# Potential plant growth promoting bacteria for sustainable agriculture

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## Abstract

Phosphate is an essential nutrient for plants, but most soil-borne phosphate is in insoluble forms and hard for plants to access. Unfortunately, soluble forms of phosphate applied in NPK fertilisers are easily lost in surface water runoff and through leaching, which is not only wasteful but a big source of pollution. Phosphate-solubilising bacteria are able to promote plant growth by dissolving insoluble metal phosphates and phytates for easy absorption. In the rhizosphere, microbes with the ability to solubilise phosphate may not only improve crop yields but also help to reduce dependence on artificial sources.

In this project, soil samples from Hong Kong were screened for likely plant-associated bacteria using CMC-agar, colloidal chitin agar or Pikovskaya agar. Selected colonies were assessed for phosphate-solubilising activity using Pikovskaya agar and pure isolates obtained by passaging on Luria agar. Under sterile conditions, plant growth-promoting activity was tested on seedlings grown on Pikovskaya agar inoculated with each isolate. DNA was extracted from isolates using a Qiagen DNeasy PowerSoil Pro kit for later identification and analysis.

Five isolates (CMC_1b, CMC_2, Piko_2, Chi_2, and Chi_1) showed strong activity on Pikovskaya agar and all five isolates were able to promote the growth of eggplant (*Solanum melongena*), but only CMC_1b and Piko_2 were also able to promote the growth of turnip (*Brassica rapa*). All isolates had high resistance to metal ions and it is likely that locally-sourced bacteria is likely to be best suited to soil and plants in Hong Kong.

## Introduction

Phosphate ($PO_4^{3-}$) is very important for plant growth because it helps to make DNA and RNA, ATP for energy, and it is also used in lots of other reactions in the cells. But inorganic phosphate and organic forms of phosphate, like phytic acid from rotting plants, easily form very insoluble compounds with iron, aluminium and calcium ions in the soils which cannot be used directly by plants. Phytic acid can account for up to 80% of phosphate in soil (Liu *et al.*, 2022).

In agriculture, phosphate is usually supplied in a soluble form in NPK fertilisers. But this is expensive and not very sustainable because easy sources of phosphate for fertilisers are running out (McKie, 2023). Also, any phosphate that is not used by the crops and does not become insoluble in soil is quickly washed away into streams, rivers and into the sea where it can damage ecosystems with algal blooms and eutrophication (Hart *et al.*, 2004).

Bacteria in the soil and close to plant roots (the rhizosphere) can help plants by making phosphate soluble so that plants can absorb it (Chen *et al.*, 2021; Elhaissoufi *et al.*, 2021). These kinds of "plant growth-promoting bacteria" could be very useful because they can help crops to grow without having to apply extra soluble phosphate fertiliser. Rhizosphere bacteria can also help plants by fixing nitrogen, collecting useful metal ions like iron and magnesium and by controlling the microbes, viruses and pests that can cause plant diseases (Olanrewaju *et al.*, 2017), so if there are more of these bacteria in the soil then they could be a sustainable bio-fertiliser.

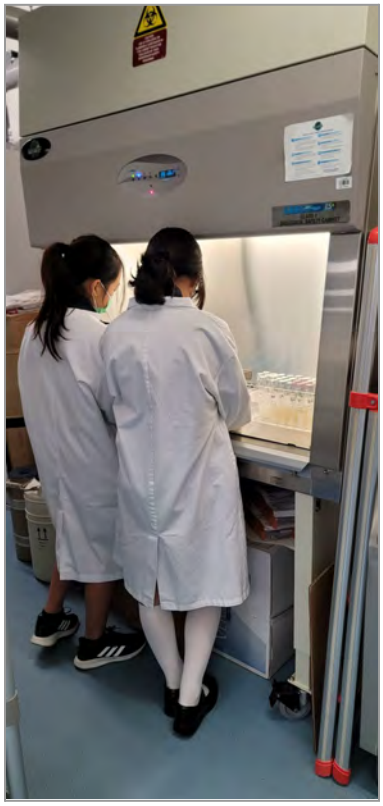
In this project, we have been screening for plant growth-promoting bacteria that can solubilise phosphate which is in the form calcium phosphate in Pikovskaya's medium (Pikovskaya, 1948). Bacteria in the plant rhizosphere can also have other activities like the ability to digest cellulose and chitin (Ali *et al.*, 2020), so we have also used CMC (carboxymethylcellulose) (Kasana *et al.*, 2008) and colloidal chitin agar (Souza *et al.*, 2009) plates to look for rhizosphere bacteria that we can also test using Pikovskaya's medium.
We have also tested our isolated bacteria on plant seedlings grown on Pikovskaya agar to see if they can promote growth. But different bacteria may be useful for different species of plants (Dawson *et al.*, 2017), so we have tried to test a number of different types of seedlings.

