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DNA Barcoding and Biocontrol strategies for the improvement of Traditional Paddy varieties (TRVs) of Tamil Nadu, India

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ABSTRACT

Totally 25 Traditional rice varieties (TRVs) were collected from selected places of Tamil Nadu. Germplasms were maintained in the department of Botany, Thiagarajar College, Madurai, TN with their passport data. Percentage of germination assessment showed highest percentage of germination in poongar variety. The un germinated seeds were treated under embryogenesis yielded recordable germination. In seed born infection test most of the pathogens observed is *Helminthosporium oryzae* and *Magnaporthe grisea*. The biocontrol assay with Pseudomonos against *Maganporthe grisea* recorded up to 3cm zone of inhibition. The DNA barcoding results were impetrative.

Key words: Poongar, *Magnaporthe grisea* and *Pseudomonos*.

INTRODUCTION

Currently the growth rate of global population is reached 10 billion people by 2050 (UNDESAP 2017) due to this reason increase will occur in the poor, highly populated region of the world that are already highly depended on rice, which emphasize the fundamental role rice will play in the blooming humanitarian crisis. Nowadays the use of Traditional Rice varieties TRVs has been considerably increased because of its low sugar content, good for diabetics, reduces weight, high amount of glutamic acid and vitamins. Report also supports that the TRVs improves eyesight, fertility vocal clarity and mitigating rashes (Caius 1999). Besides the health benefits the TRVs aslo has anthocyanins, anti-oxidative, anti-inflammatory and anti carcinogenic effects (Shipp et al. 2010). In many Asian countries the coloured rice is taken as a functional food (Kim et al. 2008). The compounds like Cyanidin-3-O-β-D-glucopyranoside are adequate in pigmented rice (Ryu et al. 1998). These compounds posses cytotoxicity (Chen et al. 2005), anti neuro degenerative activity (Kim, et al., 2008), inhibition of glycogen phosphorylase (Jakobs et al. 2006). In spite of all those benefits of TRVs the major reasons for the loss of TRVs is due to 1. Disease susceptibility (Leaf Blast caused by *Magnaporthe grisea* and Brown spot caused by *Helminthosporium oryzae*) 2. Low yield 3. New varieties introduction. Apart form all major reasons the disease susceptibility caused drastic damage to the TRVs and made farmers to adopt new varieties. So our present search is to find out the resistant varieties with good yield. The another major problems of TRVs is low in germination due to dormancy, seed born infection and slow growth. According to Kauraw et al. (1987), the major seed born fungi of rice are *Magnaporthe grisea*, *Fusarium moniliformae*, *Alternaria pedwiki* and *Helminthosporium oryzae*. Among many seed born

infections the brown rust disease caused by a *Helminthosporium oryzae* is an infectious fungal disease. We collected around 25 TRVs all over the Tamil Nadu at different season in different locality. TRVs varieties were preserved for longer period by the farmers. The TRVs are ignored for very long time even though there were more nutritive, high fodder yielder, high medicinally valued and ethnic to origin. Improvement strategy: There were many works on distribution studies on TRVs and molecular strategies on improvement have been reported. The gene banks preserves the land races of both national and International levels. To transform the significant traits from TRVs to existing varieties scientist were working all around the world (Sulthan and Subbarao 2013). In 2010 Shipp and Abdel-Aal reported many health related impacts of TRVs are anti-carcinogenic, anti-oxidative and anti-inflammatory. Coloured rice is a major functional food ingredient in several countries of Asia (Kim et al. 2008). The anthocyanins of red and black rice varieties varied in several nutritional facts (Hou et al., 2010) Biological control: Compare to all disease control strategies the biological control strategies gains more important because the beneficial microorganisms control the plant disease and also stimulate the plant growth. Rice blast biocontrol with Pseudomonos (Krishnamurthy- 1998); Bacterial blight of rice biocontrol (Gnanamanickam - 2009). All these reports were from the commercial varieties of rice. Up to our knowledge there were almost no previous report were made on boon control of TRVs disease with Pseudomonos because these varieties were not domesticated. attempt were made to use bio control agents (*Pseudomonas* and *Bacillus*) against rice blast disease of TRVs. The sideropores production of Pseudomonas and 2,4-D production promotes more disease suppression. Our present research findings are aimed towards Collection of traditional rice varieties from Madurai, Thanjavore, Sivagangai, Ulundurpet and germplasm maintenance. Assessment of seed germination percentage. Traditional rice varieties trails in field and pots. Seed dormancy breaking steps. Collection and isolation of rice blast fungi (*Magnoporthe grisea*) from infected rice leaf. Isolation of plant growth promoting *Rhizobacteria* from rhizosphere soil of TRVs. Screening of potential biocontrol agent through dual plate assay. Embryogenesis of dormant TRVs in tissue culture technique. For species characterizing DNA barcoding technique is widely used nowadays only very few DNA fragments is enough to recognize diverse species (Kress et al. 2005). Chloroplast maturase K gene (mat K) is a candidate gene for angiosperms DNA barcode is widely used.

