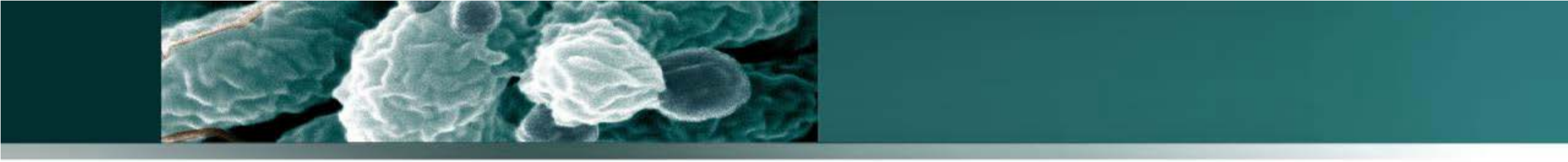

Morphological, Biochemical and Molecular Characterization Of Gibberellic Acid- Producing Bacteria, Isolated from *Saccharum* sp. and *Eleusine* sp. and Their Effect On The Growth Of Corn and Lettuce

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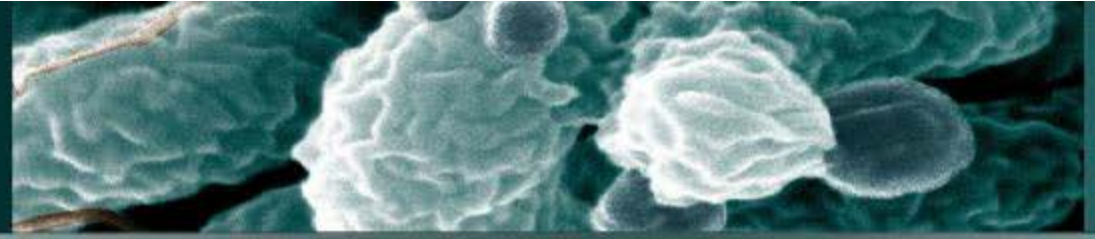
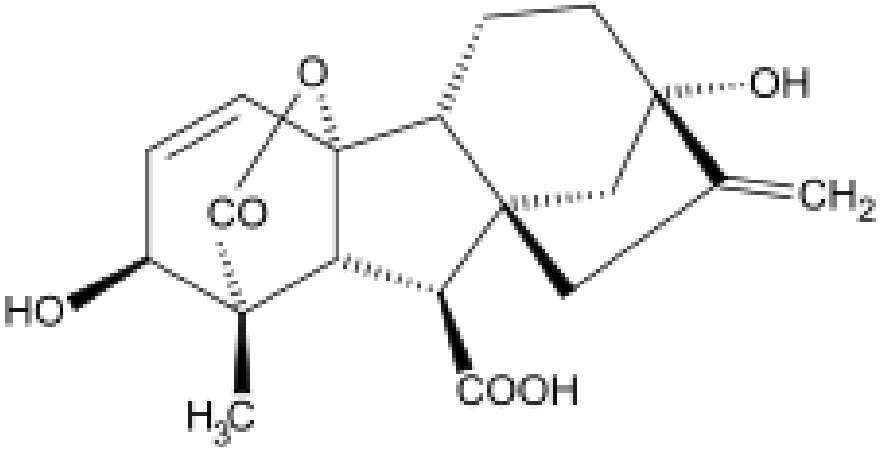
College, Laguna, Philippines



Introduction

Gibberellic Acid (GA)

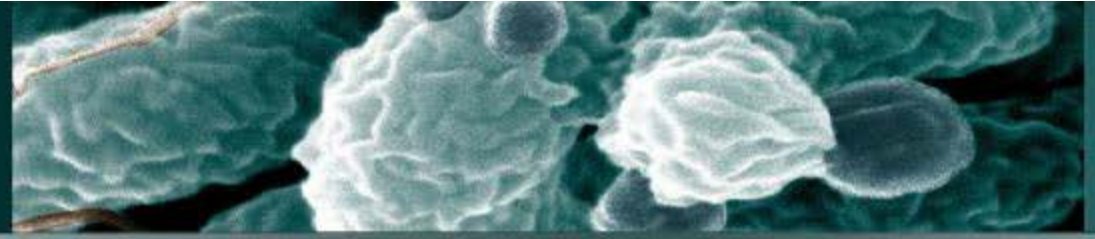
- Break seed dormancy
- Enhance stem and root elongation
- Delays senescence
- Produced as secondary metabolite by bacteria that functions as signaling factor to the host plant



136 known chemical structures

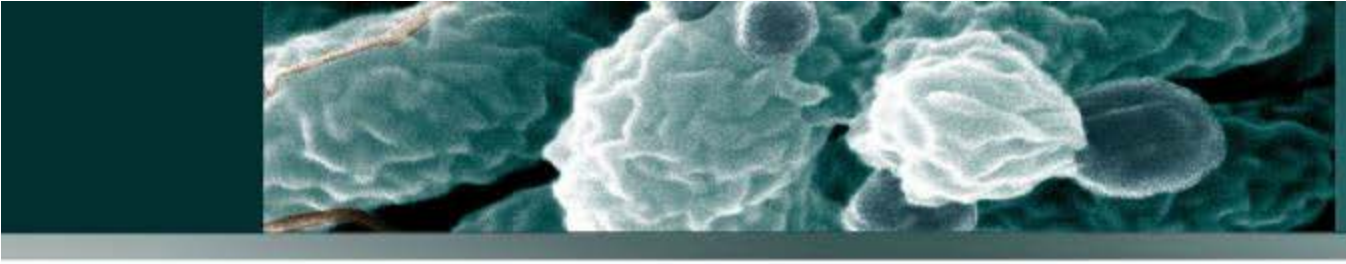
GA_1, GA_2 and GA_4 for shoot elongation

GA_3 removed inhibition of sugarcane growth due to salt stress



Objectives

- Characterize GA secreting bacteria from sugarcane and grass roots
- Quantify GA production
- Identify high GA producing strains through 16S rDNA sequencing
- Determine effects of bacterial isolates on plant development