Soils in different places may be very different chemically, so that the best plant growth-promoting bacteria for Hong Kong plants will probably be in local soils (Saad *et al.*, 2020). Hong Kong soil can have quite high levels of heavy metals (Ho and Tai, 1988), so we have also tested isolates for their ability to tolerate high levels of various metal ions.

## Materials and Methods

*Safety*

Because this project works with bacteria from soil, we must be careful to work with them safely. We must also make sure our materials and equipment and plants are not contaminated with bacteria and fungi from ourselves and from the air. We can use lab coats, gloves and face masks for ourselves and keep the area clean with 75% ethanol and the UV light. We can keep the materials clean with a flame and by autoclaving medium and any waste. We also use the biosafety cabinet in the molecular biology laboratory at school.



*Materials*

*Pikovskaya (Piko) medium previously published in Nautiyal, 1999, Chen 2021. Cellulose medium (CMC) previously published in Kim et al., 2012. Colloidal chitin agar previously published in Ispub & Com, 2012. Luria agar. Sterile 0.9% Saline. 5% Bleach. 75% Ethanol. 250 mM Co2+,Pb2+, Ni2+, Ag+, Mn2+,Cu2+, Li+. 100 mg/ml 8-Hydroxyquinoline. 20mM sulfanilamide. Qiagen DNeasy PowerSoil Pro kit. Inoculation Loop. seeds (aubergine, turnip). Aluminium foil. Boiling tubes. 50ml Centrifuge tubes. 1.5ml Microcentrifuge tubes. Incubator. Auto pipette and tips. Centrifuge.*

Bacteria plates and boiling tubes were filled with agar made using Pikovskaya's medium (Pikovskaya, 1948). CMC (carboxymethylcellulose) agar (Kasana *et al.*, 2008) and colloidal chitin agar (Souza *et al.*, 2009) was also used for plates. Luria agar (LB) was used for streaking (MacWilliams and Liao, 2006).

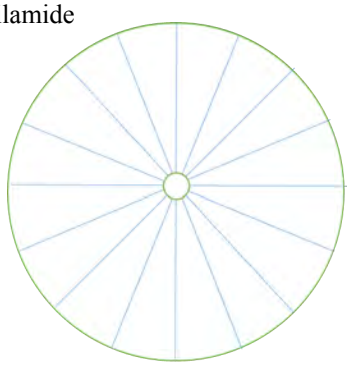
Cobalt(II) chloride, lead(II) nitrate, nickel(II) chloride, silver(I) nitrate, manganese(II) chloride, copper(II) sulphate and lithium chloride were used to prepare 250 mM solutions of $Co^{2+}$, $Pb^{2+}$, $Ni^{2+}$, $Ag^{+}$, $Mn^{2+}$, $Cu^{2+}$, $Li^{+}$.
The 8-hydroxyquinoline sulphate solution was 100 mg/mL and sulfanilamide was 20 mM. Eggplant/aubergine (*Solanum melongena*) and turnip (*Brassica rapa*) seeds were from SeedTEC.