MATERIALS AND METHODS

Collection of traditional rice varieties and germplasm maintenance

Traditional rice varieties were collected from traditional rice breeders and from private NGO. From Madurai, Thanjavore, Sivagangai, Ulundurpet. The collected rice variety were stored in a appropriate containers with code TNKYM (TN – Tamil Nadu; KYM-KattuYanam), TNPG (TN – Tamil Nadu; PG - Poongar), TNSS (TN – Tamil Nadu; SS - Seeragasemba), TNVS (TN – Tamil Nadu; VS - Valansamba) passport data were maintained.

Assessment of seed born infection, percentage of germination in both field and pot trials

To assess the seed borne infection the seeds were placed in PDA medium (without any antibiotic and observed after 48 hours). The percentage of germination was accessed by placing rice seeds. Its in moist growth chamber (petri dish with sterile distilled water on blotting sheets). Three replicates were maintained in petri plates, pot and field. In order to identify seed born infections the rice seeds were put in potato dextrose agar medium. Using small pots TRVs were maintained with regular watering and sunlight. In pots and in field the percentage of germination was cross checked by the following formula.

$$\textbf{Germination (\%)} = \frac{\text{Number of seeds that germinate}}{\text{Number of seed on the tray}} \times 100$$

Traditional rice varieties have been planted in field and pots.

In order to assess the percentage of germination and seedling trails the TRVs were planted in Pots and field. Watered regularly and maintained without addition of any urea and other growth substances.

Seed dormancy breaking steps were processed.

Due to the dormancy of seeds the TRVs were difficult to germinate this dormancy were broken up by 1.Hot water treatment 60 C for 30 minutes, 2.UV treatment for 10-15 minutes. 3. *Pseudomonos* treatment.

Collection and isolation of rice blast fungi (*Magnaporthe grisea*)from Infected rice leaves:

Blast infected rice leafs and neck sample were collected from the different regions in and around Madurai. Data sheets were used to record the details such as cultivar, place of collection, date of collection etc.

Isolation

Blast infected leaf tissues cut into 5 cm sections, washed in running water, surface sterilized with 0.01% HgCl2, incubated for 48 hours at 28ºC under fluorescent light for sporulation. Lesions examined under the microscope and single conidia were picked up with capillary tube and transferred to PDA.

Storage

M.grisea cultures allowed to grow over the filter disks. The colonized filter paper discs were removed slowly and kept for desiccation for 10-15 days and stored at –20ºC until further use.

Isolation of plant growth promoting rhizobacteria from rhizosphere soils of commercial traditional rice varieties.

