Plant Tissue Culture

Focusing on Micropropagation

A Paper by Victor Enrique Sanchez De Sa

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Preface:

I am a very interested gardener, I have my own plant on my balcony of which I take care and it fascinates me how plant live and grow, how quickly they adapt to new environment and how they produce what we need out of what we regard as waste. So, while looking for a suitable topic I knew that plants were something that would interest me in regard to Biotechnology or Genetic Engineering. I quickly found the topic of plant tissue culture and after some quick research I was fascinated. That is why

My main questions regarding this topic were if the method had any appliable methods to the industry or if it was just purely an experimental technique. I wondered what difference the plant cells had that they could turn into an entire plant on their own. How easy is it to perform tissue culture and if there are any negative aspects. And I asked myself what the possibilities of plant tissue culture were in the future.

Introduction:

To understand the connection between Plant tissue culture and Biotechnology, we must first understand the definition of Biotechnology. Biotechnology is "the manipulation of living organisms or their components to produce useful usually commercial products" (https://www.merriam-webster.com/dictionary/biotechnology (08.04.2020)). In the case of plant tissue culture, we are culturing cells or tissue in vitro and aseptic in a controlled environment, with different aims. In order to understand this paper some basic information needs to be given.

„Websters online dictionary" (the link is given in the reference section)

Plant tissue culture is a form of maintaining and growing tissue extracted from a plant. The tissue is called the explant and the plant from which it is extracted is the mother plant. The tissue is grown in vitro, hence in a controlled environment, abiotic factors are optimized, and biotic factors are eliminated, by the sterilization of the explant and materials. Plant tissue cultures has many different applications and ways with which to perform the method, this paper focuses mainly on micropropagation as well as somatic hybridization which will be briefly explained. Micropropagation is the true to type propagation of a selected genotype using in vitro techniques. Sometimes forming a callus, which is a mass of unorganized and undifferentiated cells, which can be regrown into an entire plant by the right supply of pgr's, short for plant growth regulators. Genetic Engineering is possible with the method of plant tissue culture and the most used technique is somatic hybridization, in which two protoplasts are fused together hence fusing together the nucleus and the genetic material, this is useful to mix traits from different plant and therefore improving the crop.

Applications

Plant tissue culture is widely used for Agriculture, especially now with the growing demands due to bigger population. The uses of plant tissue culture in agriculture are many but with the main objective of satisfying the demand, to achieve that the crops need to be improved, they need to resistant to pests, be able to cope with a more extreme environment, such as drought, heat and salinity and they need to produce more and bigger fruits. What agricultures have been doing for a very long time was to select and isolate specific geno- and phenotypes

and planting seeds from that plant in the next year and the applied that method over and over, slowly improving the outcome. With plant tissue culture the process of isolation and propagation to several thousand plants has been drastically improved and simplified and opening new possibilities. Through the application of plant tissue culture new phenomenon were observed, by cloning plants using Micropropagation a genetic variation has been seen anyways, that is called somaclonal variation, somaclonal variation is when a random mutation occurs in the process. Somaclonal variation has been used in agriculture to find better geno- or phenotypes of the plant. A more controlled way of genetically modify plants is the process of somatic hybridization, in which the cells of two plants are fused together. The cell wall is removed from the cells leaving us with protoplasts, these protoplasts are then fused together via somatic hybridisation, because the method does not require fertilisation, the process can be made between almost any plant. These are the techniques used in Agriculture to improve their crops and provide more and better food.

A further application of plant tissue culture is the in vitro storage of endangered plant species. Therefore, this is not a seed bank but rather a “clone bank”. The advantage is that in this kind of storage we know exactly the plant material that is being conserved.

Plant tissue culture is also used for the production of pharmaceuticals, because many pharmaceuticals contain substances which originate from plants. These substances could be synthesised but sometimes that process is very delicate and expensive, so extraction from the plant is much easier, but with grown plants a lot of waste can be produced and that’s were plant tissue culture comes into the equation. The tissue of the plant is being cultivated in vitro and a callus culture is formed. Extracting the substance from a big mass of cells is easier and more profitable than from an entire plant.

Alternative treatments include the usual form of cloning, which is transferring a branch into water or soil and hoping for roots to come out. The usual process takes much longer and is not as effective and consistent.

