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In-Vitro Cultivation of Paphiopedilum

Select Orchids
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Paphiopedilum has been considered to be a difficult genus to successfully grow in-vitro, both from seed and as micropropagation. In fact it is not the case, and here are the guidelines along with some explanations to be successful.

Preliminary concepts

In many cases, tissue culture research does not make thorough discoveries, but just copies previous technics and media without careful testing. One blatant example is the widespread use of Murashige and Skoog in most publications, therefore few new media or concepts are ever designed. Regarding orchids, it is boring to read the various publications, where one hormone is slightly increased, and the other one is slightly decreased compared to the thousands of previous publications.

There are concepts that are clear, most scientist dealing in tissue culture are poor chemists, and most have a poor understanding of what really happens when designing new tissue culture media. As such, they use a former one, try different rates, and when they get slightly better results, they just publish something for the sake of it in many instances.

Here are some logical, science-based concepts that are required to understand why I do the things the way I do as described further in my paper:

- Sugar and media. The amount of sucrose or other sugars added to a mineral base is not determined by the plant's needs in most cases. The sucrose serves in small part for the plant, but most of it is used to raise the osmotic pressure of the media, hence avoiding the damage of a too high mineral salt concentration.

- The optimal concentration of a mineral base in a media is NOT related to the optimal concentration of all of its components, but to the optimal concentration of its 'key' element. For some plants it could be calcium, for others it could be molybdenum. As a result, we can use too much of a standard MS, just to supply one nutrient at the proper rate. In this case, a vicious process starts, where we need to use too much sucrose to keep the plant healthy that has too many salts.

- If the mother-plant is properly grown, with the proper load of macronutrients and micronutrients, the seeds will be mostly viable and easy to sow. Wild collected Paphiopedilum seeds usually have a very high germination rate. Wild collected plants of Paphiopedilums produces good quality seeds for their first couple of years in cultivation, then the seed viability decreases with the number of years the plant has been grown. It is known that some crops can produce sterile seeds when they are nickel deficient, it may well be the case for Paphiopedilum with another micronutrient. Grow good plants, get good seeds and therefore good flasks.

- Paphiopedilum seeds do not take up water readily, and the coat of many species releases some brown color compounds for a while. As long as the seeds are not wet, and the compounds are still present, no proper germination will occur. The brown compound does not appear to be phenolic, though more chemical analysis would be required to identify it properly. When there is a determined concentration of that brown compound, no more is released, though the embryo will be stunted. It is

important to clean the culture from that brown compound before hoping to see good germination. Some species do not release the compound, some do extensively, such as Paphiopedilum micranthum and hangianum.

- Green capsules gives better results, only because the seeds can take up water faster, and there is less of that brown compound released. Otherwise, the seedling quality is best when the seeds are ripe with the proper media.

- Pre-packed media. It is possible to buy pre-packed tissue culture media powders. There is a wide offer for many slight variations, and oddly the pre-packed media is accepted even by peer reviewed journals, which is heresy. First, the media is not homogenous, and taking enough to make 1L out of 10L pack ensures that the prepared media is of unknown composition. Second, precipitate do occur most of the time, and can be observed. Those precipitate make something unavailable to the plants, but it is impossible to know exactly why and in which quantity. If possible prepare the media from the chemicals, and for publication, always prepare the media from the basic individual salts.

- Older media were designed with a higher rate of impurities in the components. In many cases the impurities were sufficient to supply the plants with some nutrients not present in the written formulation. Agar can contain a high rate of nutrients, iron sulfate used to contain higher rates of nickel, and sometimes even manganese. It is hard to reproduce correctly a media published 50 years ago, but with some imagination, it is feasible.

- Chemicals on a solid media are diffused. In a liquid media, they are diluted immediately, that's basic physics. It works both ways. The brown compound released by the Paphiopedilum seeds is diluted in the liquid media, which can be changed to remove it. On a solid media, the brown compound does not diffuse much, which suggests a high molecular weight, and stays close to the seeds. That's one of the causes of 'non germination' and death of the seeds.