Soil samples were collected from rhizosphere of traditional rice variety KattuYanam. Dilution plate techniques were followed, with nutrient agar medium (NA). The soil suspension were taken and diluted to 10^3–10^4x in distilled water. The diluted soil suspensions poured on petri dishes containing Kings B agar medium and incubated at room temperature for 24°C/48h. At the end of the incubation, different types of bacterial colonies that appeared on the medium will be selected and re - streaked for pure culture.

Composition of Kings B agar medium: g/litre

Protease peptone -20g, Dipotassium hydrogen phosphate-1.5g, Magnesium sulphate heptahydrate-1.5g (Mgso4.7H2O), Agar -20g, Glycerol-15g, pH-7.2

Screening of potential bio-control agents through dual plate assay

Using petri dishes the collected bacterial strains will be examined for their antagonistic activity against *M. grisea.* The strains which shows the zone of inhibition more than 2cm were taken to further studies.

Embryogenesis of dormant TRVs in tissue culture:

Though there were many rice varieties were tried for germination in pot and field. The TRVs of Kullakar, illupaipoo sample were 100% failure in germination. In order to success this we tried to tissue culture embryos germination method. In MS medium with proper rooting and shooting hormones.

DNA barcoding

Total DNA was extracted using the cetyltrimethyl ammonium bromide (CTAB) method (Doyle & Doyle, 1987). The PCR reaction mixture consisted of 1× PCR buffer, 0.5 mmol/L dNTPs, 0.25 µmol/L each primer, 1 U Taq polymerase, and 5–50 ng template DNA. Thermal cy- cling conditions were as follows: 94 ◦C for 3 min, followed by 40 cycles of 94◦C for 30s ,48◦C for 40s, 72 ◦C for 1 min, and a final extension at 72°C for 10 min. The PCR products were verified by electrophoresis in 1% agarose gels stained with ethidium bromide. Following primer sequence were used (Sang et al., 1997, Tate and Simpson 2003):

psbA3 - GTTATGCATGAACGTAATGCTC
trnH - CGCGCATGGTGGATTCACAATCC
matK472F-
CCCRTYCATCTGGAAATCTTGGTTC
matK1248R-GCTRTRATAATGAGAAAGATTT CTGC

RESULTS

Collection of traditional Paddy variety

The TRVs were collected from farmers, private NGOs and from rice breeders. The informations were collected from the farmers about their nutritional values, seedling, nursery, harvest time and medicinal values were documentated. These informations from their practical knowledge and from their ancesteral inheritance. The rice varieties were stored in gunny bags with proper ventilation. (Table-1) (Fig. 1).

Table 1. Collection data of TRV

S. No.	Place	Name of the varieties	Code and Storage		Siginifance
	Madurai, Sivagangai	Kattuyanam	TNKYM	Dept. of botany	Controls diabetics
2.	Tanjavur, Ulundoorpettei	Swarnamanjuri	TNSM	Dept. of botany	Aromatic rice, low in starch
3.	Sivagangai, Tanjavur	Poongar	TNPR	Dept. of botany	Contains more antioxidants and promotes heart health
4.	Madurai, Sivagangai	Valan samba	TNVS	Dept. of botany	Improves haemoglobin and aids digestion
5.	Tanjavur, Madurai	Karudan samba	TNKM	Dept. of botany	It possess anti-cancer and anti-skin diseases
6.	Ulundoorpettei ,	Seeraga samba	TNSS	Dept. of botany	Selenium content prevents cancer in colon and intestine.
7	Erode	Kalanamak	TNKN	Dept. of botany	Cures skin diseases, blood pressure. Eaten by monks of Buddhist
8	Erode	Mappilai Samba	TNMP	Dept. of botany	Increases Stamina. High amount of carbohydrates and crude fibre

9	Erode	Aathur Samba	TNAS	Dept. of botany	Low in Glycemic index, ood for diabetics
10	Thanjavur	Ilupai poo Samba	TNIS	Dept. of botany	It cures swellings , joint pains paralysis and immunity deficiencies
11	Thanjavur	Kaivarai Samba	TNKS	Dept. of botany	lowering cholesterol
12	Thiruvaroor	Sivapoo Kavuni	TNSK	Dept. of botany	cures anaemia, prevents asthma skin ageing, cures for bone relat problems

Assessment of seed born infection, percentage of germination in pot trials:

The seed born infection were assessed by plating technique in that the majority of seeds were infected by *Helminthosporium oryzae, Aspergillus niger, Alternaria alternate.* (Fig. 2).

