA).Then plates were kept in an incubator at 35◦c for 7 days. After the incubation colonies were counted and calculated.

2.5. Isolation of total soil microbial DNA

Soil samples were selected for DNA isolation based on salinity levels. DNA was isolated following a SDS-based DNA extraction method (Soil sample of 5g were mixed with 13.5ml of DNA extraction buffer and add with 100µl of proteinase K (10mg/ml) in Oakridge tubes by horizontal shaking tubes by horizontal shaking at 225rpm for 30min 37 C.
After the shaking treatment, 1.5ml of 20% SDS was added, and the sample were incubated in a 65 C water bath 1 h with gentle end-over-end inversions every 15 to 20 min. The supernatants were collected after centrifuging at 6,000X g for 10 min at room temperature and transferred into 50ml centrifuge tubes.
The soil pellets were extracted two more times by adding 4.5ml of extraction buffer and 0.5ml of 20% SDS, vortexing for 10s, incubating at 65 C for 10min, and centrifuging as before. Supernatants from the tree cycles of extractions were combined and mixed with an equal volume of chloroform-iso-amyl alcohol (24:1,vol/vol). The aqueous phase was recovered by centrifugation and precipitation with 0.6 volume of isopropanol at room temperature for 1h. The pellet of crude nucleic acids was obtained by centrifugation at 16,000X g for 20 min at room temperature, washed with cold 70% ethanol, and resuspended in sterile deionised water, to give a final volume of 500µl.

2.6. Purification of DNA
A DNA purification kit was used for this purpose (Eppendorf). DNA resolved on a TBE agarose gel, excise DNA band in gel slice and weighed the gel slice (max, 400mg). Added 3 volumes (750µl) of Binding Buffer for every 1 volume of gel slice, incubated at 50ºC for 5 to 10 minutes in an Eppendorf Thermomixer at 1000rpm, vortexing every 2 to 3 minutes. After the gel slice was completely dissolved, added 1 volume of isopropanol equal to the original gel slice volume. Mixed by inversion or pipetting up and down. Placed a spin column in a 2ml collection tube. Added up to 800µl, of sample to the spin column. Spin column at 6,000-10,000xg was centrifuged for 1 minute. Discarded the filtrate and replaced the spin column in the same collection tube. If the sample is larger than 800µl, reloaded and spun again. Added 750µl of Dilution buffer to the spin column. Centrifuged the spin column for 1 minute at 6,000-10,000xg. Discarded the filtrate and replace the spin column in the same collection tube. Centrifuged the spin column for an additional minute at 6,000-10,000xg. Placed the spin column in a new 2ml collection tube. Added 30µl of elution buffer to the center of the spin column and centrifuged for 1 minute at 6,000-10,000xg. Discarded the spin column and the collection tube. The purified DNA was then ready for use in downstream applications or could be stored at -20ºC for further use.

2.7. Polymerase Chain Reaction: (PCR)
PCR amplification of the extracted DNA was performed using the primer combination of Forward primes PLTC1a and Reverse primer PLTC2b (synthesized by Bangalore Genei, Pvt, Ltd, India).
Forward Primer PLTC1a 24mer 5'A ACAGATCGCCCCGGTACAGAACG 3'
Reverse Primer PLTC2 24 mer 5' AGGCCCGGALACTCAAGAAACTCG 3'
PCR amplification was carried out for 25µL reaction mixture in sterile PCR tubes containing 1µl of Primer I (10X), 1µl of Primer II (10X), 2.5µL 100mMdNTPs (each datp, dctp, dctp, & dttp), 2µl of DNA samples, 0.2µl of 1.4U of Taq DNA polymerase enzyme, 18.05µl of double sterilized H_2O was added to make up to 25µl. The reaction mixture in the PCR tubes was then amplified.

2.8. PCR Amplification Conditions:
Initial denaturation 95ºC for 2 min followed by 30 Cycled of 95ºC for 1 min, annealing at 67ºC for 1 min extension at 72ºC for 1 min. The agarose gel electrophoresis was carried out. 1µl of marker and 5µl of the PCR product (amplified gene fragment for Plt) samples were mixed with 1µl of gel loading dye respectively and were loaded to the gel. The gel was visualized on UV transilluminator and examined for the presence of bands.

