"Analysis of Biofertilizer and future plan for Biofertilizer Laboratory"

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Outline

- Biofertilizer
- Registration Process
- Analysis of Biofertilizers
- Researches
- Future Plan
- SWOT Analysis

Usage of Fertilizer and Pesticides

Biofertilizer

Biofertilizer

- Biofertilizer means the product containing carrier based living microorganisms which are agriculturally useful to increase the productivity of the soil and/or crop. (Fertilizer Control Order 1985)
- It promotes growth by increasing the supply or availabe of primary nutrients to the host plant. (Vessey, 2003)
- It is also one of the interesting technology to increase crop yield by using microbes. (Biofertilizer project FNCA, 2014)

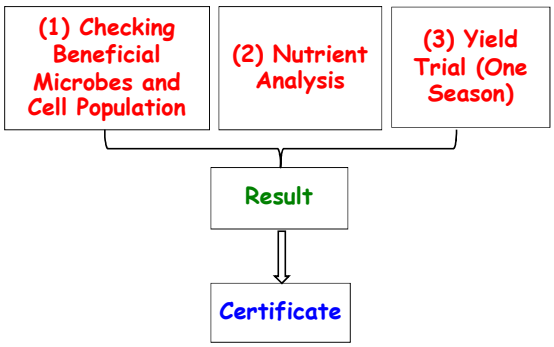
Example of Biofertilizers

- Rhizobium
- Plant growth promoting rhizobacteria (PGPR) (Azospririllum, Azotobacter, Bacillus, Burkholderia, Enterbacter, Klebsiella, Pseudomonas, Beijerinkia, Streptomyces)
- Phosphate-solubilizing bacteria
- Potassium- solubilizing bacteria
- Microbial Inoculum for Composting
- Blue-green algae
- Mycorrhiza
- Trichoderma

Why checking of the products?

- Commercial biofertilizers are increasingly produced and sold for crop productivity
  but some of them don't have any effect at all
- To be efficient and effective, biofertilizer must contain the right microbes for the the right crop
  but there is no scientific evaluation of inoculants to prove that the biofertilizer contain the right microbes or not
- Contaminants can represent a danger for plants, animals and humans during manipulation and after inoculation
  But very few countries have implemented regulation about inoculants (contained strains and contaminants)

Biofertilizer Registration



(Ref:Fertilizer Control Order 1985)

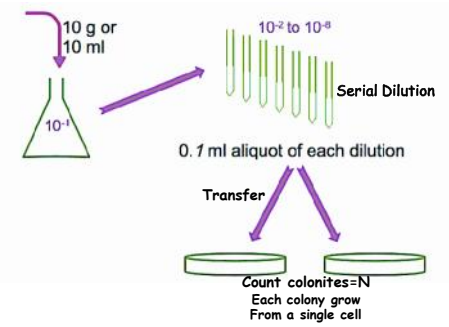
Analysis of Biofertilizer

(1) Checking Beneficial Microbes & Cell Population

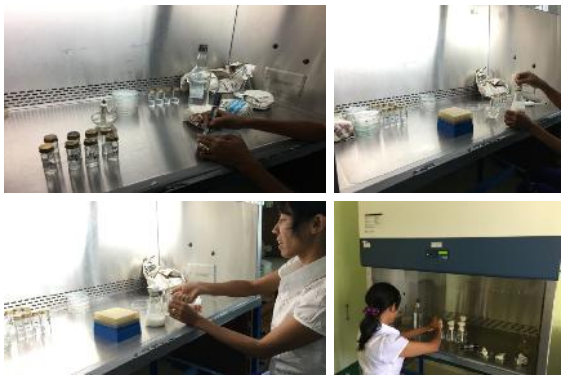
- Biofertilizer sample dilution technique for microbial count
- Quantification of microbes in biofertilizer by the plate counting technique