- Liquid media is not more difficult to use than solid media, the contamination risks are the same. In fact liquid media is very easy to replace periodically, empty the flask and refill, where scratching the solid media off the explants is more difficult.

- I have used for years the following sterilization, manual autoclave, but the metrology is checked regularly, both temperature and pressure. My autoclave has a capacity of 90 Liters, with a pressure gauge graduated in kg/cm2, which was quite the standard in Europe 30 years ago for autoclaves, especially from Germany. There is a thermometer to check the temperature in the core, I give the measurement I get in Hanoi, Vietnam, and another thermometer to check the steam temperature shall this be needed. All the gauges are checked by a metrology service periodically.

    - Liquid media, 50mL in a 100mL Duran Erlenmeyer, rubber cap with a hole and water repellent cotton plug (1 drop of picric acid dissolved in an alcoholic solution on the cotton to prevent contamination through the cotton). 1.2kg/cm^2 for 11 minutes.
    - Solid sowing media, 50mL in a 100mL Duran Erlenmeyer. 1.2kg/cm^2 for 15 minutes.
    - Solid re-plating media, 700mL in a 1L Duran Erlenmeyer, dispatched after autoclaving in sterile plastic boxes or eventually pre-sterilized Erlenmeyer flasks. 1.6kg/cm^2 for 20 minutes
    - Solid re-plating media with green banana, 700mL in a 1L Duran Erlenmeyer, 1.8kg/cm^2 for 25 minutes.

- The autoclave is loaded to its maximum capacity with all flasks of the same model. Note that prepared media with agar or gelling agents must never cool down and harden prior to sterilization, it must be poured as hot liquid, and then the flasks are closed and autoclaved immediately. If the media cools down, it needs to be reheated, and the gelling matrix will not be as strong furthermore than media that has never gelled before.

- Sterilization time will depend on the media composition. Its viscosity (water based liquid media, solid media with agar and gelrite, or solid media with a lot of banana) will determine the optimal time. It is impossible to trust the publications of 121 degrees Celsius and 15p.s.i. quoted everywhere. Whilst it is technically quite accurate, a viscous media may need a lot of time to reach such conditions at its core, compared to a liquid media. Experience first with your autoclave. Media with banana if improperly sterilized will contaminate after 3 weeks, with a milky haze appearing.

- Agar needs to be tested on a batch basis. Plant Tissue Culture agars are, with few exceptions, processed by food companies in less than ideal conditions sometimes. They test each batch of agar, and according to the analysis report, decide whether it will be Plant Tissue Culture or food grade. The more expensive grades, up to the agarose are usually more pure, but some contaminants still can remain. The processing of the agar after harvesting is of utmost importance for its future behavior, therefore test each batch of agar prior to use. I use now Kelcogel from Kelco. It is a not too pure type of Gelrite/Gellan Gum, but its composition is remarkably consistent, and because of the impurities it contains, that is not detrimental to plant tissue culture as far as I tested, is easy to prepare and gel. It gives better gels than Gelrite, and is cheaper per liter of media than most agar brands.
- Sucrose is best from sugar cane. Sugar beet sucrose can be contaminated, for the food grade, by minute amounts of herbicides, a risk I do not want to take. Double refined sucrose can be used as a safety in real tissue culture protocols. Herbicides used on sugar cane are different from the defoliant used on sugar beet, and seem to be less problematic so far.

- Peptone/Tryptone/Edamin/Casein hydrolisate/Yeast extract are said to be ‘similar’ by people in the plant tissue culture field. However for bacteria intensive bioreactor production, they are indeed very different, and some, like yeast extract, when used, can result in a higher mutation rate of the bacteria. In my extensive experience with those compounds, I use different types according to the genera and for which I want to achieve. It is very important to follow exactly the protocols, as, for example, meat peptone induces more mutation in Cattleya tissue culture than Soja Peptone. Tryptone can induce heavy mutations in Phalaenopsis, where edamin will result in no mutations with the same media and other ingredients. All the results from my research have been based on growing and blooming huge amounts of plants for the pot plant trade, though not published in detail for obvious reasons.