Methodolology

Isolation of Endophytic plant growth promoting Bacteria

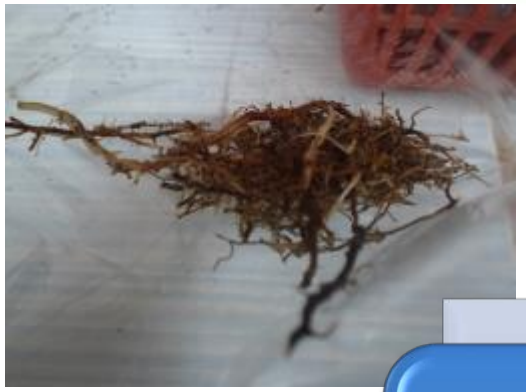
Qualitative and Quantitative Analysis

Biochemical Partial Characterization

16S rDNA isolation and sequencing

in *vivo* Assay in corn and lettuce as host plants

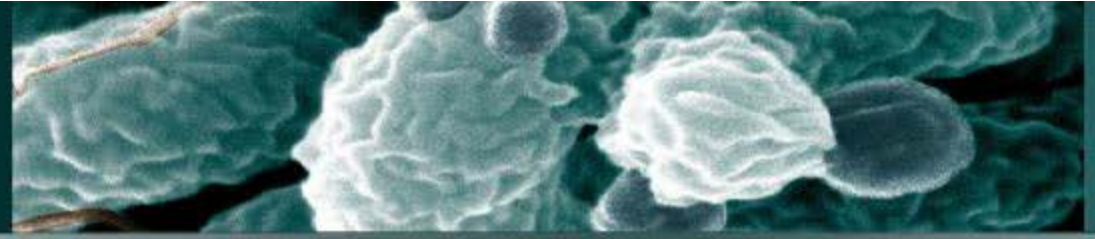
Isolation of endophytic PGPB



Extraction from root tips of sugarcane and grass

Serial Dilution

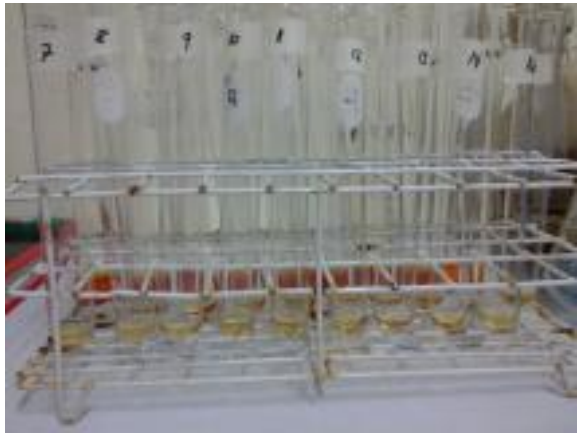
Spread Plating on Congo red-Nitrogen Free medium and *Azotobacter* medium



Qualitative and quantitative analysis of Gibberellic acid

GIBBERELLIC ACID PRODUCTION ASSAY (Graham and Thomas, 1961)

- Centrifugation: 6000rpm 12 minutes
- Addition of 2, 4-DNP to supernatant
- Boiling to 100°C
- Addition of Alcoholic KOH



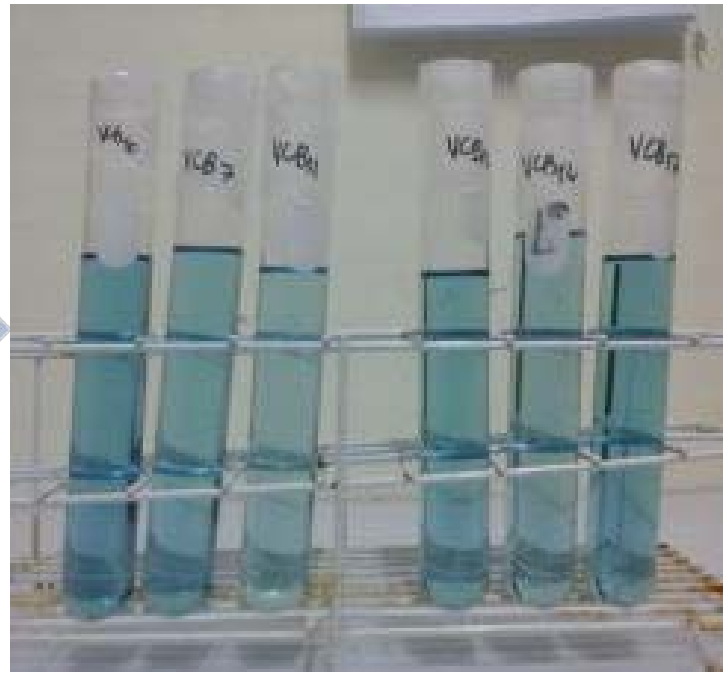
GIBBERELLIC ACID EXTRACTION

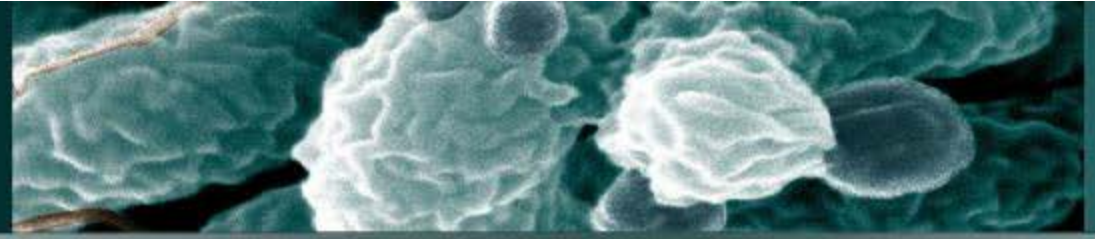
- Centrifuge: 10,000rpm, 10mins
- Acidification to pH2 of supernatant
- Extraction with Ethyl Acetate
- Redissolve GA residue in 0.05% Tween80

Qualitative and Quantitative Analysis of Gibberellic Acid

Phosphomolybdic Acid Reagent (Graham and Henderson, 1961)

1. Addition of 15ml Phosphomolydic acid reagent to GA extract
2. Incubate in boiling water for one hour
3. Addition of dH_2O up to 25ml
4. Measure absorbance at A780nm



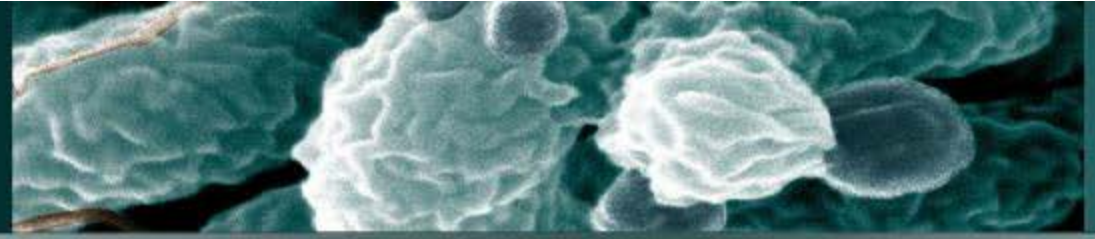


Partial Characterization of Bacterial Isolates

Colonial Morphology → Cell Morphology → Biochemical Characterization

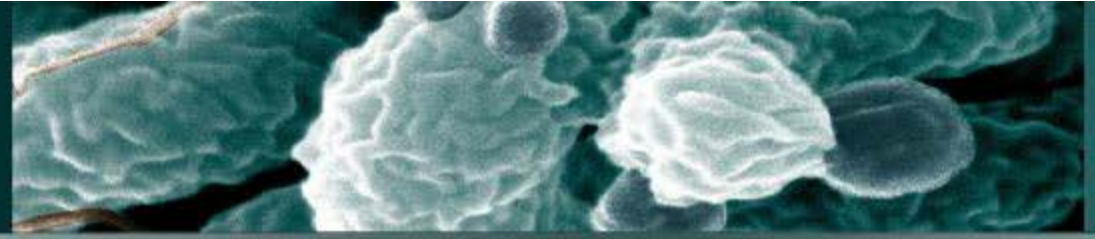
Gram Staining Method

- Crystal violet (1min)
- Gram-iodine (1min)
- 95% alcohol (30secs)
- 0.25% safranin (30secs)



Biochemical Tests

- Test for Oxygen Requirement
- Catalase Reaction
- Utilization of Citrate
- Starch Hydrolysis Test
- Tween80 Hydrolysis
- Gelatin Liquefaction test
- Acid Production from Carbohydrates



Molecular Identification of Bacterial Isolates

Genomic DNA Extraction

- (CTAB Method)

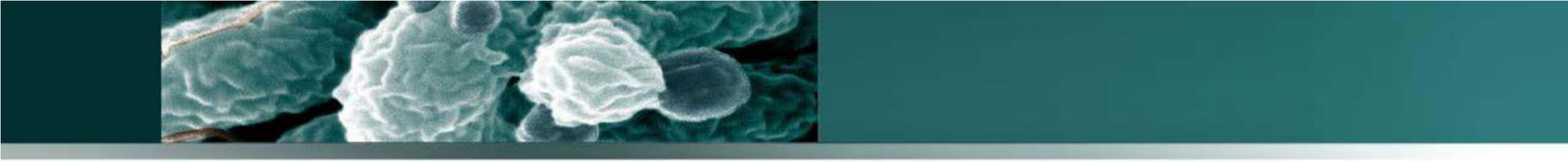
Gene Amplification

- **FC/RC: 30 cycles**
- **FC27 (5’-AGAGTTTGATCCTGGCTCAG-3’)**
- **RC1492 (5’-TACGGCTACCTTGTTACGACTT-3’)**
- **Annealing Temp: 55°C**

16S rDNA Sequencing

- Basic Local Assignment Tool (BLAST)

In Vivo Assay



RESULTS AND DISCUSSION

Isolation of Plant Growth Promoting Bacteria

Table 1. Number of bacterial isolates obtained from CRNF and ATB media and their assigned codes.