Biofertilizer sample dilution technique for microbial count

- Isolation of all the strains contained in the products



Biofertilizer sample dilution technique for microbial count



Quantification of microbes in biofertilizer by the plate counting technique

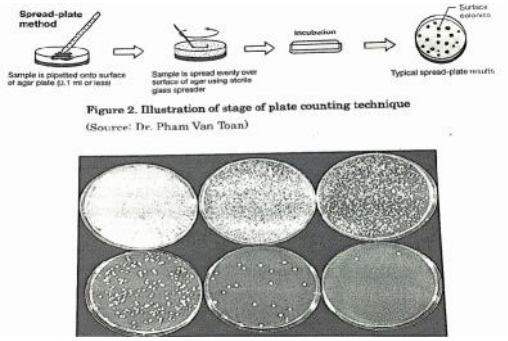


Figure 2. Illustration of stage of plate counting technique
(Source: Dr. Pham Van Toan)

(Ref: Fertilizer Control Order 1985)

Incubation and observation of new colonies ever day: description of the colonies

Beneficial Microbes

Micro-organism-specific media

Micro-organism-specific media

Micro-organism	Media	Author
Bacteria (general)	Thornton's agar medium	Thornton (1922)
Rhizobium	CRYEMA (Congo red yeast extract mannito agar)	Vincent (1970)
	Norris and Date medium	Norris & Date (1970)
Azotobacter	Jensen's N-free medium	Jensen (1942)
	Ashby's medium	Ashby (1907)
	Beijerinckia's medium	Becking (1956)
Azospirillum	Semi solid malate medium	Baldini & Dobereiner (1980)
	Nitrogen free bromothymol blue medium	Dobereiner, Marriel & Nery (1976)
	Okon's modified medium	Okon, Albrecht & Burris (1977)
PSMs	Pikovskaya medium	Pikovskaya (1948)

Staining

Checking Morphological characters Eg. arbuscular mycorrhizal fungi

Shape:	(i.e. globular, spherical, irregular etc)
Size:	Globular: diameter (minimum – average – maximum) Irregular shape: length x width (minimum – average – maximum)
Colour:	(compare with Standard Colour Chart)
Hyphal attachment:	(i.e. sporiferrous saccule, bulbous suspensor etc) sporiferrous saccule = *Acaulospora, Entrophospora, Archaeospora* bulbous suspensor = *Gigaspora, Scutellospora*
Auxiliary cell:	(presence = *Gigaspora, Scutellospora*, none)
Sporocarp:	(presence, none)
Germination shield:	(presence =*Scutellospora*, absence)
Surface ornamentation:	(i.e. smooth, rough, reticulate etc)
Vesicle:	(presence or absence in mycorrhizal roots)

* These characters should be recorded with careful observation of many spores.

(Ref:Biofertilizer Mannual, 2006)

Specification of Biofertilizers

1. Rhizobium

(i)	Base	Carrier based* in form of moist/dry powder or granules, or liquid based
(ii)	Viable cell count	CFU minimum $5x10^7$ cell/g of powder, granules or carrier material or $1x10^8$ cell/ml of liquid.
(iii)	Contamination level	No contamination at 10^5 dilution
(iv)	pH	6.5-7.5
(v)	Particles size in case of carrier based material.	All material shall pass through 0.15-0.212mm IS sieve
(vi)	Moisture percent by weight, maximum in case of carrier based.	30-40%
(vii)	Efficiency character	Should show effective nodulation on all the species listed on the packet.

(Ref:Fertilizer Control Order 1985)

(2) Nutrient Analysis

Objectives of Fertilizer Analysis

- Quality control for fertilizer registration
- Quality control for user
- Quality control for inspection

Quality Control For Registration

- Inorganic (Chemical) Fertilizer
- Organic Fertilizer
- Biofertilizer

References for Analytical Methods

- Association Of Official Analytical Chemists (AOAC)
- The FAO Soil Bulletins (Physical and Chemical
- Methods of Soil and Water Analysis)
- Soil Chemical Analysis (Jackson)
- The Fertilizer (Control) Order 1985
- A Text Book of Soil Chemical Analysis (P.R. HESSE)