- Iron was considered as very important in the early days, as manganese, zinc and copper were present as impurities in in-vitro (and in the nursery, they were present both as impurities and as mancozeb, a very commonly used fungicide, as well as Kocide, a copper hydroxide compound). However, in Paphiopedilum, and many other crops, the field and foliar analysis show clearly that manganese is the highest, followed by zinc, then iron. For some species, iron can be at the same level or slightly higher than zinc, but I never found any Paphiopedilum species where iron was even higher than half the manganese concentration, based on perfectly cultivated plants and a lot of foliar and soil analysis of many wild collected specimens.

- One can approximate a bit the macronutrients, if the formulation calls for 200mg of calcium nitrate, 180 or 210 is not going to kill the culture, and the effects will be barely noticeable for seed sowing and plantlet growing. However, no boron, or no molybdenum, or 10mg of nickel instead of 1 mg can be detrimental to some species. As a rule, the macronutrients can be a bit approximated, so can be the organic additives, peptone types, banana, even gelrite, but some of the micronutrients, specifically boron and molybdenum, as well as hormones need to be more exact. Anyway, after autoclaving, we lose some water in the media (including the condensation on the walls of the vessels), so the final formulation is always a bit different from the formulation before autoclaving. After some weeks the media usually dries out a bit too, which changes again the concentration.

- The pH for seedling re-plate is important, but can be approximated. Roughly, with some minor difference in growth, 5.5-5.9 is suitable when 5.7 is called for. For seed sowing, we need to be a bit more exact, and for tissue culture, it can make real a difference.

- The biggest secret in tissue culture is to re-plate 'on time'. This cannot be approximated at all. When the protocorms or the seedlings need to be re-plated, they must be re-plated on time, not too late. It is even better to be a bit too early as a safety. As a rule, liquid media must be changed whenever it changes color, which can be when sowing seeds twice a week, but liquid media must be routinely changed every 4 weeks. Its composition changes, the plant takes up some elements from it, and fresh media keeps the growth at its best. As for solid media, very frequent re-plating is called for. Because most molecules and ions diffuse in the gel matrix too, when a protocorm exhaust its supply near it, it can take a bit of time for the molecules to travel fast enough though the gel matrix to keep at proper levels. Ions will travel fast, but heavy molecules, amino acids, and some hormones can be much slower. It has been determined that as an example, tomato callus can exhaust BAP in the media at about a 1cm radius. The BAP, if the media is hard, will take up to a couple of days to reach again the initial, intended concentration, by that time, the tomato callus can have been deprived, and the supply of hormones in this gel matrix starts at a high close to the initial concentration, then drops, and can in fact have some peaks and stay at various levels over the culture. Frequent re-plates ensure a constant and reliable supply of hormones.

- I use disposable gamma radiated paper pads, otherwise, cupped 'watch glasses' of 10cm can be used, packed in tissue and aluminum foil, then autoclaved. Autoclaving thin cardboard in a paper envelope works as well.

- I harvest the seeds usually just before the capsule is going to open naturally. I used to use 120 day green seed capsules, but for some species it was a hit and miss, and second, the timing to produce seedlings from pollination to finishing flasks was not really different. The gain was not worth the risk to my mind.

- As for my equipment, all the stainless steel scalpels (with disposable blades), forceps are sterilized in the autoclave. I use a glass bead sterilizer in a vertical flow class II laminar hood.

- The hormone concentration and ratio to be used in a media depends largely on the mineral composition and concentration, and other ingredients of the media. A higher mineral composition and concentration in some cases can require tremendously high amounts of hormones compared to an optimized mineral composition at a low concentration, carrying higher risks of mutation than an optimized formulation.

- I work for Paphiopedilum and many things at a pH of 5.7. In fact I use that pH and adjust the mineral composition around. Some people quote much higher pH for Paphiopedilum, yet they just have to increase the pH because their mineral composition is not suitable, and can result in toxicities at lower pH. However, at pH of 6.2-6.5 I noticed micronutrient deficiencies, no matter the adjustments, so it is best to work at a pH of 5.7. For some other genera, I use even much lower pH, down to 4.6 for Phalaenopsis, but it requires really specific formulations.