Sample	Media	No. of Isolates	Isolate codes (VCB)
Sugarcane	CRNF	7	1-5sc, 16-17sc
	ATB	4	12-15sa
Grass	CRNF	8	6-8gc, 18-22gc
	ATB	3	9-11ga

22 isolates

Legend: (CRNF) Congo-red Nitrogen-free medium, (ATB) *Azotobacter* medium

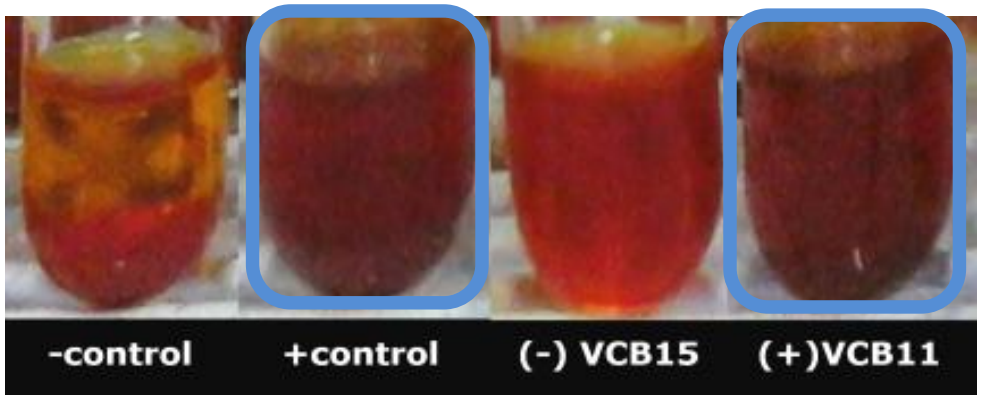