RESULTS

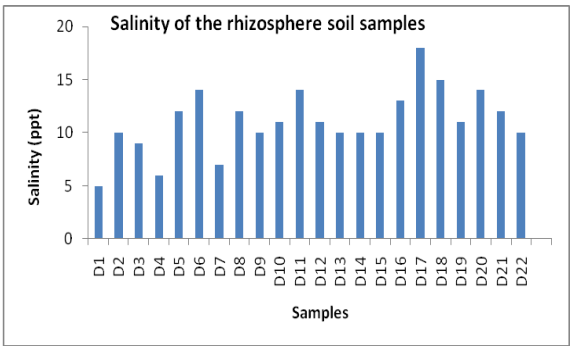

Figure 1: Salinity of the samples

Table 2: Bacterial population

Samples	THB*10^5 cfu/g)
D1	1.23
D2	1.52
D3	.36
D4	2.1
D5	2.69
D6	2.58
D7	0.88
D8	1.23
D9	0.31
D10	3.25
D11	1.25
D12	1.1
D13	1.36
D14	5.55
D15	3.56
D16	1.28
D17	1.69
D18	0.16
D19	0.25
D20	1.58
D21	1.69
D22	1.05

Table 3: fungal population

Samples	Fungal population*10^3cfu/g
D1	0.31
D2	0.11
D3	1.5
D4	1.5
D5	1.4
D6	1.6
D7	0.36
D8	0.25
D9	0.96
D10	1.23
D11	2.36
D12	0.69
D13	1.23
D14	0.89
D15	2.5
D16	2.6
D17	2.3
D18	1.0
D19	1.1
D20	2.3
D21	1.12
D22	2.36

Table 4: Actinomycete population

Samples	Actionomycete population*10^3 cfu/g
D1	0.30
D2	1.2
D3	0.35
D4	0.25
D5	0.10
D6	0.12
D7	0.41
D8	0.21
D9	0.14
D10	0.17
D11	0.11
D12	1.36
D13	0.25
D14	0.63
D15	0.26
D16	0.30
D17	0.25
D18	0.20
D19	0.30
D20	0.26
D21	0.23
D22	1.21

Lane 1

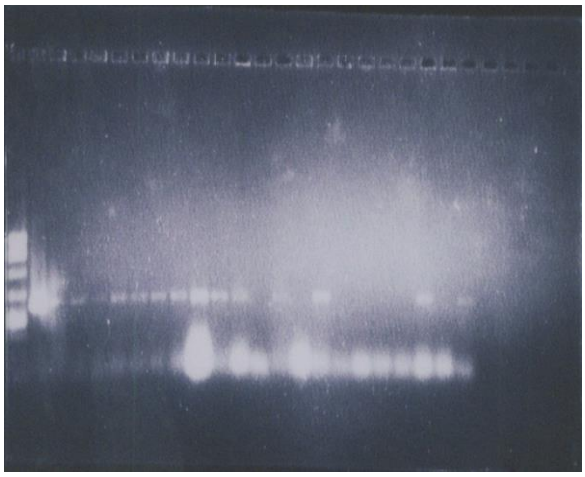

Figure 2: pyoluteorin a gene amplification
Lane 1 DNA marker; Lane 2 to 22- showing the samples D1 to D22 pyoluteorin gene amplification (236bp)

DISCUSSION

Our present study revealed that paddy rhizosphere samples were having different salinity levels (Figure 1). Thus the coastal agricultural rhizosphere soil samples having different salinity level Plants exposed to salt stress undergo changes in their metabolism in order to cope with changes taking place in their environment. This makes water unavailable to plant, which results in reduced water up take (Mwai, 2001).

Total number of bacteria (Table 1) was observed. From the result it was found D14 5.55*105 cfu/g was having maximum population of bacteria, D18 having $0.16*10^5$ cfu/g minimum number of bacterial population. The results favored the conclusion that microbiology of the soils was very complex. This may be due to organic content which would be more in clay-loam soils. It has been studied THB in different soil samples their population also varied very much. This may be due to the fact that metabolites released from rice plants may stimulate soil microbial biomass and populations in the rhizosphere (Bai *et al*., 2000; Lu *et al*., 2002).

Soil fungi can occur free-living or in association with plant roots. The best-known function of fungi is decomposition of complex compounds of plant and animal origins, such as cellulose, lignin, and chitin (Vig *et al*., 2003). Fungi are mainly primary decomposers feeding on carbohydrates. Maximum

population of fungi was observed with D16 sample population which was $2.6*10^3$ cfu/g; minimum was recorded in D2 sample population, $0.11*2.6*10^3$ cfu/g.

The results favored that microbiology of the soils was very complex and therefore, no correlation between population dynamics and habitat could be obtained. Fungal contribution to the decomposition of easily degradable substrates is highest in acid soils. This pattern has been attributed to the ability of fungi in its superior osmatic stress tolerance capabilities in comparison with those of the bacteria (Griffiths *et al.*, 1998; De-Boer *et al.*, 2005)

Biosynthesis of pyoluteorin is initiated from proline or a related molecule, which is condensed serially with three acetate equivalents, with chlorination and oxidation at unidentified stages (Cuppels *et al.*, 1986). Actinomycetes are known to emerge late in the microbial succession, possibly because of their capacity to degrade complex organic polymers (Lacey, 1973).

In our observation among the soil samples, maximum population of actinomycetes was $1.36*10^3$ cfu/g recorded in D12; Minimum w as observed with D11 population, $0.10*10^3$ cfu/g The actinomycetes are better competitors for structural substrate than for easily degradable substrate (Watson and Williams, 1974; Thirup *et al.*, 2001).