Media preparation

If you have no understanding of chemistry, to make 1 liter of media from scratch, risk-free from any formulation:

- Check in the formulation what is present. Usually chloride and nitrate can be dissolved in the same vial. Phosphate separately, sulfate separately (including micronutrients sulfates).
- Boron is dissolved in hot water separately.
- Chelated iron is dissolved separately. Depending on the supplier, it is always best as a safety to dissolve it, and then boil it. The solution must never be cloudy.
- Vitamins can be dissolved along with the sucrose. Heat if required until everything is completely dissolved.
- Hormones are to be dissolved individually, especially in stock solution.
- Banana, whether green or yellow, and tomato need to have their skin and seeds removed (and the core for tomato), then they are frozen, it is very important, as it will make it easier to mix them in the media, and apparently it lowers the risks of contamination after sterilization. Freeze them, even if you plan to make the media a couple of hours after peeling the banana or tomato.

1) Measure the total amount of water required.
2) Keep about 1/3 for dissolving the gelling agent.
3) Another 1/3 will be used to blend the banana, tomato, and other organic compounds.
4) The last 1/3 is split to dissolve the macronutrients. Dissolve in one vial the sulfate, one vial the phosphate, one vial the nitrate and chloride.
5) Blend the banana, tomato, etc… with a blender. Add the coconut water, eventually the peptone types, and the vitamins. Check the pH, and correct it around to 5.5.
6) Add the 1/3 containing the macronutrients to the banana and tomato, adjust the pH. Add the micronutrients stock solutions, eventually the hormones. Warm up the soup.
7) Dissolve the gelling agent thoroughly. It is best to dilute the powder in the cold water, and then warm up when stirring. Keep it just below the boiling point. A magnetic stirrer, or hand stirring nonstop is required, as the bottom of the cooking pan can be too warm, and crystallize some of the gelling agent (it leaves a kind of sticky residue that does not dissolve). When everything is dissolved, add step by step the remaining 2/3 containing all the salts and organics. When it is hot, add the charcoal if required, and stir. At that stage, check and adjust the pH.
8) For Paphiopedilum I use potassium hydroxide to adjust the pH for those media. In the case of some potassium hungry crops, such as Phalaenopsis type dendrobiums last re-plate, and the media containing there pineapple juice and added citric acid, I prefer to use sodium hydroxide, which makes a more stable buffer, as the sodium will not be taken up by the plant.

Seed Sterilization

I use 1g/L of sodium dichloroisocyanurate for the disinfection of both seeds and capsules. To this solution is added' tween 20' or dishwashing liquid, at a rate of a few drops before use.

- The seeds are put in a 10mL test tube, the solution is added, and they are shaken thoroughly. The sterilization is less efficient if they are not shaken. I usually sterilize the seeds for 15 minutes, and then remove the liquid.

- The seed capsules are washed with dishwashing agent and tap water, then put in a test tube, the solution is added, and I leave them like that for about 20-30 minutes, ensuring that the seed capsules are immersed in the solution. If any bubbles appear on the side of the seed capsules, shake a bit, or add some more dishwashing agent, to ensure that no bubbles are present. Bubbles will protect the sides of the capsules from the sterilizing solution, and some parts will not be sterile, resulting in contamination. Another option for the seed capsules is to wash with straight bleach, dry with a clean tissue, dip in ethanol, and flame them. Be careful, sometimes some hairy seed capsules can accumulate so much ethanol that they can instead end up roasted when flamed. I put them on the paper pad or glass watch to dry in the laminar hood. When they are dry, they then can be opened.

- They are then placed in the liquid media flask. I use a scalpel to remove the seeds from the test tube, or to open the sterile seed capsule. The dry seed sowing test tubes can be cleaned with a syringe and some liquid media when all the removable seeds have been sown. The seed capsules are opened, their contents removed with a scalpel and sown, and then the remaining spare parts can be soaked straight in the liquid media to remove any remaining seeds. There is a slight risk of contamination when bathing the seed capsule, though this has happened very rarely to me.

- Alternately, they can be sown on the solid media, though the results for some species will not as good as the liquid media.