*Bacteria screening*

We have collected soil samples from the back of our school for this project. To identify phosphate-solubilising bacteria, soil is shaken with 0.9% w:v saline and this solution diluted and spread onto Pikovskaya agar. Clear zones around colonies show that calcium phosphate is being dissolved. Clear zones around colonies on colloidal chitin agar show chitinase activity and clear zones around colonies on CMC agar (after staining with iodine solution) show cellulase activity.

1. Measure 1 g of soil and put it into the 15 mL centrifuge with 9 mL of 0.9% w:v saline. (10x)
2. Vortex for 3 seconds
3. Change the auto pipette tip
4. Use the 200 µL auto pipette to take 100 µL of the diluted soil sample into a 1.5 mL microcentrifuge tube with 900 µL of saline. (100x)
5. Vortex for 3 seconds
6. Change the auto pipette tip
7. Use the 200 µL auto pipette to take 100 µL of the 100x diluted soil sample then put it into another 1.5 mL microcentrifuge tube with 9 ml of saline. (1000x)
8. Vortex for 3 seconds
9. Change the auto pipette tip
10. Use the 200 µL autopipette to take 100 µL of the 1000x diluted soil sample then put it into another 1.5 mL microcentrifuge tube with 9 ml of saline. (10000x)
11. Vortex for 3 seconds
12. Change the auto pipette tip
13. Use the 200 µL autopipette to take 100 µL of the 10000x diluted soil sample and spread it to an Pikovskaya plate using the L-shape spreader.
14. Parafilm the Pikovskaya (Piko) plate and put it to the 27 °C incubator for 48 hours
15. Observe the Pikovskaya plate and use the inoculation loop to select one colony with an obvious clear zone and inoculate the colony on the colloidal chitin and CMC agar plate for an additional function test.
16. Parafilm the plate and put it to the 27 °C incubator for 48 hours
17. Use the inoculation loop to streak the colony on a new LB plate using the quadrant streak method
18. Parafilm the LB plate and put it to the 27 °C incubator for 48 hours
19. Repeat steps 19-20 until generation 10 to produce pure isolated colonies.
20. Streak the selected colony from generation 10 using continuous streak method
21. Extract the bacteria genomic DNA with DNeasy PowerSoil Pro Kit according to the manufacturer's instructions. Store the DNA extract at –20 °C before sequencing.

*Functional Star test* – $Co^{2+}$, $Pb^{2+}$, $Ni^{2+}$, $Ag^{+}$, $Mn^{2+}$, $Cu^{2+}$, $Li^{+}$, 8-hydroxyquinoline and sulfanilamide



1. Label the LB agar plate as shown
2. Pick a well isolated colony from streaking plate
3. Inoculate the isolate from the external to internal follow the line
4. Repeat step 3 for all isolates.
5. Use sterile forceps to add one paper disc in middle
6. Transfer 10 µL of the chemical solution to the paper disc
7. Parafilm the agar plate and put it to the 27 °C incubator for 48 hours
8. Measure the distance between the bacteria growth and paper disc.

*Plant growth assay*

*Seed-surface sterilisation.*

1. Put around 50 Eggplant/aubergine (*Solanum melongena*) seeds into the 50 mL Falcon tube
2. Pour 35-40 mL of 75% ethanol into the 50 mL Falcon tube.
3. Place the centrifuge tube into the vortex and shake it for 3 minutes
4. Pour out the ethanol
5. Add 35 mL of sterile water into the Falcon tube
6. Close the lid of the tube
7. Set a timer for 3 minutes
8. Put it in the vortex and shake it for 3 minutes
9. Pour out the water
10. Repeat step 5 - 9 for 2 more times
11. Add 5% bleach to the centrifuge tube
12. Set a timer for 3 minute
13. Put it in the vortex and shake it for 3 minutes
14. Pour out the bleach
15. Repeat step 10-13 for 2 more times
16. Take a Petri dish
17. Put moistened cotton wool into the plate
18. Add the surface sterilised seeds on top of the moistened cotton wool
19. Close the lid of the plate and parafilm the plate
20. Put it into the 27 ºC incubator for 2 days to germinate
21. Repeat step 1-20 with turnip (*Brassica rapa*) seeds
22. Transfer of a young well isolated colony to 1 mL 0.9% w:v saline as bacteria suspension.
23. Vortex the bacterial suspension for 30 seconds.
24. Repeat steps 22-23 for each isolate.
25. Use disposal inoculation loop to transfer the surface sterilised seed from the petri dish to each plant assay tube with 15 mL Pikovskaya medium.
26. Inoculate each plant assay tube with 5ul of bacterial suspension.
27. Repeat steps 25-26 for each bacteria isolate and plant seeds, 5 replicate for each type of seed and bacteria isolate.
28. 5 replicate without bacteria inoculation as a control group.
29. Put the assay tubes in the plant growth cabinet, incubate at room temperature with 16 hours of light and 8 hours of darkness cycle
30. Measure plant height on Day 3 and Day 10.