Fig. 1. Representative solution color from result of the Gibberellic Acid Assay

Quantification of GA produced

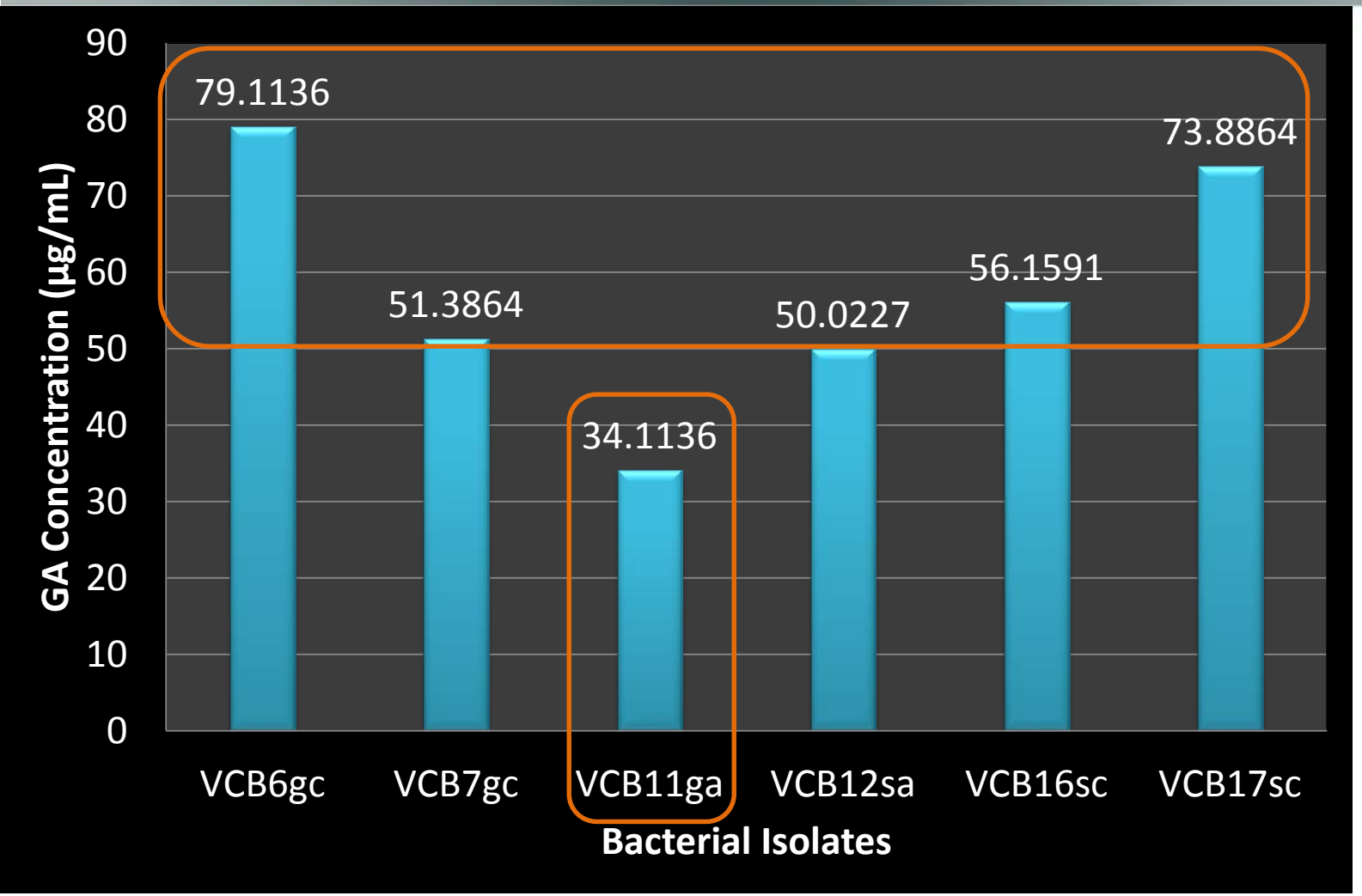
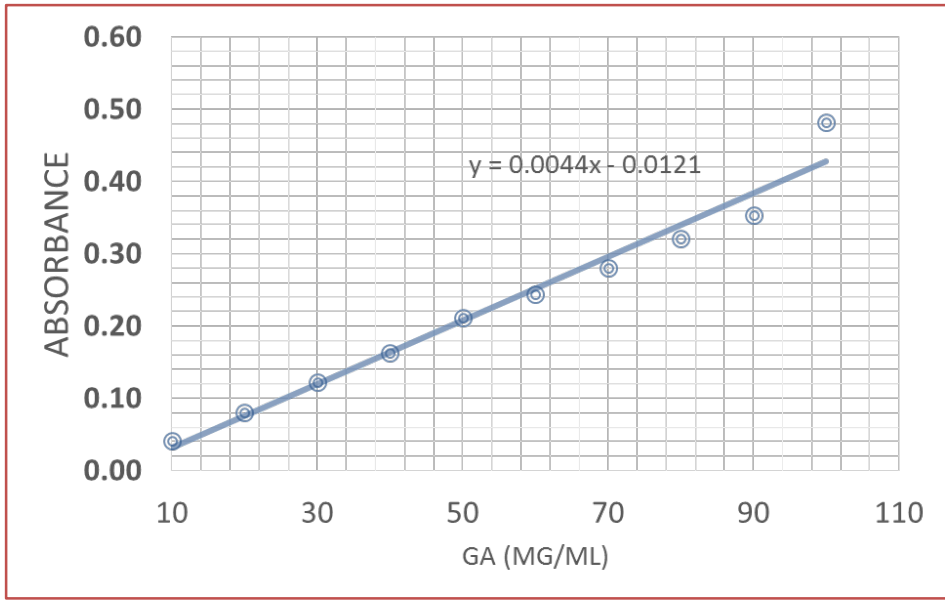
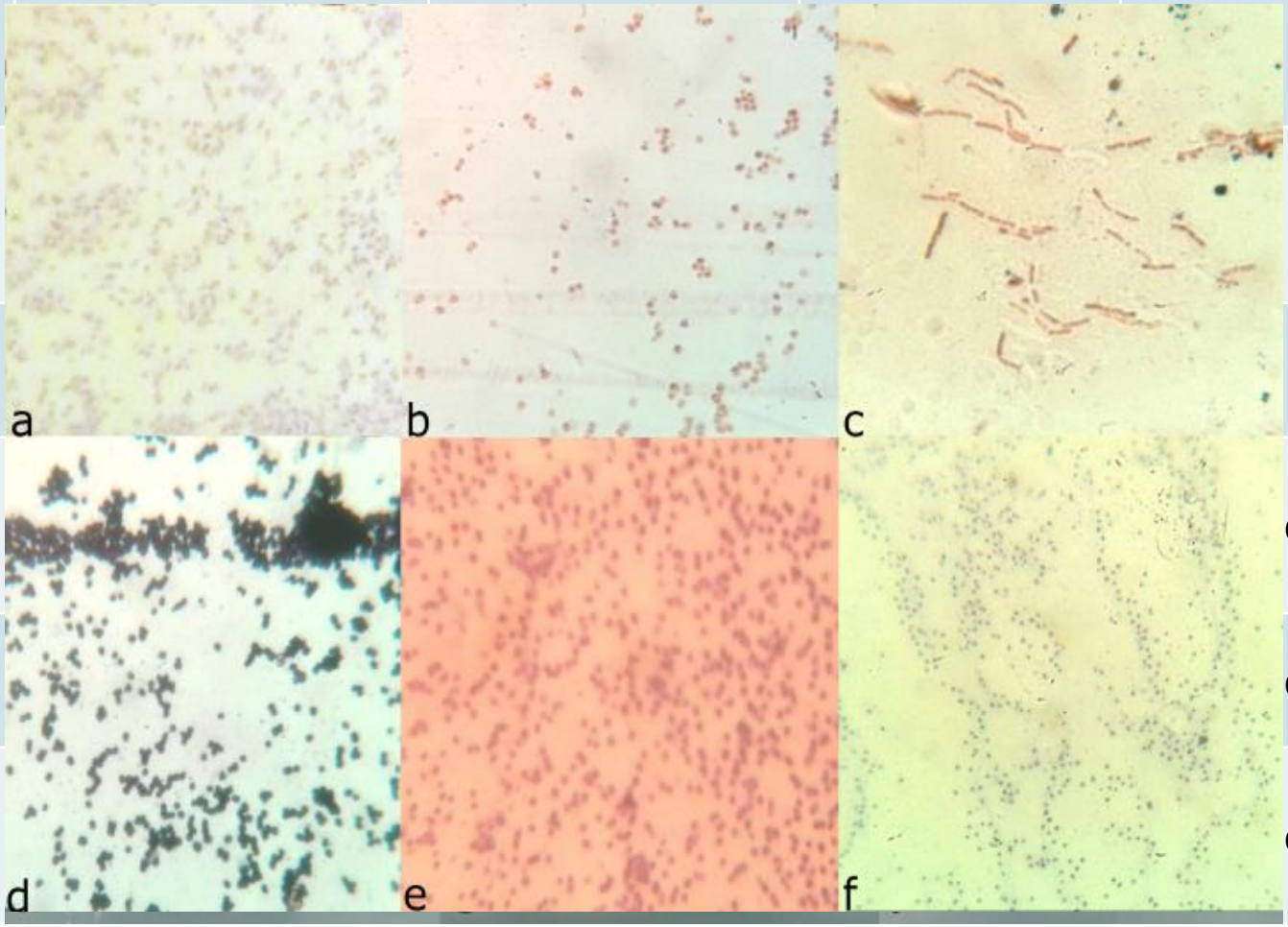