Storage of the flasks

They are simply stored at around 21/25 degrees Celsius, in very dim light. I did not find, with my media, that they require darkness. In fact I think that the light sensitivity is related to the media composition, as one media I tested (all organic nitrogen media) required the seeds to be placed in complete darkness.

Care of the flasks

Whenever the liquid media starts to turn slightly brown, the media is changed in the flask. It can mean twice a week in the early stages. I usually hand shake the flask a couple of times per week for a few seconds, and after a while all the seeds will sink, sometimes 2 days after sowing, sometimes 3-4 weeks. Replacing the media frequently is the key. When all the seeds sink, and there is no more brown color, usually they will start to germinate after a few days, 2 weeks maximum, for all the species and hybrids.

On the solid media, some species will not germinate properly, some will germinate readily, and some will have some trouble. In all cases, the seeds need to be scrapped off the media and re-plated every 3-4 weeks on fresh media, whether they germinated or not. It is important, and they will usually germinate readily. Some

species however will not germinate easily on solid media, such as Paphiopedilum volonteanum, bougainvilleanum, hangianum… There are solid media formulations, that are more complicated, and can result in germination of all species, but to my mind it is way more complicated than using the right liquid media and the germination will be slower, so it is not worth it.

From liquid to solid

When the protocorms are quite big, and start to show a leaf tip, they can be re-plated on the solid media. They will need to be re-plated again after 4-6 weeks, no matter their stage (but usually they will have grown pretty well), and when they have 2 tiny leaves and a root nearly coming. When they have 2 roots coming, they can be re-plated in the last re-plate media. The last re-plate flask do not need frequent re-plating anymore, as the roots will grow through the media, gathering what the plant needs. In the earlier stage, the protocorm has to rely on its own structure for assimilation, the tiny 1 leaf 1 tiny root seedling can only use a very small part of the media plate, where the seedling with actively growing, good roots, can use the complete media plate within a short time. Usually, from a 2 leaf 2 root seedlings to a 7-8 cm leaf-span seedling, there will be no more than 6 month, though some complex Paphiopedilum will require 9-10 months for that last stage.

The two last stage media

I will give two very different formulations for the last stage. One is used for everything, but it can be a bit mild for Maudiae and Complex pot plant Paphiopedilum. In this case, the stronger formulation with stronger mineral and sucrose concentration is preferred. I did not find any benefit using that stronger formulation for species Paphiopedilum however, and some might not like it at all. Re-plate only seedlings with 2 leaves and about 2 roots coming. Do not overcrowd the flask, as some species definitely do not like it.

Tissue culture of Paphiopedilum

This is a very sensitive subject. It can be done, however there are some problems, especially the low proliferation rate. Many papers have been published, that are nothing more than garbage using high hormones to produce obviously muted seedlings, according to the pictures provided. A recent success in Taiwan seems slightly more promising in a commercial lab however.

I have completed tissue culture of Paphiopedilum species; however it requires first to grow, second the financial profit out of such a technic is not obvious. The people will not believe it until the plants are bloomed, and second the price of seedlings or young plants with supposedly ‘selected’ parents is so low that it would not be possible to get enough profit out of a commercial application. Another reason is that, for the commercial pot plant trade, hybrids flasks are cheap, and efficient, as many breeding lines are stable enough to produce a crop of homogenous enough plants for the pot plant trade.

The keys:

- Starting from a growth can be done if the potting mix is really clean. The growth must be harvested when it has one leaf emerging, not earlier as it would be difficult to disinfect, and not too late, or it might well be contaminated by external bacteria’s, whilst retaining soft tissues that will not like too much strong sterilization required to disinfect them. Two three scales and one emerging leaf is the perfect stage. Harvest the growth as down as possible, to keep extra tissue. Let it dry non-sterile under the laminar hood, to seal a bit the vascular tissue, otherwise the sterilant can enter through the wound and kill the explant.

- The other option is to grow the plant in a very specific way. Use a lot of calcium carbonate in the mix, and feeding it exclusively with ammonium dihydrogen phosphate at 0.5g/L, very occasionally with a normal feeding once per month to keep the growth from being deficient and follow the treatment for some months. It will make an elongated growth, with a stem and leaves forming around the stem, a bit like a stolon. Maudiae types respond readily to that treatment, but even multiflorals and rothschildianum will, within a few months. The result is something that looks like a Phalaenopsis flower spike, with the scales being replaced by leaves. One just has to cut the leaf at about 1 cm from the stem, cut the stem in segments, and it will be processed as indicated below.