Instruments in Laboratory

- Flame Photometer (Na, K)
- Spectrophotometer (P, Fe, Mo)
- Atomic absorption spectrophotometer (Cu, Zn, Mn, Fe)
- Digester Block (Nitrogen Determination)

ITEMS FOR SOIL ANALYSIS AND METHOD

Sr. No	*Items*	*Method*
1.	Soil Moisture	Gravimetric Analysis
2.	pH	1:2.5 (Soil: Water w/v suspension)
3.	EC	1:5 (Soil: Water w/v suspension)
4.	Soil Texture	Mechanical Analysis (Pipetting Method)
5.	Organic Carbon & Humus	Walkley & Black
6.	Total N	Kjeldahl's Method
7.	Available P	- Olsen Method for Alkaline & Neutral soil - Bray Method for acid soil
8.	Exchangeable K, Available K_2O & Exchangeable Na	Flame Photometric Method. {1M Ammonium Acetate (CH_3COONH_4)}

ITEMS FOR SOIL ANALYSIS AND METHOD

Sr. No	*Items*	*Method*
9.	Exchangeable Ca & Mg	EDTA titration Method {1 M Ammonium Acetate (CH_3COO NH_4)}
10.	Exchangeable Al & H	Titrimetric Analysis {1M KCl extracting solution}
11.	Water Soluble SO_4	Turbidimetric Method
12.	Mobile Iron	colordeveloped solution with a a - dipiridile solution.
13.	Soil Water Extraction (CO_3, HCO_3, Cl, SO_4, Na, K, Ca, Mg)	

ITEMS FOR WATER ANALYSIS AND METHOD

Sr. No	*Items*	*Method*
1.	pH	pH Meter
2.	EC	EC Meter
3.	NH_4-N & NO_3-N	Kjeldahl's Method
4.	Available P	Spectrophotometric Method
5.	Available K_2O & Na	Flame Photometric
6.	Ca, Mg, CO_3, HCO_3, CL, SO_4,	Titration Method
7.	Sodium Absorption Ratio (SAR)	
8.	Total Dissolved Solids (TDS)	
9.	Residual Sodium Carbonate (RSC)	

ITEMS FOR FERTILIZER ANALYSIS AND METHOD

Sr. No	*Items*	*Method*
1.	Moisture	Gravimetric Analysis
2.	**Nitrogen** (1)Total N (2)Water soluble N (3)NO_3N (4)NH_4N	Kjeldahl's Method
3.	**Phosphorus** (1)Total P_2O_5 (2)Water soluble P_2O_5 (3)Available P_2O_5 (4)Citric Soluble P_2O_5 (5)Water soluble P_2O_5	Spectrophotometric Method

ITEMS FOR FERTILIZER ANALYSIS AND METHOD

Sr. No	*Items*	*Method*
4.	**Potassium** (1) Total K_2O (2) Water Soluble K_2O	Flame Photometric
5.	Water Soluble Sodium (Na)	Flame Photometric
6.	**Calcium & Magnesium** (1) Total Ca & Mg (2) Water Soluble Ca & Mg	EDTA Titration Method
7.	**Sulphur** Total & Water Soluble S	Turbidimetric Method
8.	Organic Matter	Walkley & Black
9.	pH	pH Meter

ITEMS FOR MICRONUTRIENTS FERTILIZER ANALYSIS AND METHOD

Sr. No	*Items*	*Method*
1.	Molybdenum (Mo)	Spectrophotometric
2.	Boron (B)	Volumetric Method
3.	Chloride (CL)	Titration Method
4.	Manganese, Copper, Iron & Zinc (Mn, Cu, Fe, Zn)	Atomic Absorption Spectrophotometric (AAS)

(3) Yield Trial (One season)