Traditional Paddy varieties planted pots.

Traditional paddy varieties were grown in pot culture with out any addition of urea or any other growth hormones. (Fig. 3).

Collection and isolation of rice blast fungi *(Magnoporthe grisea*) from infected rice leaves:

Sucesseptive cultivars were selected from the collection sites of Keeladi, Silaiman, Sakkudi and Thoothai. Totally 32 samples were collected and their passport data were tabulated (Fig. 4) (Table:2,3).

On PDA medium the proliferating *M.grisea* fungi were identified by observing its pyri formed spores under microscope.

Table:2. Collection data of *M.grisea* isolates

S. No.	Place of Collection	Rice varieties	No.of samples Collected	No.of isolates
1.	Pulankulam	Super ponni	11	8
2.	Sakkimankalam	Aishwarya	12	8
3.	Udankundu	LRR Swarna	11	7
4.	Thoothai	Karnadakaponni	10	9

Table 3: Passport data *M. grisea* of isolates

S. No	Place of Collection	Rice varieties	No.of Isolates	Isolates
1.	Pulankulam	Super ponni	8	Py-1, Py-3, Py-5, Py-6, Py-8, Py-11, Py-12, Py-13.
2.	Sakkimankalam	Aishwarya	8	SI-1, SI-3, SI-4, SI-5, SI-6, SI-7, SI-9, SI-10.
3.	Urankundu	LLR Swarna	7	U-2, U-3, U-5, U-6, U-8, U-11.
4.	Thoothai	Karnadakaponni	9	TO-1, TO-3, TO-4, TO-5, TO-7, TO-8, TO-8, TO-9, TO-11.

Fig. 1. Traditional Paddy varities

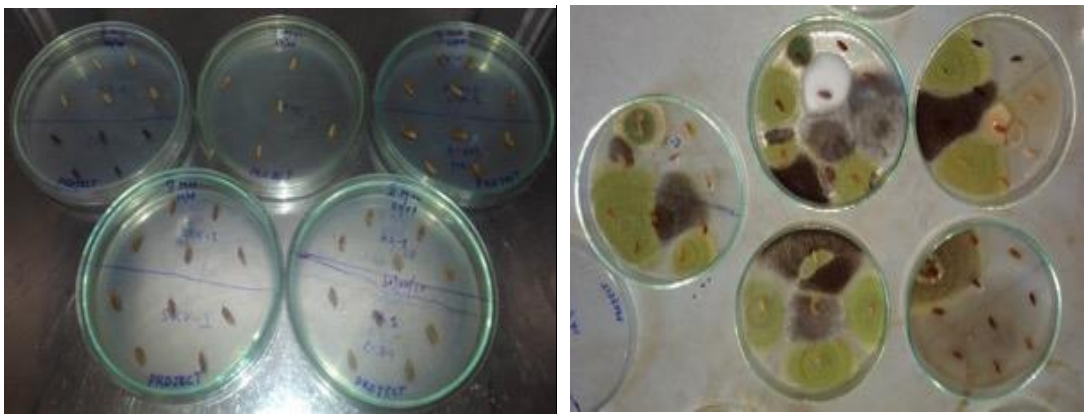

Fig. 2. Assement of seed born infetions in TRVs

Isolation of plant growth promoting rhizobacteria from rhizosphere soils of commercial rice varieties: Totally 19 samples were collected from the rice growing regions. The isolated strains were observed under fluorescent light for confirmation because of the production of 2,4,diacetylphloroglucinal the fluoresces colour appearance was noted under UV light illumination. These culture were grown in Kings B selective medium for *Pseudomonas fluoresces* (Fig. 5)