- The stems with their cut leaf, or the harvested growth are placed for 10 minutes in the sterilizing solution, made with a double concentration (2g/L of sodium dichloroisocyanurate), and stirred periodically.

- Under the laminar hood, remove a bit more of the unwanted material. On the stem, cut the leaves at about 5mm from the stem. For the shoot explants, cut the scales at about 1-2mm from the explant itself. It requires skill and sometimes a binocular lens can be used. One does not need absolutely sterile conditions at this stage.
- Sterilize again the explants in the seed sterilization solution (1g/L of sodium dichloroisocyanurate), and under the laminar hood, in sterile conditions this time, finish to remove the leaf stump and scales bases, to leave only the explants. Use the solid seed sowing media containing tomato. Dip the explant below the media surface to remove a bit of the sterilant, and plant it on surface of the media a few centimeters apart. Do not bury it. After about 1-2 weeks, the explant will restart, without any necrosis.

Note well the total absence of any strange compounds or hormones. They are not required for those two processes at all, and the tomato supplies enough compounds to keep the explants alive and thriving.

Using proper media, it is possible to elongate again the explants, and cut small sections with one leaf and a stem, to propagate in a slow but safe way. When the proper quantity is reached, they can be grown a bit more to get a couple of roots, and re-plated like seedlings from seed.

I have done so many times, though I do not understand, as it is so simple, why so many people failed in propagating Paphiopedilum. It is true however that too high mineral concentration or too high sucrose rates will kill the explants, like it is true too that many people do not know how to sterilize properly the explants, resulting in contamination.

I did not find, ever, any endogenous contamination, and I tend to think that many contaminations reported in the research were from improper processes regarding explant sterilization. In many cases, people do remove too soon the scales or bracts in the sterilization process, or leave too much tissue. Even in some commercial tissue culture laboratories dealing in Phalaenopsis tissue culture on a really large scale, I am amazed that they can leave so much of the stem and eventually the scale on the node, whilst complaining at their tremendous contamination rate.

There is a way to re-induce a PLB without high risky concentrations of hormones. After a couple of subculture on the solid seed sowing media, they can be placed on a proprietary proliferation media to accelerate the process, however they will never reach the propagation speed of Cymbidium or Phalaenopsis. The research is ongoing, though I can say that it involves quite a low mineral concentration media, Soja Peptone at 200mg, and the early media contained 0.1mg Indole Butyric Acid, 0.1 mg Indole Acetic Acid and only 20 mL of coconut

water. Embryogenic calluses were obtained on Paphiopedilum using this system. Now more research is ongoing to fine tune this system, and to propagate some more mother plants in order to have more seed capsules bearers rather than to make a real commercial breakthrough. Paphiopedilum pot plant is not a huge market compared to Phalaenopsis, but most important, there is no demand for Paphiopedilum mericlone from the pot plant market, the hybrids flasks being more than enough. The hobby market would not welcome so many such mericlones, and to be profitable, one would have to make a couple of hundred plants per mother plant. For this there is no market at a price that would compete with even seedlings from the same said mother plant.

It is clear however, that the protocols published using high rates of TDZ or BAP are totally useless, as the mutation rate will be, inevitably and invariably, tremendously high.

I hope this part will help people achieve better flasks of Paphiopedilum, and try new ideas or concepts around this work.

LM-1 - Liquid seed sowing media

Component	Quantity	Note
For 1 Liter of Water		
KNO3	300mg	
Ca3(PO4)2	100mg	dissolve in HCl
KH2PO4	200mg	
(NH4)2 SO4	200mg	
MgSO4, 7H2O	100mg	
NaFe EDTA	12.5mg	
MnSO4, H2O	5mg	
ZnSO4, 7H2O	3mg	
H3BO3	0.05mg	
Edamin S	0.6g	
Thiamine	5mg	
Pyridoxine	1mg	
Riboflavine	1mg	
Biotin	0.5mg	
Sucrose	15g	
Distilled water	1L	
pH before autoclaving	5.7	adjust before autoclaving

This formulation has been adapted from the modified Tsuchiya used by Donald Wimber for his seed sowing and colchicine treatment of Paphiopedilum.
Edamin S can be replaced by Soja peptone, never by tryptone or meat peptone.