History of plant tissue culture

In 1838, Schleiden and Schwann proposed the theory that each cell in a plant could be regrown into an entire plant by itself, meaning that each cell in the plant is totipotent. But it wasn’t until 1902 when Gottlieb Haberlandt described the first attempt in plant tissue culture. He failed in his attempt to clone a plant, but he managed to keep the plant alive for a month and it even grew a bit in size, his attempt lacked a proper culture media. Regardless of his failed attempts he is regarded as the father of plant tissue culture. From that point the method of plant tissue culture was improved, and new things were discovered. The first plant growth hormone, Indole acetic acid, was discovered, as well as the introduction of Vitamin B to the culture media. Furthermore, the cell division hormone kinetin was discovered and the concept of hormonal control for organ formation was born. The next big step was the degradation of cell wall and the development of the MS medium, which is still used today. The so called pomato, a plant that produces tomatoes from the stem of the plant and potatoes on the roots of the plant, was also made via plant tissue culture performing somatic hybridization. Some of the latest historical improvements was the development of biolistic gene transfer.

Again, where are alle the references in the text?

Description of Technique:

Micropropagation

To perform micropropagation some materials are needed, which will be listed. The technique consists of five phases, which will be explained.

Stage 0: Preparation of donor plant

In this stage we need to ensure that our donor plant, the mother plant is healthy and growing vigorously and at best free from pests and diseases. To optimize the success, the mother plant should be grown in a glasshouse, with regular fertilisation and controlled for bugs. A healthy and vigorous plant, free from pests and disease and grown in a glasshouse provide the best chance of success.

Stage 1: Initiation Stage

In this stage the culture medium and the explant are prepared. The culture medium usually contains all the micro- and macronutrients, vitamins, further organic substances, a carbon source, plant growth regulators and gelling agents if the media should be solid. For the vegetative stage or the multiplication, the MS medium, developed by Murashige and Skoog in 1962, is used. The growing medium should have a ~~PH~~ of 5.4-5.8 and aseptic. The preparation of the explant is an important process to prevent the contamination once in vitro. The explant is firstly washed under running tap water for at least 20 minutes, then rinsed with a 70% ethanol solution for 30 seconds and then put into a chlorine solution with a bit of a non-ionic tenside for up to 20 minutes. After that, the explant is rinsed two to three times with distilled water. The explant is then prepared to be incubated, the explant is cut into pieces and transferred into the culture vessel. The used material such as, culture media, culture vessel, tweezers and scalpel are autoclaved and the scalpel is dipped in ethanol after each cut, to prevent contamination. This procedure should optimally be done in a laminar flow hood, to prevent further contamination.

„pH“

Stage 2: Multiplication Stage

In this stage the focus lies on producing multiple shoot development. This is achieved by providing the plant with the right combination of pgr's, for the shoot development a culture medium with a higher concentration in cytokinins provides that. In this stage we can also initialise the formation of the callus by having a ratio of auxin and cytokinin that cancels each other out, in that form they do act as antagonists.

Figure 1: A callus formed by micropropagation.

Stage 3: Rooting Stage

In this stage the plant is being prepared for the introduction to ex-vitro. For that the plant needs to begin to produce roots. The rooting stage is induced with a higher concentration of auxin in the culture medium. The plant is transferred to another culture vessel with the right medium, in which the ~~pgr~~ levels are adapted.

PGR

Stage 4: Acclimatisation Stage

The acclimatisation Stage is a very delicate stage that should not be rushed. The plant up until now has had perfect conditions and almost did not have to do much because it was al provided for, therefore some mechanism as the stomata and photosynthetic machinery is not fully developed yet. Once plant reaches a stable level in vitro, it is ready to be transferred into ex vitro. The plant is being transferred into a potting mix with sand, peat and vermiculite. The plant is then placed in a humid area without direct sunlight, the humidity is maintained by a humidifier and a shade cloth protects the sensible plant from strong sun light, alternatively a rather dim lamp, with around 30% of sunlight intensity, can be used. In the following weeks

light intensity is increased and humidity lowered until normal conditions are achived. Fertilizers should not be added until the third or forth week of ex vitro conditions. The acclimatisation stage can take up to 4-6 weeks

It needs to be added that the duration of different stages and the media used varies from plant to plant. This is a general description of the technique, and details vary from plant to plant.