Fig. 3. Concentrations of GA produced by bacterial isolates



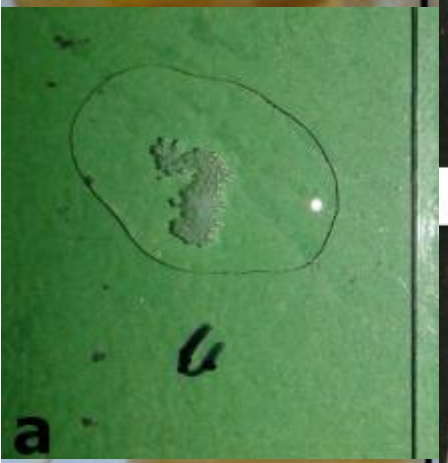
Standard Curve of Gibberellic Acid

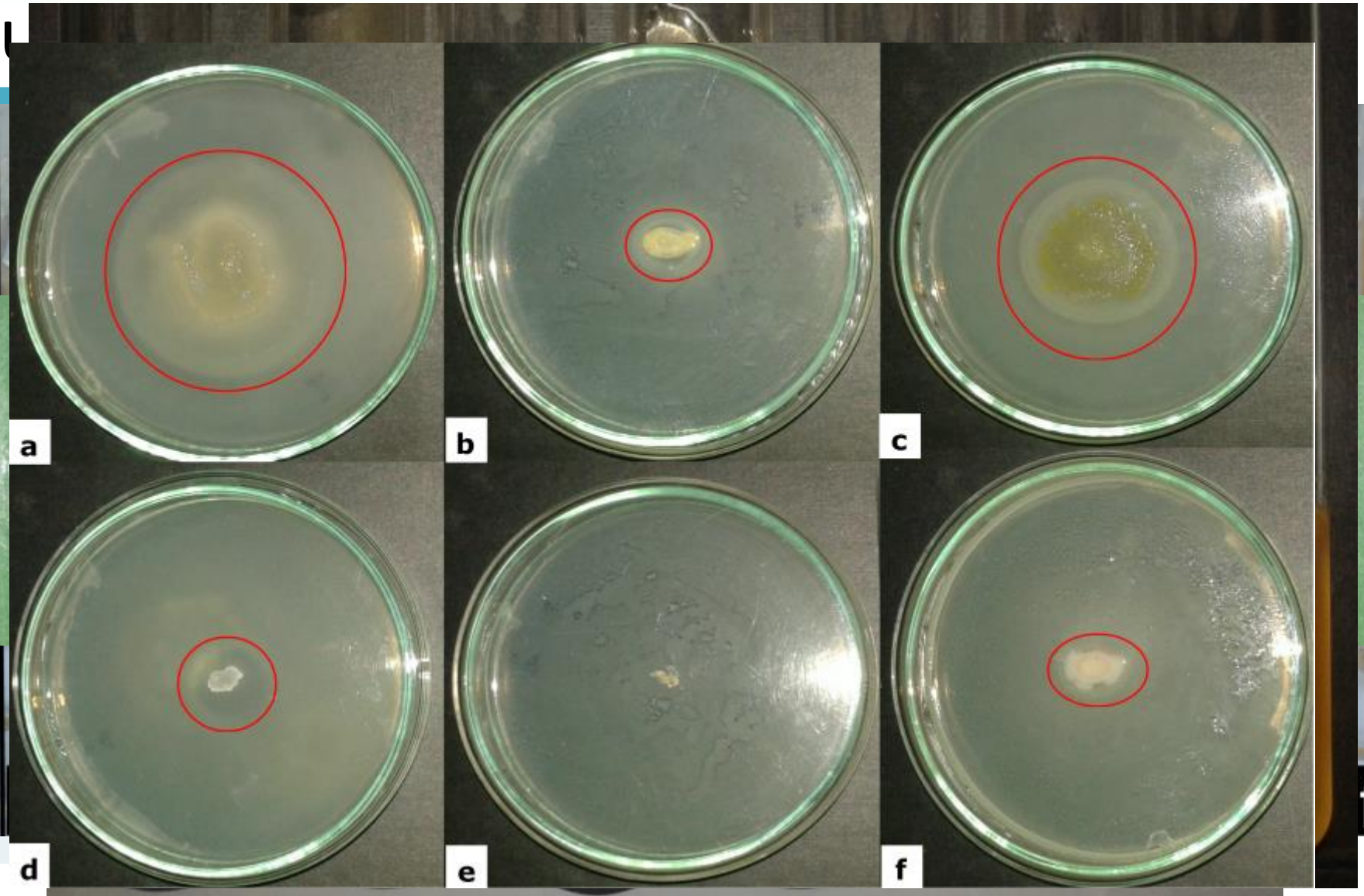
Partial Characterization of Bacterial Isolates

Bacterial Isolate	Colony Morphology	Colony size	Gram Reaction	Cell Morphology	Cell size
VCB6gc				rod	1-1.5µm
VCB7gc				rod	1-1.5µm
VCB11ga				rod	3-5µm
VCB12sa				cocci	1-2µm
VCB16sc				cocci	1-2µm
VCB17sc				cocci	1-1.5µm

Biochemical Characterization

Table 3. Su

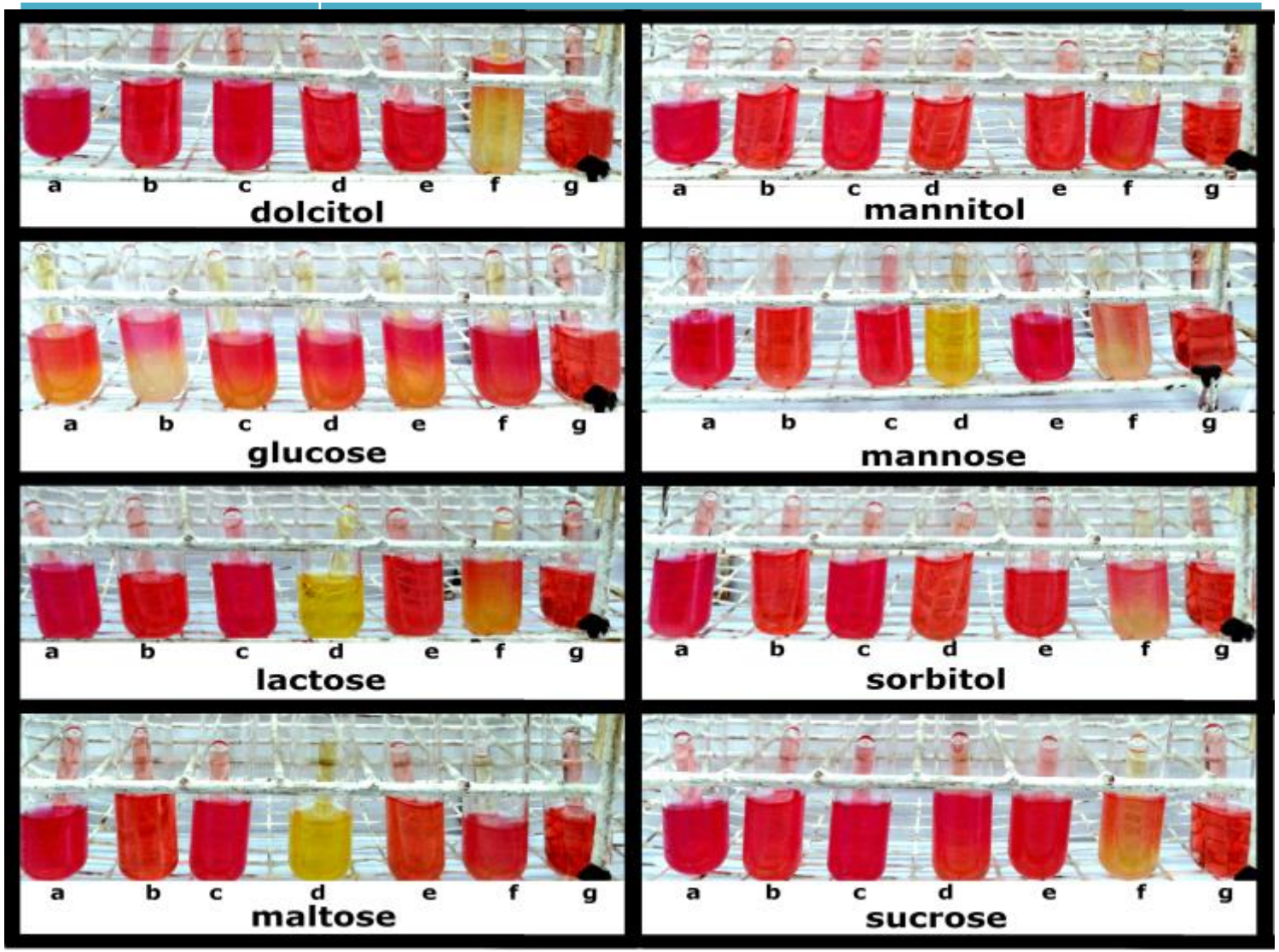


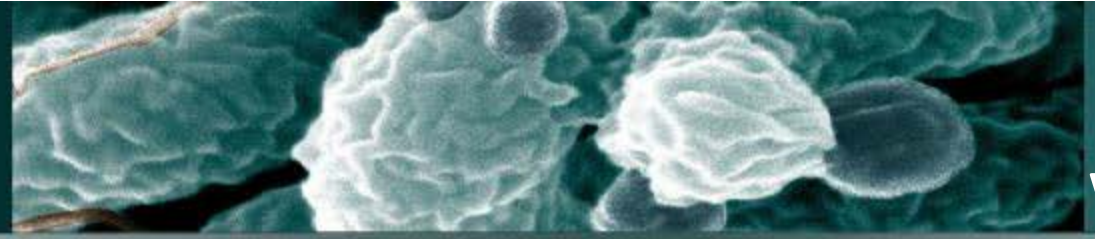


Biochemical Characterization

Acid Production from Carbohydrates

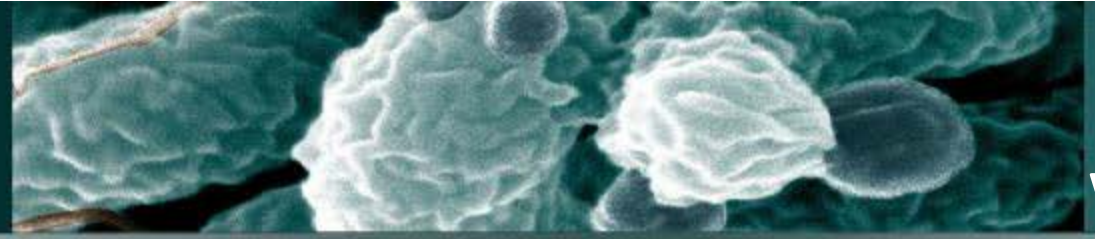
Table 4. Acid and gas production of isolated bacteria from carbohydrates.