## Results and Discussion

*Bacteria screening*

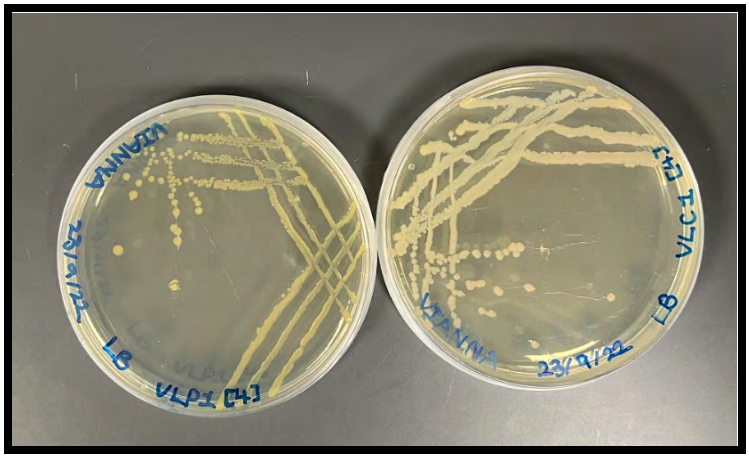
Five isolates (CMC_1b, CMC_2, Piko_2, Chi_2, and Chi_1) were able to solubilise the calcium phosphate in Piko agar. These isolates were also tested for the ability to digest chitin and cellulose, as well as for their resistance to antimicrobial agents and to high concentrations of (heavy) metals.CMC_2, Pilo_2, Chi2 show resistance to sulfanilamide.

| | CMC_1b | CMC_2 | Piko_2 | Chi_2 | Chi_1 |
|---|---|---|---|---|---|
| Phosphate solubilising activity (Piko) | +++++ | ++ | +++++ | +++++ | +++++ |
| Chitinase activity (colloidal chitin) | +++++ | +++++ | +++ | +++ | +++ |
| Cellulase activity (carboxymethylcellulose or CMC) | + | + | – | – | – |
| Antimicrobial agent (8-hydroxyquinoline ) | ❌ | ❌ | ❌ | ❌ | ❌ |
| Antibacterial agent (sulfanilamide) | ❌ | ✅ | ✅ | ✅ | ❌ |
| Heavy metal ($Co^{2+}$) | ❌ | ❌ | ❌ | ❌ | ❌ |
| Heavy metal ($Pb^{2+}$) | ✅ | ✅ | ✅ | ✅ | ✅ |
| Heavy metal ($Ni^{2+}$) | ✅ | ✅ | ✅ | ✅ | ✅ |
| Heavy metal ($Ag^{+}$) | ✅ | ✅ | ✅ | ✅ | ✅ |
| Heavy metal ($Mn^{2+}$) | ✅ | ✅ | ✅ | ✅ | ✅ |
| Heavy metal ($Cu^{2+}$) | ✅ | ✅ | ✅ | ✅ | ✅ |
| Metal ($Li^{+}$) | ✅ | ✅ | ✅ | ✅ | ✅ |