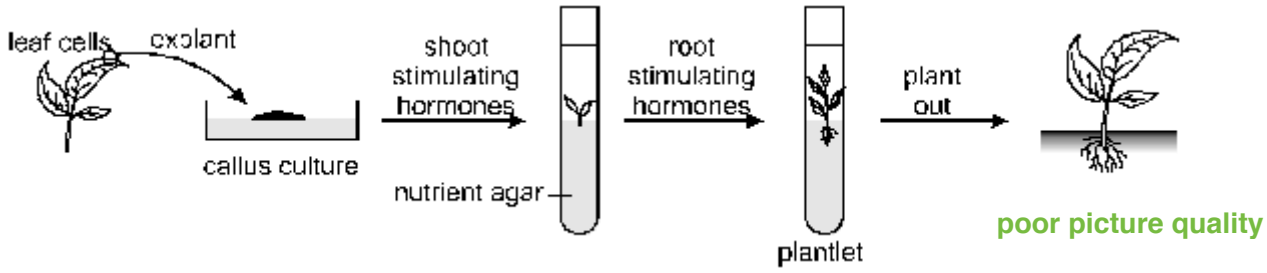

Figure 2: This Image shows the basic micropropagation from a leave as an explant and with the formation of a callus.

Somaclonal variation

Somaclonal variation is random mutation that occurs in the process of plant tissue culture. We can not fully control somaclonal variation, but we can try to minimize the possibility of it to happen. Nevertheless, somaclonal variation can have positive effects and it can be used as a way of improving your crop by genetic mutation.

It is shown that the occurrence of somaclonal variation can differ in regard to the parts of the plant used as explants. Using axillary buds directly from the mother plant reduces the chances of somaclonal variation, because it stems from the mother plant and the tissue culture with axillary buds is regardes as big one step and as it stems from the mother plant it did perform sexual reproduction, so the tissue has less chance of somaclonal variation.

On the other hand, using cells derived from somatic embryogenesis are more likely to undergo somaclonal variation, because the tissue does not stem from the mother plant directly but from an embryo produced by somatic cells of the mother plant, it therefore did not undergo sexual reproduction and in not undergoing that process not every gene was expressed, in addition somatic embryogenesis is not one big step but rather two, further increasing the chane of somaclonal variation.

Somatic hybridisation

Somatic hybridisation or protoplast fusion is the process in which two protoplasts are fused together, therefore fusing together their nucleus and their DNA. Somatic hybridisation is a form of genetic engineering that is carried out for various reasons, but mainly for agriculture. With somatic hybridisation you can fuse together two protoplasts of completely different plants. Somatic hybridisation is therefore a good way in fusing together plants which in nature could not cross with each other providing us with a big chance to further improve crops, for example a resistance that is present in one plant can now be transferred to all other plants. The problem of somatic hybridisation is that the hybrid plant has some features that

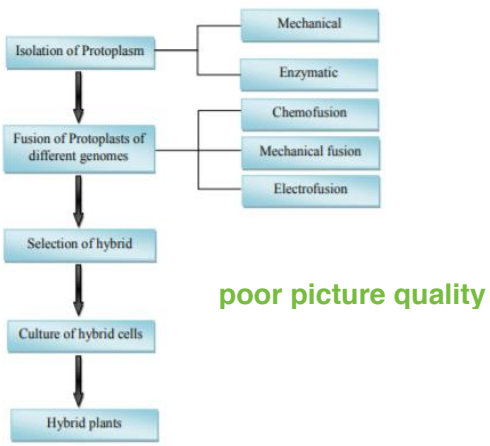

Figure 3: The procedure of somatic hybridisation.

make it less viable for commercial use and often the hybrids are sterile and can only be propagated by tissue culture.

For somatic hybridisation, we need cells that we can strip from their cell walls, a callus is perfect, that means that in micropropagation in stage 2 we need to provide the right pgr's for a callus formation to occur. The cell walls are usually destroyed in an enzymatic way using cellulase, to destroy the cellulose cell walls. Once the protoplasts are free, they are fused together via chemofusion, electrofusion or mechanical fusion. To differentiate the hybrids form the cells that did somatic hybridisation with a cell from the same plant as they are, polymerase chain reaction and protein analysis can be made to differentiate them and isolate the hybrids. The new cells are then introduced in the tissue culture and you continue on stage 2. The problem is, that after somatic hybridisation the result is a tetraploid cell which is not always stable if we want to propagate the plant leaving us with different phenotypes. But somatic hybridisation has also created some interesting new plant like the pomato, a plant that grows potatoes and tomatoes.