X-PSM - Solid seed sowing media

Component	Quantity	Note
For 1 Liter of water		
• 200mL		
NH4NO3	400mg	
KNO3	200mg	
Ca(NO3)2, 4H2O	150mg	
Peptone Soja	1g	
Sucrose	12g	
Thiamine	10mg	
Pyriodoxine	5mg	
Biotin	0.5mg	
Inositol	100mg	
NaFe EDTA	15mg	
Nicotinic acid	5mg	
• 200mL		
MgSO4, 7H2O	100mg	
MnSO4, H2O	10mg	
ZnSO4, 7H2O	5mg	
• 100mL		
KH2PO4	150mg	
• 200mL		
Tomato peeled	40g	
Ripe banana	30g	Yellow Banana
• 300mL		
Kelcogel	3g	
Activated charcoal	200mg	
pH before autoclaving	5.7	Adjust with KOH
pH after autoclaving	5.8	Check and record.

Dissolve each batch in the indicated quantity of water (approximately, measure 1L of water, then split approximately according to the quantities indicated here), adjust separately the pH of the tomato and banana finely blended to ca. 5.5 using KOH, dissolve the kelcogel as indicated before, still heat and add the 3 batches of minerals and organic compounds, then add the tomato and banana blended. Stir well, add the activated charcoal, and then adjust the pH to 5.7

X-PRM - Solid re-plate standard media

Component	Quantity	Note
For 1 Liter of water		
• 200mL		
NH4NO3	400mg	
KNO3	400mg	
Ca(NO3)2, 4H2O	200mg	
Peptone Soja	0.2g	
Sucrose	15g	
Thiamine	10mg	
Pyriodoxine	5mg	
Biotin	1mg	
Inositol	100mg	
NaFe EDTA	20mg	
Nicotinic acid	5mg	
• 200mL		
MgSO4, 7H2O	100mg	
MnSO4, H2O	20mg	
ZnSO4, 7H2O	10mg	
• 100mL		
KH2PO4	150mg	
H3BO3	1mg	
• 200mL		
Tomato peeled	40g	
Ripe banana	20g	Yellow banana
Green banana	50g	
• 300mL		
Kelcogel	3g	
Activated charcoal	500mg	
pH before autoclaving	5.7	Adjust with KOH
pH after autoclaving	5.8	Check and record.

Dissolve each batch in the indicated quantity of water (approximately, measure 1 L of water, then split approximately according to the quantities indicated here), adjust separately the pH of the tomato and banana finely blended to ca. 5.5 using KOH, dissolve the kelcogel as indicated before, still heat and add the 3 batches of minerals and organic compounds, then add the tomato and banana blended. Stir well, add the activated charcoal, and then adjust the pH to 5.7

X-HSRM - High strength re-plate media

Component	Quantity	Note
For 1 Liter of water		
• 200mL		
NH4NO3	600mg	
KNO3	400mg	
Ca(NO3)2, 4H2O	300mg	
Peptone Soja	0.1g	
Sucrose	10g	
Fructose	5g	
Thiamine	5mg	
Pyriodoxine	1mg	
Biotin	1mg	
Calcium panthothenate	0.5mg	
NaFe EDTA	20mg	
Nicotinic acid	1mg	
• 200mL		
(NH4)2 SO4	100mg	
MgSO4, 7H2O	100mg	
MnSO4, H2O	20mg	
ZnSO4, 7H2O	10mg	
• 100mL		
KH2PO4	400mg	
H3BO3	0.5mg	
• 200mL		
Tomato peeled	20g	
Green banana	150g	
• 300mL		
Kelcogel	3.5g	
Activated charcoal	1.5g	
pH before autoclaving	6.2	Adjust with KOH
pH after autoclaving	5.7	Check and record