Molecular Characterization with 16s DNA sequencing

Bacterial Isolates	Description	Length (bp)	Score (bits)	E value	Identities %
VCB6gc	*Pseudomonas hibiscicola* strain ATCC 19867	1191	1827	0.0	97%
VCB7gc	*Paenibacillus lautus* strain NBRC 15380	1181	730	0.0	80%
VCB11ga	*Stenotrophomonas pavanii* strain LMG 25348	1166	372	2e-102	69%
VCB12sa	*Micrococcus yunnanensis* strain YIM 65004	1015	901	0.0	83%
VCB16sa	*Staphylococcus hominis* sbs. novobiosepticus strain GTC 1228	1180	1918	0.0	98%
VCB17sa	*Staphylococcus aureus* (identified through biochemical characteristics)				

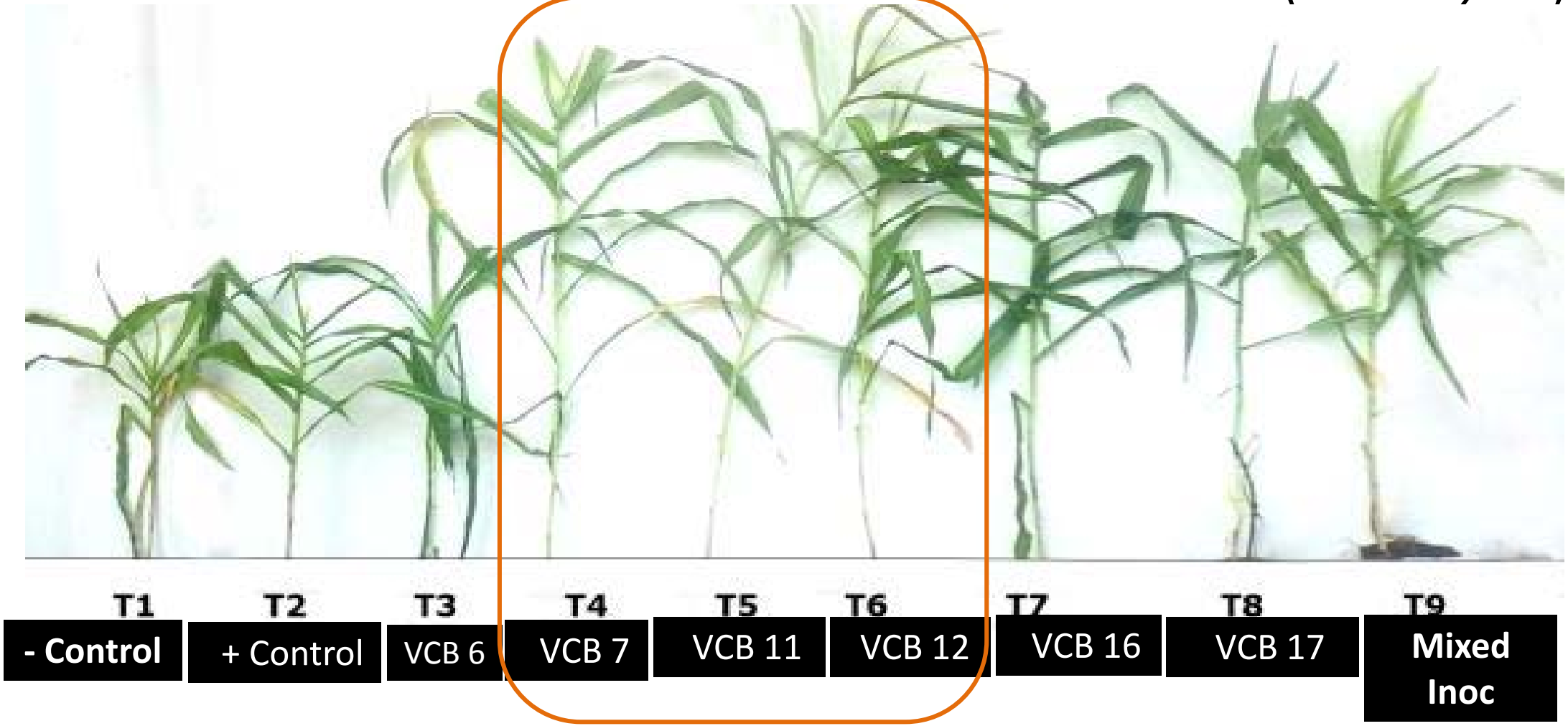


Molecular Characterization with 16s DNA sequencing

Isolates	Description	Related Literature Review
VCB6gc	*Pseudomonas hibiscicola* strain ATCC 19867	Type strains of *P. hibiscola* belong to *S. maltophilia,* a Gram neg; catalase +; use in biotechnological applications; may cause nosocomial infection: http://www.bacterio.net/stenotrophomonas.html
VCB7gc	*Paenibacillus lautus* strain NBRC 15380	PGPB with P removal and N2 fixation properties; biocatalyst for functional health food (World J of Microbiol and Biotech, 2008; (http://gcm.wfcc.info/speciesPage.jsp?strain_name=Paenibacillus%20lautus
VCB11ga	*Stenotrophomonas pavanii* strain LMG 25348	Endophytic N_2 Fixer from Brazilian sugarcane variety used in Organic farming (Ramost et al Int J Sys Evol Microbiol 2011 61(4):926-31)
VCB12sa	*Micrococcus yunnanensis* strain YIM 65004	Catabolically versatile; produce restriction enzymes and vitamins; isolated from Polyspora axillary roots; (https://en.wikipedia.org/wiki/Micrococcus)
VCB16sa	*Staphylococcus hominis* sbs. novobiosepticus strain GTC 1228	Coagulase negative, found in the skin, blood etc, antibiotic resistant; biofilm former; (http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0061161)