Somatic embryogenesis

Somatic embryogenesis is applied to make embryos out of somatic cells, the advantage somatic embryogenesis provides is that usually if we want to regrow a plant from tissue or callus, we have to determine the specific and exact amount of different pgr's a specific plant needs. It is a very delicate process and inducing somatic embryogenesis saves time and work. For somatic embryogenesis we need a callus, the callus is transferred to a medium with high amount of auxin, to promote growth. The next step is to put the callus in a medium with more gibberellic acid and no auxin, after that the callus will mature and go from a globular shape into a heart shape and finally into a torpedo shape, from which the plant is grown.

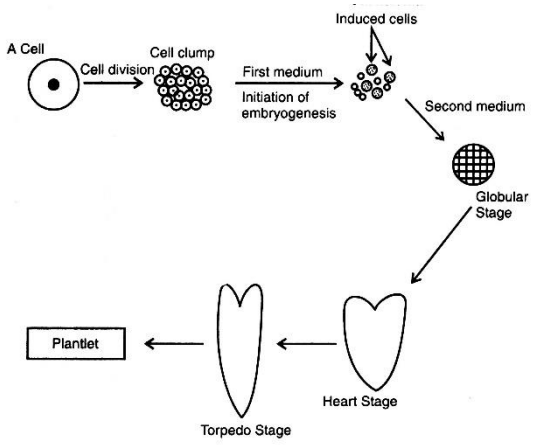

Figure 4: Somatic embryogenesis step by step.

Plant growth regulators

Plant growth regulators are essential substances to provide the growth of the plant in the different stages and to induce one stage or another. The most common pgr's are Auxin, Cytokinin and Gibberellin. Providing an explant with a high concentration of Auxin leads to the formation of roots on the other hand providing an explant with a high concentration of cytokinin leads to the formation of shoots. If both are present in a relatively low concentration and in ratio that they cancel each other out, then a callus will be formed.

Interview

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For this paper two Interviews have been made, one with Mr. Adrian Weber, Dr. Pascal Schläpfer and Dr. Anjanappa Ravi Bodampalli and the second one with Mr. Yuta Kawakami. The full Interview will be found in the appendix, here only their views on the future are listed.

Interview with Mr. Adrian Weber, Dr. Pascal Schläpfer and Dr. Anjanappa Ravi Bodampalli

-Where do you personally see the most potential for plant tissue culture in the future?

There are several plants that are hard to breed. Plant tissue culture can help to combine beneficial genes and thus beneficial traits into one single plant. Clonally

propagated plants (Strawberry, Banana, Cassava, …) also typically produce chimeric plants that have unique traits. Plant tissue culture can allow us to separate the different genotypes and thus study the effect of mutations in different layers. Grafting then could let us to re-create chimeric plants that similar to hybrid vigour then allow for a chimeric vigour.

-What will be the next step for plant tissue culture and where do you personally see the most potential for plant tissue culture in the future?

Even though plant tissue culture is widely used for mass propagation in clonally propagated crops, the biggest advancement we expect to see is in genetic improvement of crop species. This is particularly true in crops species with longer and complex breeding cycles (e.g. apple, cassava, citrus). Also, tissue culture along with genetic engineering will be and to some extent already is a powerful tool for generating improved varieties along with better response to biotic (disease and pest resistance) and abiotic (drought, salinity etc.) stresses.
Since the plant tissue culture involves a number of steps that needs to be optimized from crop to crop, I think we should develop a robust and general protocol that could be used in all crop species and this would give us all the advantages without necessarily having to going through the optimization of tissue culture steps. In combination with a robotic setup, a lot of tedious, very precise work could potentially be sped up so that we can produce more, and more consistent output without the involvement of highly trained and thus usually very expensive personnel.

Interview with Mr. Yuta Kawakami

-Where do you personally see the most potential for plant tissue culture in the future?
-What will be the next step for plant tissue culture and where do you personally see the most potential for plant tissue culture in the future?

I see a good potential of plant tissue culture in producing plant-based meat alternatives. Another promising area is use of plant cells as bioreactors to produce medicinal secondary metabolites or vaccines.