Screening of potential bio-control agents through dual plate assay:
Fifteen strains of *P. fluorescens* from our selected locations inhibited the rice blast pathogen, and the zone of fungal inhibition ranged above 3cm in diameter. The 3 cm of this zone of inhibition yielded about 90% of dis. Suppression. This could be due to the production of 2,4 diacetylphloroglucinol in the inhibition zone region. Among 15 strains, two isolated from Urankundu (TNTM, URU5, URU7) of sakkimangalam [Fig. 6]

Preparation of bacterial consortium of PGPR:
The two *Pseudomonas fluorescens* strains of Urakundu village of sakkimangalam was potential in disease suppression. Potential bacterial bio-control agents were, pooled in a kings B agar medium and Kept for mass proliferation. After 48 hours the pooled cultures were mixed with CMC + gelatine. For field application of *pseudomonas fluorescens* .We pooled the two strains and prepared bio-consortium.

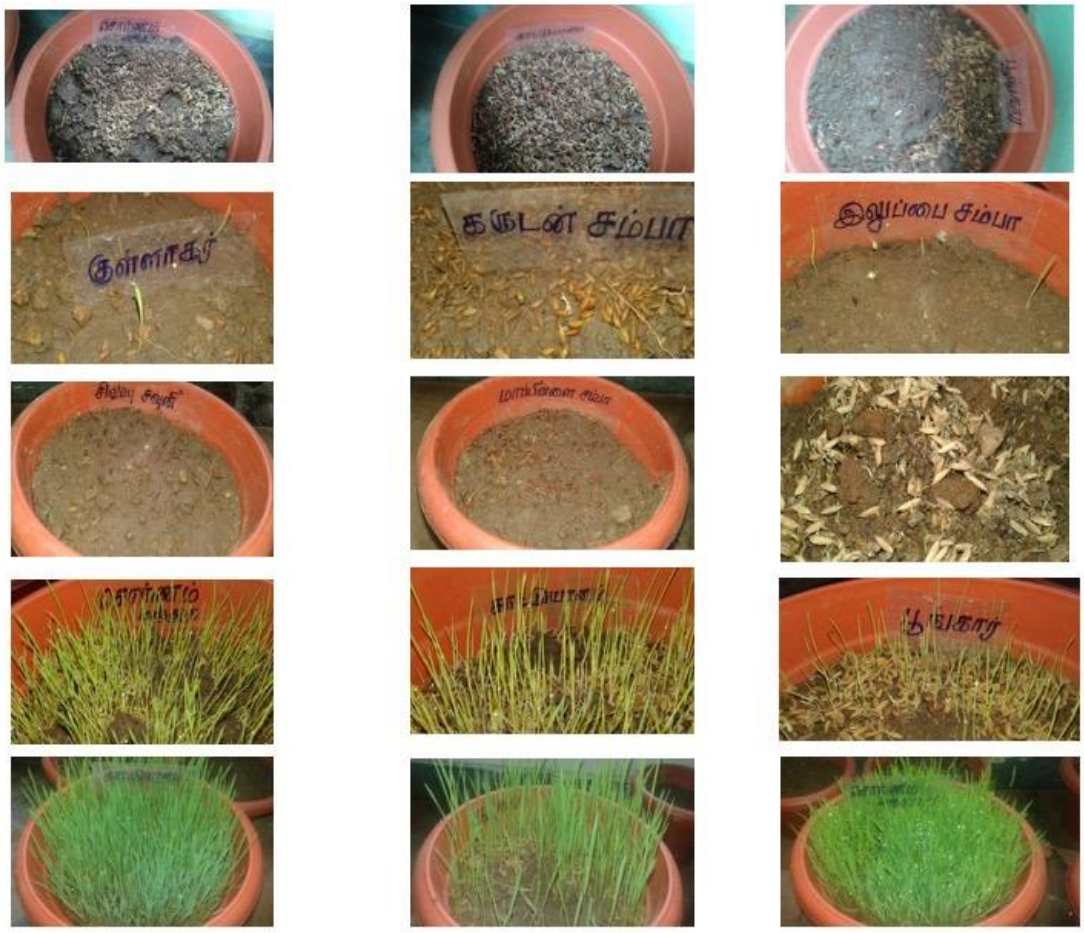

Fig. 3. TRVs in Pot culture

Embryogenesis of dormant TRVs in tissue culture:
Though there were many paddy varieties were tried for germination in pot and field. The TRVs of Kullakar, illupaipoo sample were 100% failure in germination. In order to success this we trial to tissue culture embryos germination method. In this method 2 varieties were started their germination. (Fig. 7)

DNA barcoding of TRVs
Chloroplast based evolutionary studies resulted complementary with nuclear genomic information (Figs 8 a,b).

DISCUSSION