List of Registered Biofertilizer in Myanmar

Sr.	Company Name	Name of Biofer	Microbes
1	United Nilar Agribusiness	Bio Organic Fertilizer (BioGro) (500 g)	Bacillus azotofixans Bacillus megaterium Bacillus subtillis Lactobacillus aciclophillus Saccharomyces cerevisiae
2	Supreme Enterprise Limited	Bio-Organic Fertilizer (Bio Supreme)	Bacillus spp Bacillus sphaericus Streptomyces spp
3	Zayar, Zarni, Sithu Int Trading Co., Ltd.	GP Growth Promoter Fertilizer (EM Tec) (18g)	Bacillus subtilis Bacillus latenspores Bacillus licheniformus Bacillus megaterium Trichoderma harzianum Trichoderma viride

List of Registered Biofertilizer in Myanmar (Cont.)

Sr.	Company Name	Name of Biofer	Microbes
4	TSUBAKILONG LIFE Co., Ltd.	Bio Active Water (Kankyo Daizen) (20 L)	Yeast Lactic acid bacteria
5	Ayeyar Pathein Rice Paddy Trading Co., Ltd.	Biofertilizer (30 kg)	Bacillus subtilis
6	United Nilar Agribusiness Co., Ltd.	Bio Organic Fertilizer (Bio Gro) (20 g)	Bacillus azotofixans Bacillus megaterium Bacillus subtillis Lactobacillus aciclophillus Sucharomyces cerevisiae

List of Registered Biofertilizer in Myanmar (Cont.)

Sr.	Company Name	Name of Biofer	Microbes
7	Ministry of Science and Technology	Shwe Ziwa Biofertilizer (50 kg)	Azotobacter sp Lysobacter sp Saccharomyces Cerevisiae
8	Ministry of Science and Technology	Shwe Thi Pwint (25 kg)	Bacillus megaterium Yeast Pseudomonas spp
9	Kaung Su Co., Ltd.	Bio Fertilizer (SHUBHODAYA) (50 g)	Glomus Vesicular Arbusular Mycorrhiza

Collection of root nodule bacterial isolates from Myanmar during 2010

Shan State Site

Mandalay Site

Yangon Site

( ) indicates number of isolates

(48) root nodule bacteria – MAS1 to MAS48

Materials and Methods

Identification and genetic diversity of bradyrhizobia

- **DNA extraction**

A1E liquid culture
ISOPLANT kit
(Nippon Gene, Japan)

- **ITS Analysis (Sarr et al. 2011)**

Internal transcribed spacer (ITS) region between the 16S and 23S rRNA gene
Primer sets:
ITS1512F(5'GTCGTAACAAGGTAGCCGT-3')
ITSLS23R (5'-TGCCCAA GGCATCCACC-3')

- **Purification of PCR product**

Wizard Gel and PCR Clean-up System (Promega, Madison, WI, USA)

- **Gel electrophoresis**

- **PCR Concentration**

NIH image 1.62
(National Institute of Health, Bethesda, MD, USA)

Result

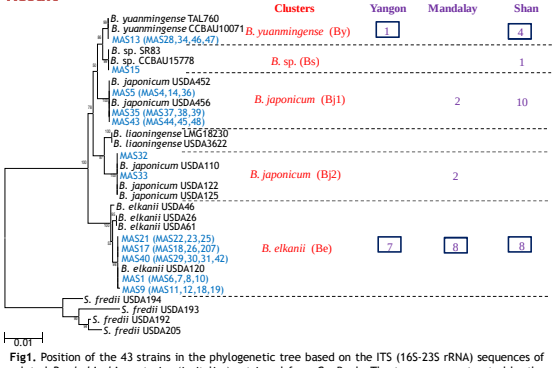


Fig1. Position of the 43 strains in the phylogenetic tree based on the ITS (16S-23S rRNA) sequences of related *Bradyrhizobium* strains (in italics) retrieved from GenBank. The tree was constructed by the neighbor-joining method and 1000 bootstrap replications. Bootstrap values above 50% are indicated at the nodes. Bar; 0.01 in nucleotide sequences. *B: Bradyrhizobium* and *S: Sinorhizobium*.