Discussion

The answers to the question in the interview above show the direction in which plant tissue culture is going to evolve, in a direction with much more optimised processes and hopefully soon be able to present a general process for all plants regarding micropropagation. But the most potential lies in the Genetic Engineering methods applied, such as somatic hybridisation and somaclonal variation. This opens up an entire new world of possibilities regarding crops an it has the potential to drastically improve the quality of life, not only for people living in a wealthy country, but especially for those how constantly suffer from hunger and need crops that are resistant to biotic and abiotic factors and provide essential nutrients without being too difficult to propagate. Plant based meat alternatives will also benefit from further research into the topic of Genetic Engineering and of course the production of medicinal secondary metabolites.

Plant tissue culture has been constantly evolving and improving, new methods have been found and already existing processes have been modified. The future of plant tissue culture is very big, especially regarding Genetic Engineering, even though if that comes with ethical conflicts for some, gmo's are not as bad as many think, this is at least regarding the scientific data we have at the moment. Apart from that some processes have been simplified and optimised to the point where special lab equipment is not needed and processes such as micropropagation can even be done at hoe or at schools, to further increase the knowledge of plant tissue culture, Genetic Engineering and of course of plants.

No disadvantages or dangers mentioned - there alway are some.

Summary

Micropropagation is a process in which, from a very small amount of tissue from a mother plant, a lot of new plant can be regrown, that process is especially interesting for agriculture. A lot of other processes can be added to one of the stages, manipulating the plant, often genetically to improve the results. Somatic hybridisation has opened new possibilities to the world and has already crated some fascinating plants. Somatic embryogenesis helps in propagate specific cultures in which inducing shoot or root growth is not an easy task. Plant growth regulators are important in every process regarding plant tissue culture and often they are the reason when happens what. Plant tissue culture has a bright future, especially in Genetic Engineering to help bring the world a step forward.

for the links: authorship and date of last visit missing

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Figure 2: https://forum.grasscity.com/threads/tissue-culture-micropropagation.998333/

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Figure 4: https://biocyclopedia.com/index/images/Biotechnology/chapter08/072_large.jpg

Appendix

Interview: Plant tissue culture with Mr. Adrian Weber, Dr. Pascal Schläpfer and Dr. Anjanappa Ravi Bodampalli

1. "The high concentration of auxins generally favors root formation, whereas the high concentration of cytokinins promotes shoot regeneration. A balance of both auxin and cytokinin leads to the development of mass of undifferentiated cells known as callus." (1)
   Why does a balance of both pgr's produce a mass of undifferentiated cells and not equal shoots and roots production?

   Good question. Plant growth hormones (pgrs) act as signalling molecules. The molecules are perceived by plant receptors that then activate or deactivate the transcription of genes which leads to differential translation of the mRNAs to proteins. This difference in signalling leading in a different protein composition and thus also metabolite and signalling molecule composition then leads to differences in morphology and thus either into roots, shoots or for that matter any cell type. So, it happens to be that a balanced concentration of both cytokinin and auxin is perceived as the signal for accelerated growth. In this case this results in a higher number of dividing cells, the higher the concentration, the more growth is initiated. This is true until a certain point where a threshold of concentrations is reached and the plant suffers from the toxicity of the hormones. Every species, even cultivars and even down to single organisms have their own thresholds. If you now keep one concentration constant but increase the other pgr, you tend to either see the formation of roots or shoots. One pgr takes over and besides more growth you get an additional signal, the differentiation into celltypes. So the two pgr have a double function. 1) together they tell the plant how much cell division you have, and 2) as antagonists they tell the plant what kind of differentiation it should make. If both hormones are at the same concentration, they cancel each other out.

2. "Fidelity is assured by the application of micropropagation by axillary buds, whereas somatic embryogenesis from long-term cultures does not guarantee genetic fidelity and leads to somaclonal variation among the regenerated plants." (2)
   What is the reason for assured fidelity in micropropagation of axillary buds, but somatic embryogenesis does not guarantee fidelity, if both methods used the same mother plant?

   Axillary buds stem from plants. Thus the tissue is sampled from a plant that likely underwent a crossing event (and thus a seed stage) and so on. Long-term cultures however do not have such a restriction. Genes only needed in very specific stages of a plant are not needed for the culture cells to grow, and thus are prone to mutate. Also cell cultures tend to express pretty much every gene. If you now produce plants out of a fresh batch of cell cultures, then these plants are likely genetically the same among each other, but they will be different from the original mother line and different from plants from a different batch. This difference is larger than when you take buds, since the mother plant is maintained and (d-) evolves slower, so to say, than a cell culture. But also axillary buds will lead to the fact that plants will eventually

differ. If you compare typical long-time favourite model plant lineages like Arabidopsis Columbia 0 from different labs with each other, you will see quite a lot of differences.