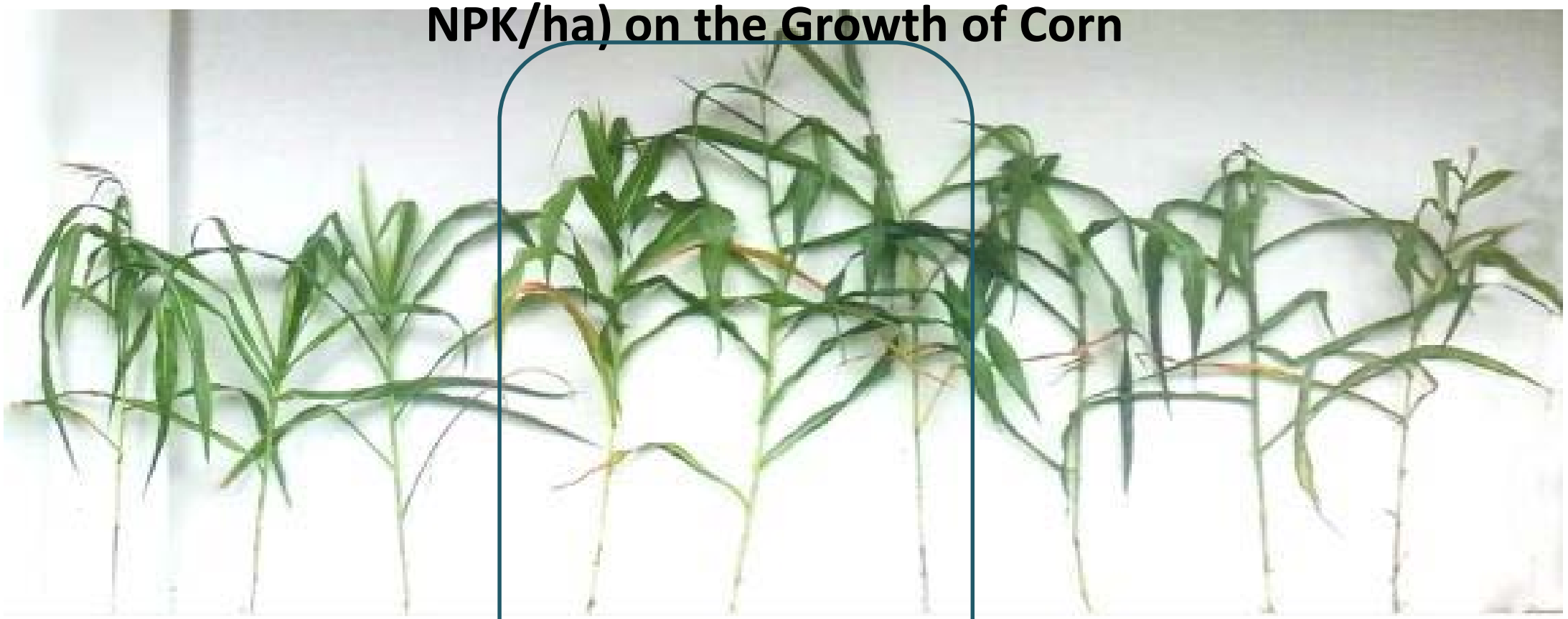
In Vivo Assay

Effect of Bacterial Isolates with on the Growth of Corn (*Zea mays* L.)



In Vivo Assay

Effect of Bacterial Isolates with Rec. Rate (RR) of Chem Fertilizer (100-40-40 kg NPK/ha) on the Growth of Corn



RR+ T1	RR+T2	RR+T3	RR+T4	RR+T5	RR+T6	RR+T7	RR+T8	RR+T9
- Control	+ Control	VCB 6	VCB 7	VCB 11	VCB 12	VCB 16	VCB 17	**Mixed Inoc**

In Vivo Assay

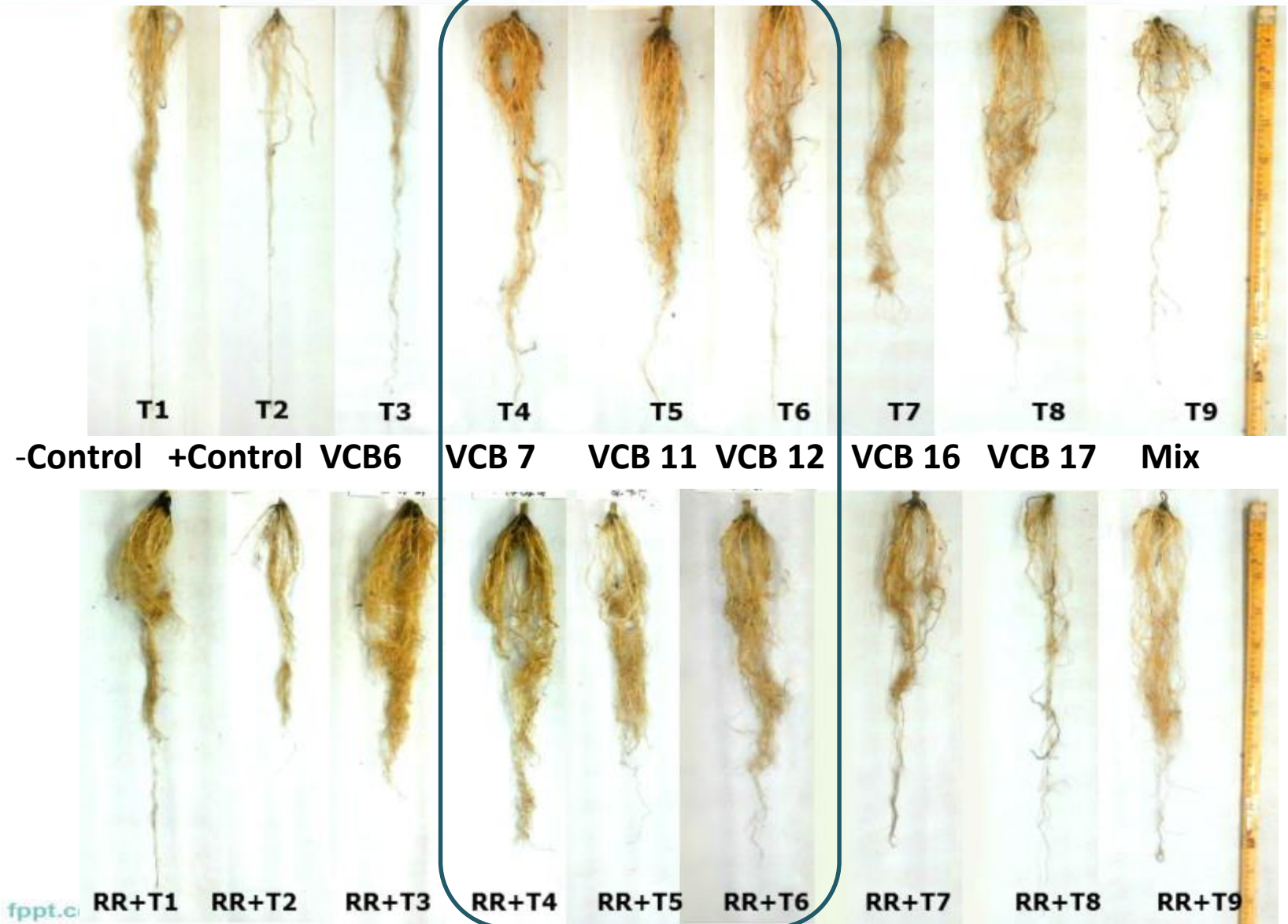


Fig. 14. Representative root samples of corn *(Zea mays* L.*)* grown for two months under screen house condition as affected by bacterial inoculation and with no chemical fertilizer (top) and with Rec. Rate of chemical (RR): T1(-control), T2(+control), T3 (VCB6), T4(VCB7), T5(VCB11), T6(VCB12), T7(VCB16), T8(VCB17), and T9(Mixed inoculants).