Our results (Figure 2) demonstrate clearly that Plt production, and expression of the Plt biosynthetic genes exist at different levels of salinity in the rhizosphere soil. This study also proved that different salinity level does not suppress the pyoluteorin synthesis. As Plt is produced by some *Pseudomonas sp*, it can be safely concluded that those *Pseudomonas sp* are also not affected by different levels of salinity. It was also concluded that *pseudomonas sp* were associated with paddy rhizosphere soil samples.

CONCLUSION

The paddy rhisozphere soil samples were having very rich microbial biota. Different levels of salinity in the paddy rhizosphere soil did not affect the production and synthesis of pyoluteorin. It can also be inferred that those *Pseudomonas sp* which produce pyoluteorin were also not affected by the different levels of salinity.

REFERENCES

Alef K. Estimation of soil respiration. In: Methods in Soil Microbiology and Biochemistry. Academic Press: New York, NY, USA 1995; pp:464-470.

Anonymous. An assessment of the resource base and the supports for the global economy. International Institute for Environment and Development, World Resources Institute. Basic Books, New York 1987.

Bai Q, Gattinger A, Zelles L. Characterization of microbial consortia in paddy rice soil by phospholipid analysis. Microb Ecol 2000; 39: 273–281.

Brendecke J, Alexson RD, Pepper IL. Soil microbial activity as an indicator of soil fertility: long-term effects of municipal sewage sludge on an arid soil. Soil Biol Biochem 1993; 25: 751-758.

Chien CT, Maundu J, Cavaness J, Dandurand LM, Orser CS. Characterization of salt-tolerant and salt sensitive mutants of Rhizobiumleguminosarum biovar viciae strain C1204b. FEMS Microbiol Lett 1992; 90: 135–140.

Cuppels DA, Howell CR, Stipanovic RD, Stoessl A, Stothers JB. Biosynthesis of pyoluteorin: a mixed polyketide-tricarboxylic acid cycleorigin demonstrated by [1,2-13C2] acetate incorporation. Z Naturforsch 1986; 41: 532–536.

De-Boer W, Folman LB, Summerbell RC, Body L. Living in a fungal world: impact of fungi on soil bacterial niche development. FEMS Microbiology Reviews 2005; 29: 95-811.

Glick BR. The enhancement of plant growth by free living bacteria. Canadian journal of microbiology 1995; 41: 109-117.

Griffiths BS, Ritz K, Ebblewhite N, Dobson G. Soil microbial community structure: Effects of substrate loading rates. Soil Biology and Biochemistry 1998; 31: 145-153.

Lacey J. Actinomycetes in soils, compost and fodders In: Skinner FA, Sykes G (Eds.) Actinomycetales: Characteristics and Practical Importance Society of Applied Bacteriology Symposium, Series No. 2, Academic Press, London 1973.

Lu Y, Watanabe A, Kimura M. Contribution of plant-derivedcarbon to soil microbial biomass dynamics in a paddy ricemicrocosm. Biol Fertil Soils 2002; 36: 136–142.

Miller KJ, Wood JM. Osmo adaptation by rhisozphere bacteria. Annual Review of microbiology 1996; 50: 101-136.

Mwai GN. Growth responses of spinderplant (Cleome gynandra L.) to salinity. M.Sc Thesis. Maseno University, Maseno, Kenya 2001.
Thirup L, Johnsen K, Torsvik V, Spliid NH, Jacobsen CS. Effects of Fenpropimorphn on Bacteria and Fungi During Decomposition of Barley Roots. Soil Biology and Biochemistry 2001; 33: 1517-1524.
Van loon LC, Bakker PA, Hand Pietesse CMJ. Systamic resistance induced by rhisospheric bacteria. Annu Review Phytopathol 1998; 36: 453-483.
Velusamy P. Biological control of bacterial blight of rice by plant associated bacteria: molecular and genetic analysis of metabolites for their role in disease control, PhD thesis, CAS in botany madras University 2003.
Vig K, Megharaj M, Sethunathan N, Naidu R. Bioavailability and Toxicity of Cadmium to Microorganisms and their Activities in Soil: a Review. Advances in Environmental Research 2003; 8: 121-135.
Watson ET, Williams ST. Studies on the Ecology of Actinomycetes in Soil-VII: Actinomycetes in a Coastal Sand Belt. Soil Biology and Biochemistry 1974; 6: 43–52.
Weller DM. Biological control of soil borne plant pathogens in the rhizosphere with bacteria. Annu Rev Phytopathol 1998; 26; 379-407.
Zahran HH, Sprent JJ. Effects of sodium chloride and polyethylene glycol on root-hair infection and nodulation of Vicia faba L. plants by Rhizobium leguminosarum. Planta 1986; 167: 303–309.