Result

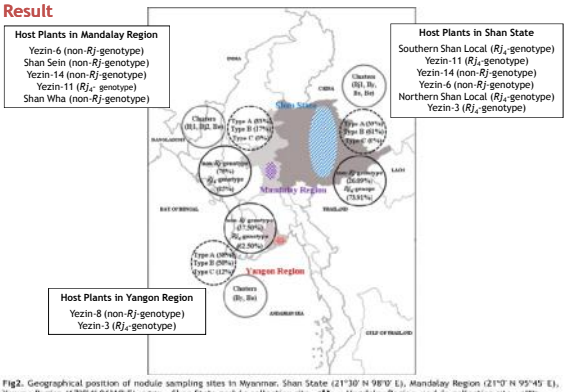


Fig2. Geographical position of nodule sampling sites in Myanmar. Shan State (21°30' N 98°0' E), Mandalay Region (21°0' N 95°45' E), Yangon Region (17°0' N 96°10' E). ≈≈ = Shan State nodule collection site, ▲ = Mandalay Region nodule collection site, ▪ = Yangon Region nodule collection site, ◔ = Nodulation type, ◯ = *Rj*-genotype of soybean, ◯ = Clusters of *Bradyrhizobium* strains, ▭ = Host plants in individual sites.

RESEARCH ARTICLE
ScienceAsia 39 (2013): 574–583
doi: 10.2306/scienceasia1513-1874.2013.39.574

Phylogenetic diversity of indigenous soya bean bradyrhizobia from different agro-climatic regions in Myanmar

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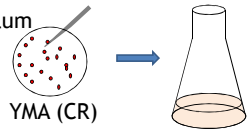
Evaluation of effective Myanmar *Bradyrhizobium* strains isolated from Myanmar soybean and effects of coinoculation with *Streptomyces griseoflavus* P4 for nitrogen fixation (Khin Myat Soe and Takeo Yamakawa, 2013)

Materials and Methods

- Preparation of inoculum



YMA (CR)

20 mL of A1E liquid medium (Kuykendall, 1987)

Incubation: 30˚C for 7 days, 100 rpm

100 times dilution with Hoagland solution

ca 10^7 cells mL^{-1}

Result: Screening of effective bacterial strains by Yezin-6 for nitrogen fixation

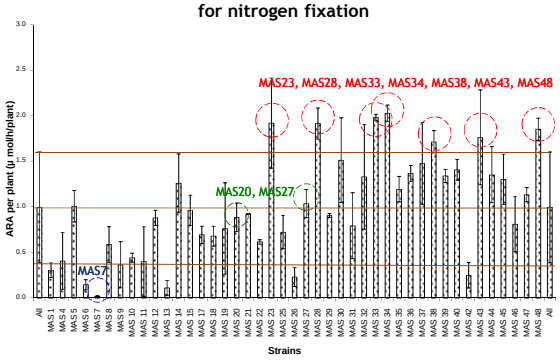


Fig. ARA per plant of Yezin-6 inoculated with 43 Bradyrhizobial strains after five weeks

Result Summary

- Synergistic effects of P4 coinocuation with some *Bradyrhizobium strains* was found.
- The positive interactions were observed under proper varieties and proper *Bradyrhizobium* strains were used with P4.
- It is worthwhile to further investigate on this selected P4 and some *Bradyrhizobium strains* for field experiment with Myanmar soybean varieties.