Traditional rice variety of Tamil Nadu (TRVs) has been not much studied. This is the first attempt on TRVs of Tamil Nadu aims towards collection of TRVs and germplasm maintenance. There were few reports from northern part of India

Through our Tamil Nadu is vast traditional state but lack in traditional knowledge documentation.
There were reports from Karnataka on genetic variability analysis of TRVs by Nandini, 2017

Likewise our traditional rice varieties also under threat of an extension. So our first aim is to collect and document the TRVs and germplasm maintenance is a effective methodology for further research.

The collected germplasm of TRVs were in future will be deposited in NBPGR.

The information cxollected from farmers about the TRVs are interesting for Ex:

- Kattuyanam
- Sarnamanjuri
- Poongar

Seed germination

Due to the prolonged storage of TRVs by the farmers to the TRVs seeds are contained by many seed born fungus. This could be the major reason for the low germination and pathological problems leads to low yield and slow growth .This results was supported by the percentage of germination test. Our bio-control strategy Pseudomonas has eliminated the seed born infection up to 90%. The strategy can be employed of TRVs

The major problems of TRVs are dormancy and low percentage of germination. Due to the longer period storage by the TRVs were induced dormancy.

Fig. 4. Rice balst symptoms and Isolation of Magnaporthe grisea form inected samples through mosit chamber technique

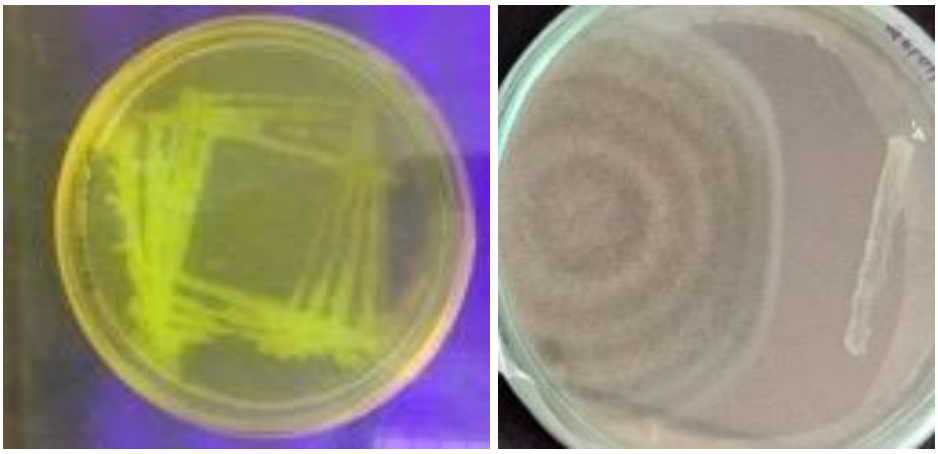

Fig. 5&6. *Pseudomonas fluoroscens* bacteria and its antagonistic activity against *Magnaporthe grisea*.