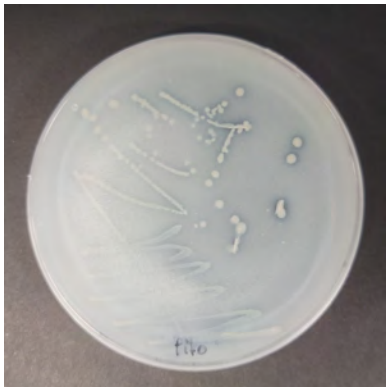
Symbols:

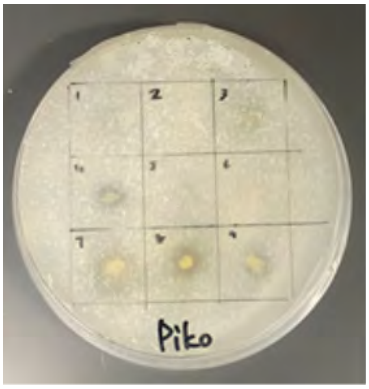
➕ / – = activity level ✅= Resistant ❌= Sensitive

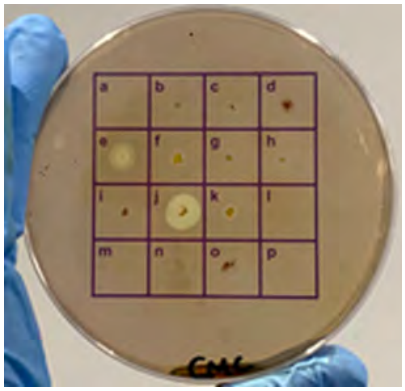
Quadrant streak method of our selected isolates