In Vivo Assay

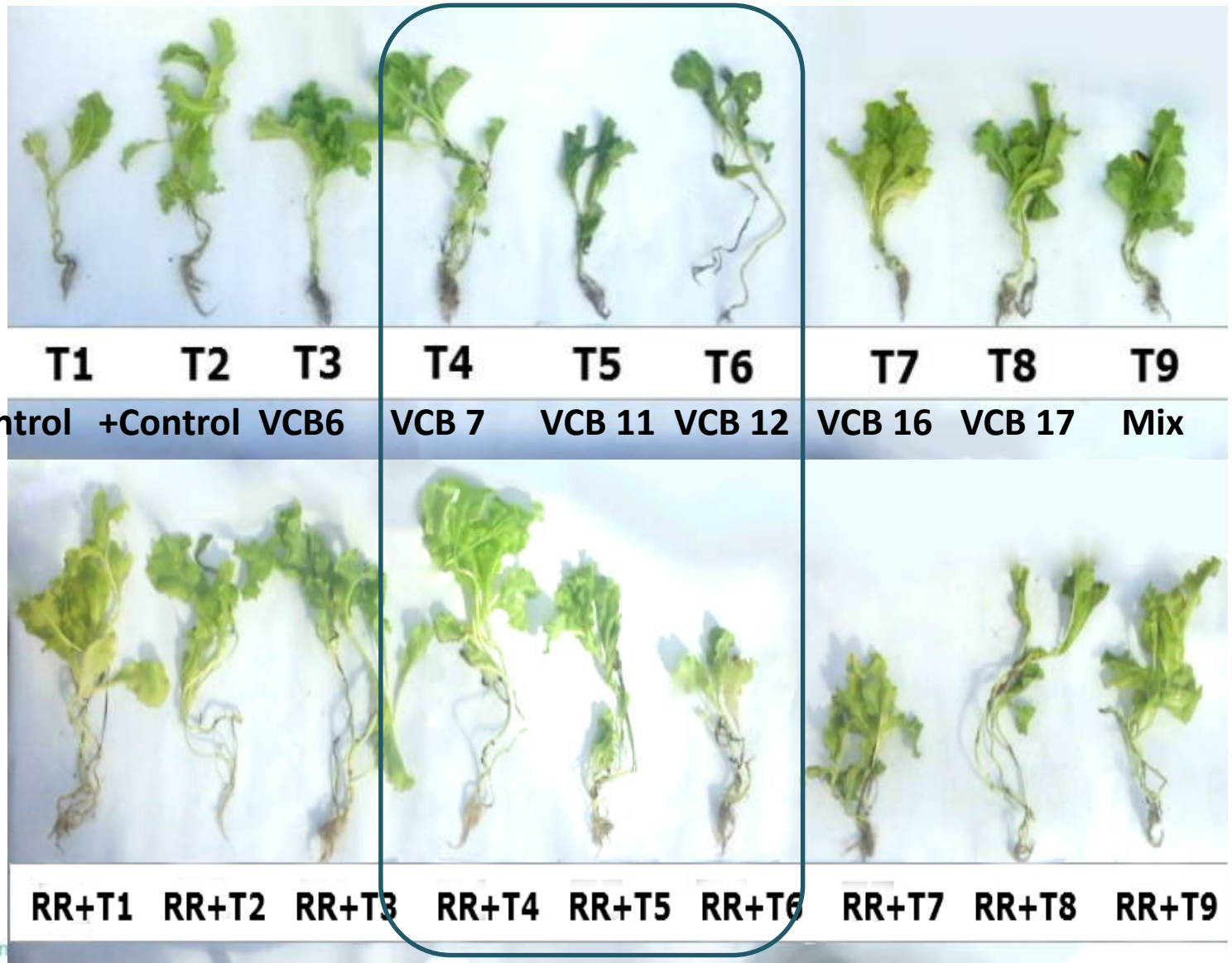
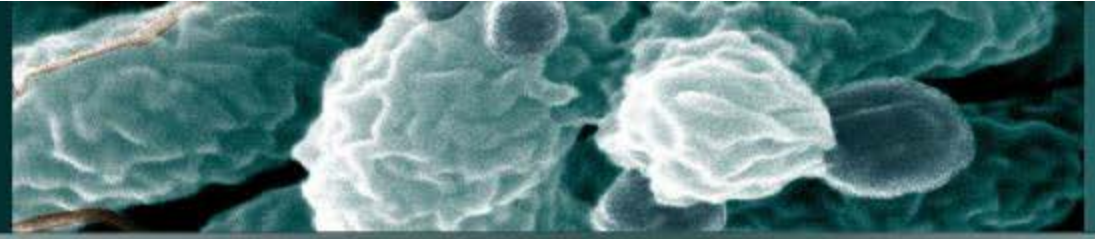
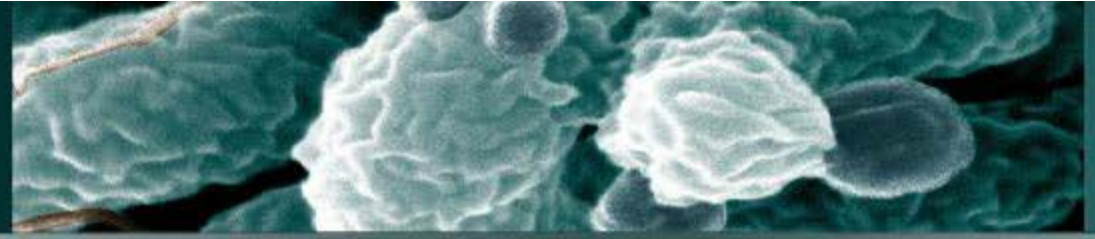


Fig. 15. Representative plant samples of lettuce (L. *sativa*) grown for two months under screen house condition with zero(up) and full(down) recommended rates of chemical fertilizer (RR) and microbial inoculation: T1(-control), T2(+control), T3 (VCB6), T4(VCB7), T5(VCB11), T6(VCB12), T7(VCB16), T8(VCB17), and T9(Mixed inoculants).



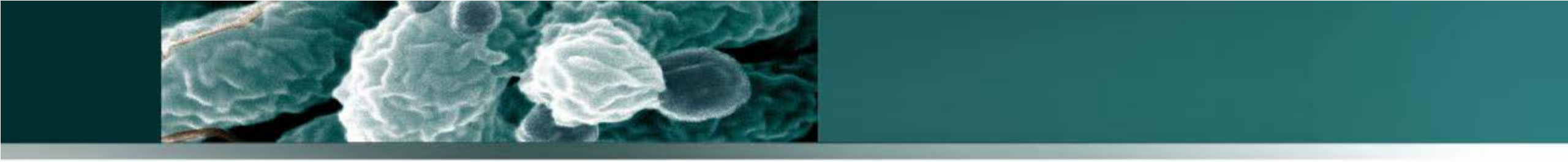
Summary

- Endophytic bacteria from sugarcane and grass roots were isolated with capacity to produce GA (6/22)
- GA concentration ranged from 34.11µg ml^{-1}-79.11µg ml^{-1} highest in strain VCB6gc; lowest in VCB11ga
- Biochemical characterization was enlightening, but tedious work
- Molecular characterization gave insight in ID of isolates
- GA production is also done by commensal or pathogenic strains as well as plant growth promoting bacteria
- GA producing isolates enhanced corn stem length and roots
- GA production has positive combination with chemical fertilizer



Conclusion

- Use of beneficial microorganisms with PGPB properties (ie GA production) should be encouraged for better plant growth
- More *in vivo* trials should be done on other crops to show positive effect of GA producing isolates
- Microbial isolates need further characterization through polyphasic analysis since percent identities were <97% suggesting that they could be possible novel strains or species.



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