Fig. 7. Embryogenesis of Kullakar, Illupaipoo samp.

Fig. 8. DNA barcoding of TRVs

The percentage of germination assessed in field and pot compared to control because of this reaction the seasonal change of present day hinders the growth rate of TRVs. So our present on growth enhance with addition of *Pseudomonas fluorescens* treatment.

The appearance of *Pseudomonas fluorescens* effectively root and shoot length. This result supports the previous work on setaria shoot and root length increase.

In current situation farmers only like the fast growing rice verities and more yields. So the farmers don't choose the TRVs. When the hybrid rice varieties are have only less quantity of nutrients. When the height of hybrid rice variety is less then 3cm and it's easily affected the diseases so the farmers use large amount of chemical fertilizers.

Among many major disease of rice, the rice blast disease was choosed because the importance of crop damage up to 60% every year. The blast isolates were taken from commercial varieties because due to the non-availability of blast pathogen been TRVs at present. The previous data about TRVs, blast. Indicated the disease occurrence and damage loss, recorded.

Seed dormancy breaking test have been processed:

TRVs seeds dormancy can be beaked by using dry heat method, Pseudomonas culture treatment, germination under different temperature. The following methods are used to break the seed dormancy, after the seeds are grow in a pots and field.

The TRVs contain high nutritive value then compared to hybrid varieties vary less. But the Farmers grow only hybrid rice varieties.

Similarly as the collection of *Magnoporthe oryzae* from commercial varieties the isolation of *Psedomponas fluorescens* from commercial varieties were subjected for rhizobacteria isolation.

Through there were many other soil bacteria disease bacillus were also obtained of *Pseudomonas fluorescens* were only used because of their previous activity against blast were reported and documented.

Disease control of pseudomonas in pathogen

Previous work on blast disease resistance gene tagging were done by Goh et al.(2018).

But there were almost no attempt was made on biocontrol of TRVs.

So we attempted the dual plate assay of *pseudomonas fluorescens* with *Magnoporthe grisea* reported by many earlier workers.

In TRVs, we use the pseudomonas culture for control of disease attack. The Pseudomonas do not cause any impact in plants. Now-a-day the farmers use the chemical fertilizers for quick results but it cause the very dangerous impact on human through plants.

The aim of preparing bio-consortium is to have better disease management strategy on fields. Our consortium productivity was prepared and it can be recommended to field trails in future. Due to time constrains and longer time needed for the growth and harvest of TRVs. Our preliminary has been pushed up to this level of success.

The attempt of embryogenesis of TRVs. We make at first time. The reported yield recordable result. It may be collected to further studies.

Plants are under serious treatment to various disease for ever. But there were tremendous effect of segregating a new resistance are still prevailing. The combination of bio-control and molecular appearance will severely safe our own method plants.

Traditional rice varieties samples were collected from different growing regions of Tamil Nadu and isolation of *M.grisea* from rice were made. Rhizobacteria were isolated from rhizosphere soils of KattuYanam (KYM). Potential bio-control agents were screened through dual plate assay. Further perceptive

were the preparation of bacterial consortium of PGPR. The results presented here indicate that mixtures of PGPR strains can enhance disease protection and improve the consistency of biological control. Therefore, it is essential to investigate microbial interactions that enhance or detract from bio-control to understand and predict the performance of mixtures of specific bio-control agents.

The both pot and field trails resulted in improving the strategies of seed germination and seed born test. The laboratory assay and field assay has also proved the greater level of disease suppression. We conclude the *Pseudomonas* bacteria will help the plant growth among with disease suppression which may assist the reintroduction of KattuYanam (KYM) variety. In our present study the TRVs namely kaatuyaanam, Sooramoorusi, and Poongar were found to be highly resistant to blast disease in net house nursery condition. Further investigations for two more seasons were needed to reconfirm the resistance.

In the barcoding approach we need to proceed further with some more available varieties.

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