Functional test of selected isolates (left and middle: Piko, right: CMC)

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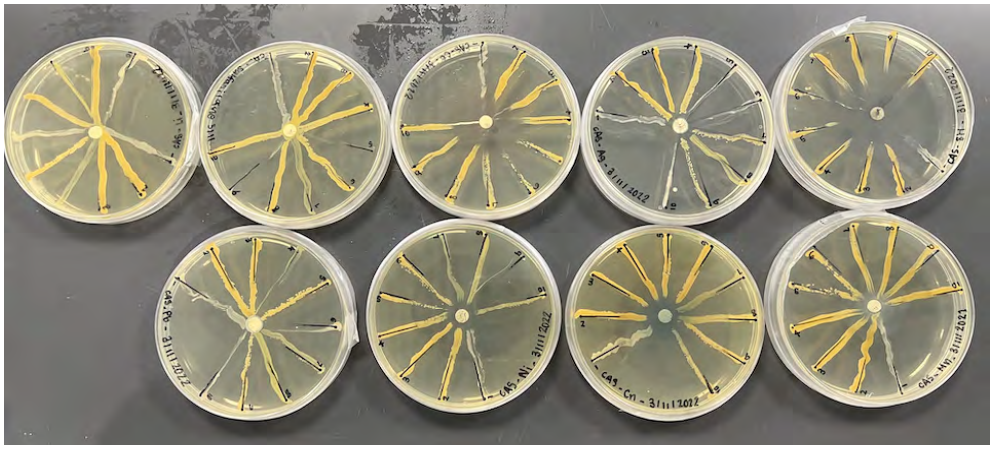
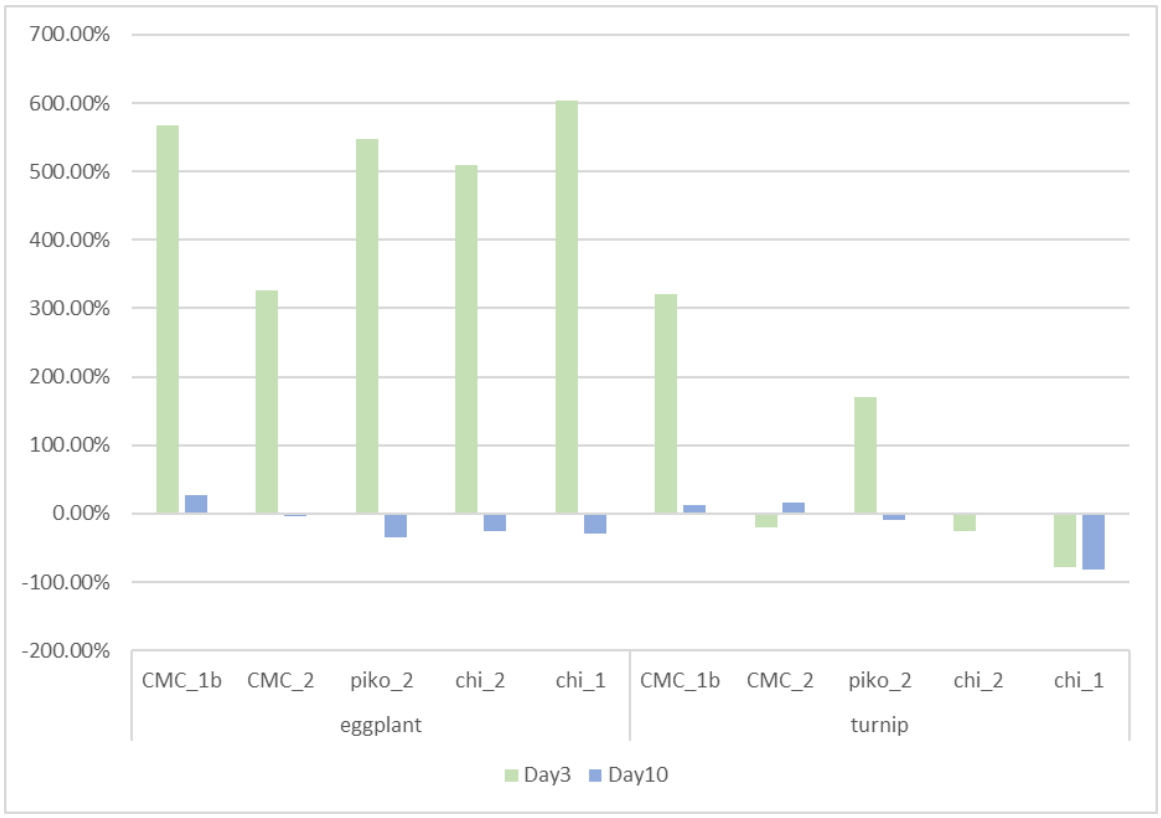