3. If the amount of auxin and cytokine is balanced in the culture medium is there a callus formation always going to happen or does it matter what part of the mother plant is being used as explant?

   No. It depends. If you were to use Xylem cells of potato parenchyma, then, since the cells are dead, nothing will happen. Also, depending on what tissue you use, the callus formation is more likely to work. A simple example that you can test on your own, if you have the hormones, and can work cleanly, is carrots. If you sterilize a carrot, slice it, then quarter the slice, and put the carrot onto the medium with one side of the fresh cut quarter on top of it, then you see that only the cambium forms a callus. The rest is too slow. But all of this depends on the plant and if one wants to form a callus from a plant where one has no previous knowledge, some trial and error has to be performed until one gets a working system.

4. How can we make sure that not two protoplasts of the same plant perform somatic hybridization with each other?

   You can't. Depending on the tissue, variety of the organism, or species this will happen. Sometimes it happens all the time and you give up that one variety.
5. How is the DNA fused together in somatic hybridization, do we get tetraploid cells if so, how is the ploidy restored or does it even need to be restored?
6.

   Typically the DNA itself stays intact as its chromosomes. But we get tetraploid cells. This in itself is not a large issue, since most plant cells are typically polyploid (look up Endoreduplication). But if you want to propagate these plants, then most of the time the tetraploidy is not stable. This then can have major effect on plant phenotype and the likelihood that you get viable offspring.

7. How can we induce embryogenesis in a callus?

   See below, question 7.

8. What is the importance of embryogenesis, doesn't a callus just develop into an entire plant with the supply of the right plant growth regulators like auxin and cytokinin?

   Embryogenesis is an important step in *in vitro* plant regeneration. We can induce embryogenesis by using different pgrs. In theory, the callus could develop an entire plant, if it can respond the right way at different sites to the correct concentrations it needs to form all organs it needs. But to determine the optimal concentrations of these PGRs is typicaly a tedious process and this is varying on the different ex-plants that are planned to be used for tissue culture, crop species, and as well as varieties with in the crop species. So, the process has to be optimized for every individual crop/variety/ ex-plant. Therefore to establish the

system, we rarely see one setup that reliably leads to callus formation and to plant formation. However, it is possible that industry has machines that allow for this for a specific variety of crops, where all information is known. If this does not exist yet, this might be one of the next steps to mass produce such plants in a much more efficient way.

9. Where do you personally see the most potential for plant tissue culture in the future?

   There are several plants that are hard to breed. Plant tissue culture can help to combine beneficial genes and thus beneficial traits into one single plant. Clonally propagated plants (Strawberry, Banana, Cassava, …) also typically produce chimeric plants that have unique traits. Plant tissue culture can allow us to separate the different genotypes and thus study the effect of mutations in different layers. Grafting then could let us to re-create chimeric plants that similar to hybrid vigour then allow for a chimeric vigour.

10. What will be the next step for plant tissue culture and where do you personally see the most potential for plant tissue culture in the future?

    Even though plant tissue culture is widely used for mass propagation in clonally propagated crops, the biggest advancement we expect to see is in genetic improvement of crop species. This is particularly true in crops species with longer and complex breeding cycles (e.g. apple, cassava, citrus). Also, tissue culture along with genetic engineering will be and to some extent already is a powerful tool for generating improved varieties along with better response to biotic (disease and pest resistance) and abiotic (drought, salinity etc.) stresses. Since the plant tissue culture involves a number of steps that needs to be optimized from crop to crop, I think we should develop a robust and general protocol that could be used in all crop species and this would give us all the advantages without necessarily having to going through the optimization of tissue culture steps. In combination with a robotic setup, a lot of tedious, very precise work could potentially be sped up so that we can produce more, and more consistent output without the involvement of highly trained and thus usually very expensive personnel.

(1): https://www.intechopen.com/books/recent-advances-in-plant-in-vitro-culture/plant-tissue-culture-current-status-and-opportunities citation from section 3 in the second last paragraph.

(2): https://www.intechopen.com/books/recent-advances-in-plant-in-vitro-culture/somaclonal-variation-in-tissue-culture-a-case-study-with-olive citation from section 8 first paragraph.