Soil Science and Plant Nutrition (2013), 59, 361–370 http://dx.doi.org/10.1080/00380768.2013.794437

ORIGINAL ARTICLE

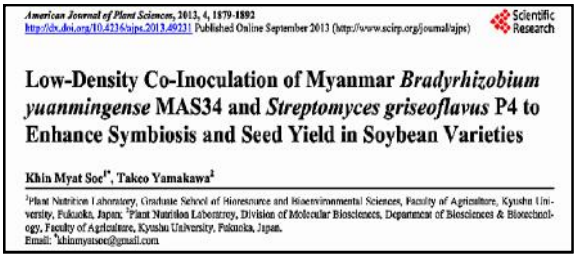
Evaluation of effective Myanmar *Bradyrhizobium* strains isolated from Myanmar soybean and effects of coinoculation with *Streptomyces griseoflavus* P4 for nitrogen fixation

Khin Myat SOE[1] and Takeo YAMAKAWA[2]

Low density coinoculation of Myanmar *Bradyrhizobium yuanmingense* MAS34 and *Streptomyces griseoflavus* P4 to enhance symbiosis and seed yield of different soybean varieties (Khin Myat Soe and Takeo Yamakawa, 2013)

Result Summary

- The single inoculation of *Bradyrhizobium yuanmingense* strain improved growth, N fixation and yield of Yezin3, Myanmar soybean variety.
- Synergistic effects of P4 coinocuation with *Bradyrhizobium yuanmingense* was found in Yezin6, Myanmar soybean variety.



American Journal of Plant Sciences, 2013, 4, 1879-1892
http://dx.doi.org/10.4236/ajps.2013.49231 Published Online September 2013 (http://www.scirp.org/journal/ajps)

Low-Density Co-Inoculation of Myanmar *Bradyrhizobium yuanmingense* MAS34 and *Streptomyces griseoflavus* P4 to Enhance Symbiosis and Seed Yield in Soybean Varieties

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Effects of *Streptomyces griseoflavus* P4 on Seven different crops of rice, maize, wheat, spinach, komatsuna, sweet pea and soybean (Khin Myat Soe, Unpublished)

Result

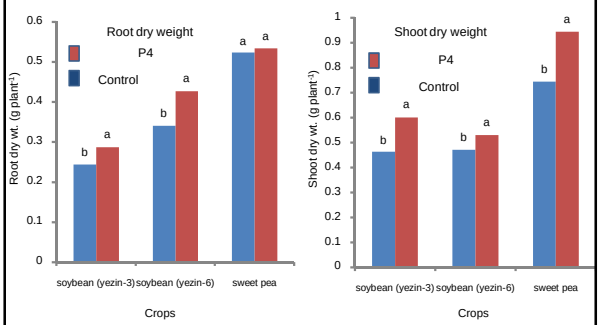


Fig. Effects of *Streptomyces griseoflavus* P4 on root and shoot dry weight of soybean and sweet pea after two weeks. Means in each crops follows by different letters differed significantly at $P<0.05$ (Tukey's test).

Result

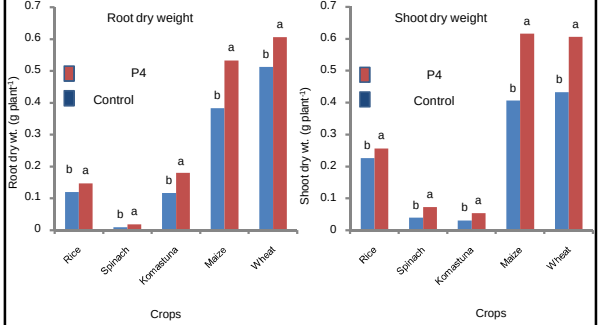


Fig. Effects of *Streptomyces griseoflavus* P4 on root and shoot dry weight of rice, spinach, komatsuna, maize and wheat after three weeks. Means in each crops follows by different letters differed significantly at $P<0.05$ (Tukey's test).