*Plant growth assay*




From the table above, the bars that are highlighted in dark green show the percentage difference on Day 3 relative to the control (0% baseline), whereas the bars highlighted in blue show the percentage difference on Day 10.

For eggplants, the average heights of plants inoculated with CMC_1b, CMC_2, Piko_2, Chi_2, and Chi_1 are much taller than the control. On Day 10, only CMC_1b inoculated plants are taller than the control.For turnip seedlings, the average heights of CMC_1b, and Piko_2-inoculated plants are taller than control on Day 3, while CMC_2 was shorter than control. On Day 10, only CMC_1b, and CMC_2 treated turnip remain taller than the turnip control.

We observed that all of the eggplants that included bacteria grew taller than the control (eggplants without bacteria), whereas not all of the turnips that included bacteria grew taller than their control (turnips without bacteria). However, at day ten, the eggplants are not tall as the control , and even grow slower.

Is is possible that the plant growth inhibition on day 10 may be due to the nutrient competition and/or the accumulation of bacterial metabolites.The results with different bacteria-plant combinations demonstrates that plant-growth promotion is not a "one size fit all" response – different plants have their unique preference for bacteria species. It is likely that bacteria isolated from Hong Kong are more suitable to soil conditions here, but further studies will be needed.

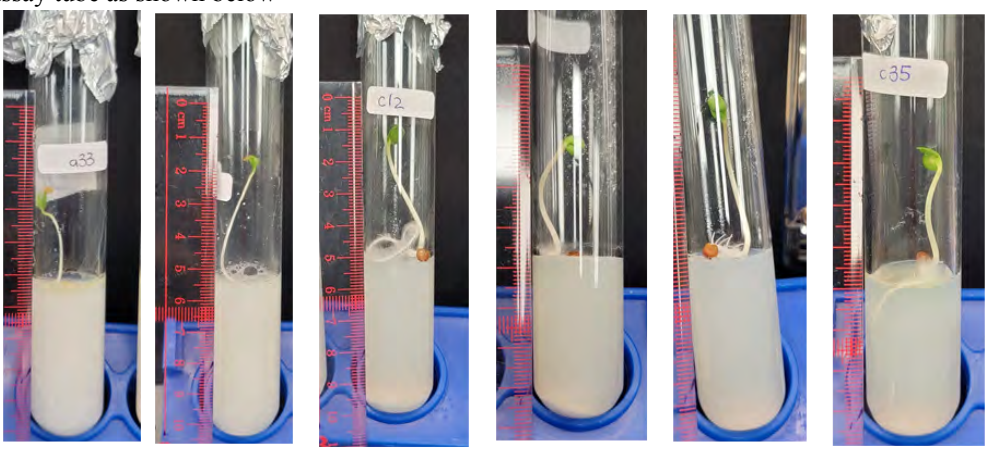
**On going ……**

Due to time limit and contamination, more dicot and monocot seeds like *Brassica rapa chinensis, Brassica oleracea, Lactuca sativa, Oryza sativa, Spinacia oleracea, Solanum lycopersicum* and *Triticum aestivum* are currently testing with all 5 bacteria for specific bacterium-plant relationship investigation.

All isolates will be sequenced, using both Illumina MiSeq and Oxford Nanopore Minion platforms, hybrid assembly by Unicycler v0.4.3 will be used to generate complete genomes in order to understand the likely mechanisms enabling the bacteria to help the plants to grow.

Selected plant assay tube as shown below

Day3

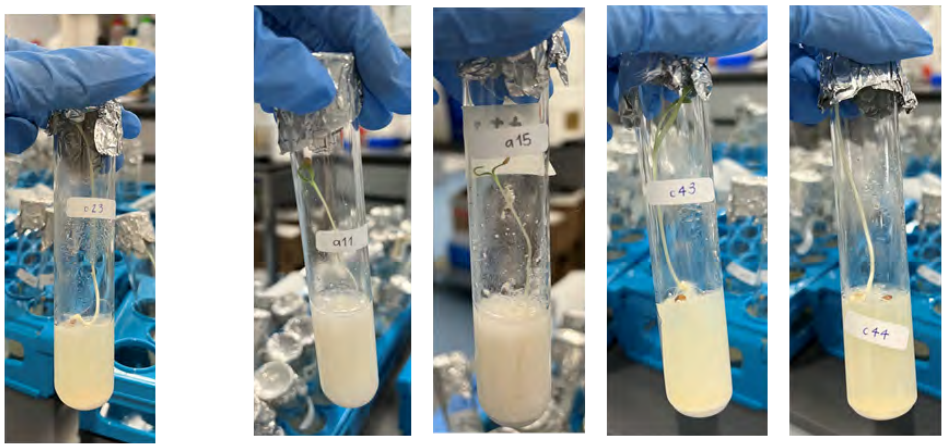



Aubergine with isolate CMC_1b has grown well.

Turnip with isolate CMC_1b has grown well.

Turnip with isolate Piko_2 has grown well.

Day10




Turnip with isolate CMC_2 has grown well.

Aubergine with isolate CMC_1b has grown well.

Turnip with isolate chi_2 has grown well.

## Conclusion

In this project, we have found the phosphate-solubilising bacteria which is able to help promote plant growth provided that the bacterium and the plant are matched but more research needs to be done on the specific bacterium-plant relationships and to understand their molecular mechanisms.

The phosphate-solubilising bacteria isolated from HK soil potentially use as biofertilizers for specific crop plants, which help the sustainable agricultural farming, less dependent on chemical fertilisers which cause water pollution and subsequent environmental problems.

In our further work, we want to know whether locally sourced bacteria are more adapted to the soil and climate of Hong Kong in our future research. Any wider use of bacterial plant growth boosters will be crucial if indigenous bacteria are better for local crops.

## Acknowledgements

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