Interview with Mr. Yuta Kawakami

1. “The high concentration of auxins generally favors root formation, whereas the high concentration of cytokinins promotes shoot regeneration. A balance of both auxin and cytokinin leads to the development of mass of undifferentiated cells known as callus.” (1)

Why does a balance of both pgr's produce a mass of undifferentiated cells and not equal shoots and roots production?

2. If the amount of auxin and cytokine is balanced in the culture medium is there a callus formation always going to happen or does it matter what part of the mother plant is being used as explant?

   The meaning of the word "balance" is rather vague here in this text. Please look at the URL below. Some auxin : cytokinin ratios indeed lead to root and shoot regeneration (= plant regeneration), whereas if auxin : cytokinin ration is higher it only leads to callus formation. It should be noted that the auxin : cytokinin ratio for callus induction/plant regeneration largely depends on plant species and even on varieties, it has to optimized experimentally. https://www.quora.com/High-ratio-of-cytokinin-to-auxin-favour-shoot-production-What-does-that-mean-in-plant-literature-Does-it-mean-that-the-concentration-of-both-should-be-high-or-only-either-one-of-them

   As for the second question, it is a "good question" for me because I have not thought deeply about that. But if I were to guess (= I can be wrong), I think the same auxin : cytokinin does not necessarily lead to the same outcome if different parts of the plants are used as a explant, because the uptake rate of plant hormones can be different among tissues. For example, auxin is transported into the cells through auxin transporters on the plasma membrane. In theory, the number of such transporters on the plasma membrane can differ between cells, depending on the tissue type that the cells belong to and the developmental stage. I would think this could lead to variation in the outcome, though I am not sure how significant such a variation is in the context of applied tissue culture. If you are more interested in it, you could look for evidences.

3. "Fidelity is assured by the application of micropropagation by axillary buds, whereas somatic embryogenesis from long-term cultures does not guarantee genetic fidelity and leads to somaclonal variation among the regenerated plants." (2)

   What is the reason for assured fidelity in micropropagation of axillary buds, but somatic embryogenesis does not guarantee fidelity, if both methods used the same mother plant?

   In my view, this is mainly due to the difference in the number of steps involved in the two methods.
   Very roughly speaking, plant propagation involving somatic embryogenesis is a "two-step" process. First, one has to derive embryonic cells from somatic cells by modulating the PGRs. Then, embryonic cells are regenerated into plants again by modulation of PGRs. In the first step, a lot of (epi)genetic modification can occur, leading to poor genetic fidelity. On the other hand, plant propagation involving axillary buds can be considered as "one-step" process. Axillary buds are by nature dormant embryonic tissues, which only requires proper modulation of PGRs for breaking dormancy and regeneration. Since this method does not require induction of embryonic cells (because axillary buds are already embryonic), it can

reduce the risk of (epi)genetic modification, leading to higher fidelity when the same mother plant is used.

4. How can we make sure that not two protoplasts of the same plant perform somatic hybridization with each other?

   If I were to make sure that the plants regenerated from protoplast fusion are actually the plants derived from the fusion of two different species (let's say species A and B), I would perform PCRs targeting genes specific to species A and those species B. If the PCR is positive for genes from both species, it means that the plant is derived from the hybrid protoplast from the two species. Similar screening is performed in the attached paper (Figure 3D), though they seemed to have used protein analysis instead of PCR in this paper.

5. How is the DNA fused together in somatic hybridization, do we get tetraploid cells if so, how is the ploidy restored or does it even need to be restored?

   It is generally considered that the number of chromosomes in the hybrid plants are the sum of that of the original plants, though some exceptions can be found as you can see in the attached paper.

6. How can we induce embryogenesis in a callus?
7. What is the importance of embryogenesis, doesn't a callus just develop into an entire plant with the supply of the right plant growth regulators like auxin and cytokinin?

   I think callus induction is often one of the steps in the somatic embryogenesis. By modulating PGRs, one can induce embryogenic callus from explants.

8. Where do you personally see the most potential for plant tissue culture in the future?
9. What will be the next step for plant tissue culture and where do you personally see the most potential for plant tissue culture in the future?

   I see a good potential of plant tissue culture in producing plant-based meat alternatives. Another promising area is use of plant cells as bioreactors to produce medicinal secondary metabolites or vaccines.