Result

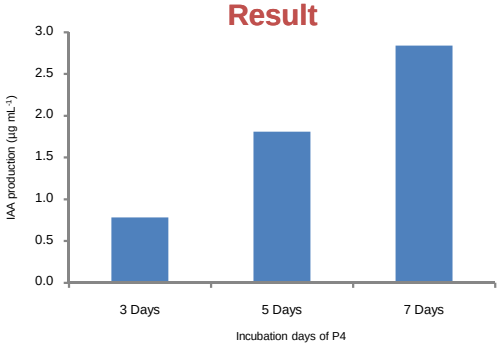


Fig. Effects of *Streptomyces griseoflavus* P4 on Indole Acidic Acid (IAA) production after 3days, 5 days and 7 days

Effects of Co-inoculation of Myanmar *Bradyrhizobium yuanmingense* MAS34 and *Streptomyces griseoflavus* P4 to enhance symbiosis and seed yield of different soybean varieties (DAR Research Farm)

Materials and Methods

- Experimental site - DAR Research Farm
- Experimental design - Split-plot design
- Treatments - 4
- Replications - 3
- No. of experimental plots - 24
- Soybean varieties - Yezin-12 and CM 6
- Sowing Date - 1.8.2016

Materials and Methods

- Soybean Varieties (as Main plots)

  V1 = Yezin-12, V2 = CM 6

- Treatments (as Sub plots)

(1) T1 - Uninoculated (Control)

(2) T2 - *Streptomyces griseoflavus* P4 (P4)

(3) T3 - *B. yuanmingense* MAS34 (MAS34)

(4) T4 - P4 + MAS34

Materials and Methods

Data Collection

- V6 stage, R3.5 stage and Maturity stage
- Dry weight determination (nodule, root and shoot)
- Seed yield at maturity stage
- Seed yield (kg/ha)= $\frac{A \times B \times C \times 100 \text{ seed wt. (g)}}{10}$

  A= number of plants/m^2

  B= number of pods/plant

  C= number of seeds/pod

- Nitrogen fixation
- Total N accumulation (Cataldo *et al*. 1974)

Effects of EM, *Trichoderma* and *Sreptomyces griseoflavus* P4 with different fertilizer levels on Sin Thu Kha, Rice Variety (DAR Research Farm)

Effects of EM, *Trichoderma* and *Sreptomyces griseoflavus* P4 with different fertilizer levels on Sin Thu Kha, Rice Variety (Pot Experiment)

Materials and Methods

- Experimental site - DAR Research Farm
- Experimental design - Split-plot design
- Treatments - 4
- Replications - 3
- No. of experimental plots - 36
- Fertilizer Level - F0, F50 and F100 (DAR)
- Sowing Date - 2.8.2016

Materials and Methods

- Microbes (as Main plots)

(1) Uninoculated (Control)

(2) EM

(3) *Trichoderma*

(4) *Streptomyces griseoflavus* P4

- Fertilizer Level (as Sub plots)

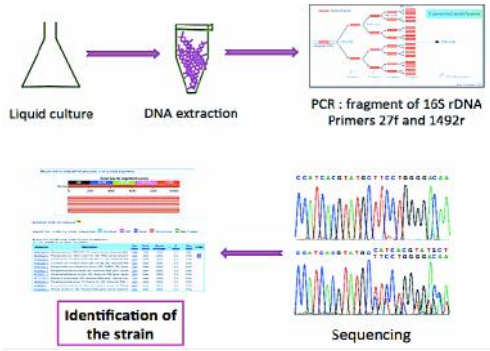
(1) F1 - F0 (Control)

(2) F2 - F50 (50% DAR recommended rate)

(3) F3 - F100 (100% DAR recommended rate)

Future Plan

Identification of the strains



Source: Didier Lesueur (Ph.D)

Future Plan

1. Isolation of microbes from roots or soils
2. Laboratory screening of microbes for plant growth
3. Greenhouse screening of microbes for promote growth in potted soil
4. Field screening of most effective microbes in cropped soil
5. Refinement of inoculum
6. Substantiation of microbes
7. Production

SWOT Analysis

Strengths ▪ Collect the indigenous beneficial microbes ▪ Strong encouragement of Department	**Weaknesses** ▪ Limited Resource Person ▪ Need Lab facilities
Opportunities ▪ Saving cost and environment ▪ Move Sustainable Agriculture	**Threats** ▪ Less percentage of Farmers' Community awareness ▪ Need International Collaboration

Thank You