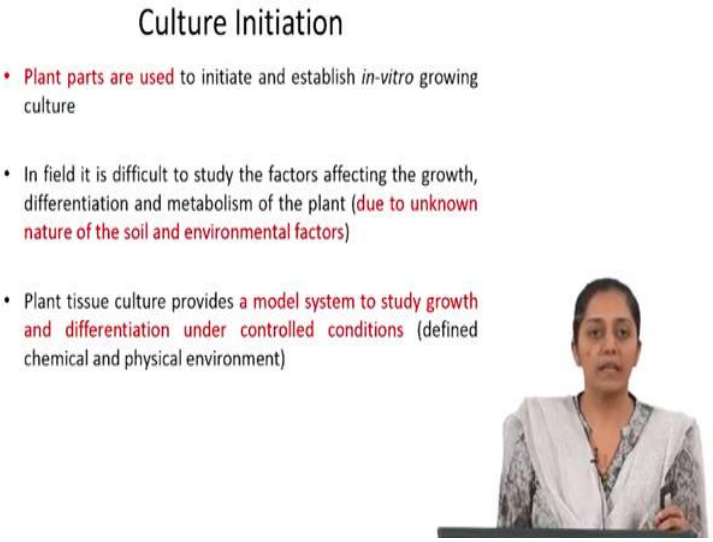
Plant Cell Bioprocessing
Dr. Smita Srivastava
Department of Biotechnology
Indian Institute of Technology, Madras

Lecture - 06
***In vitro* culture initiation**

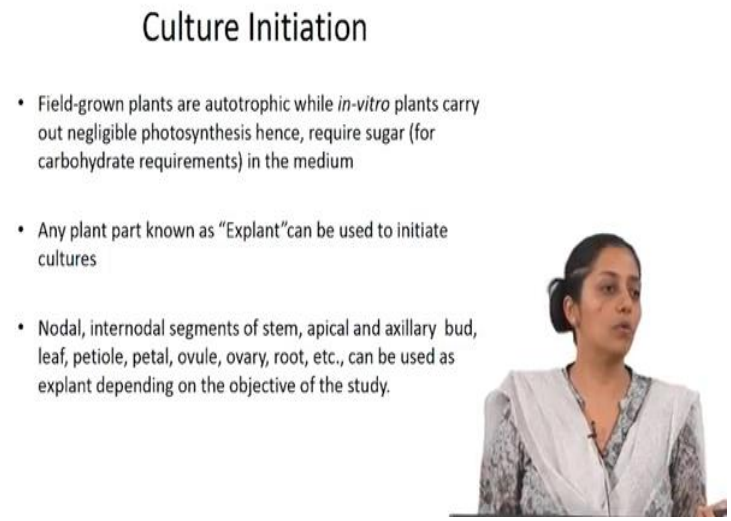
So, today we will study about different forms of cultures which are used and their applications in plant biotechnology. Also, we will study about the nutritional requirements of plant cells.

(Refer Slide Time: 00:34)



So, then starting with culture initiation we all now know that any part of the plant can be used as an explant to initiate different forms of cultures. So, these plant parts are used to initiate and establish *in vitro* grown cultures. So, why do you think *in vitro* plant cell culture or tissue culture can be of use? Because, they can act as model systems to study the effect of various environmental parameters on the plant physiology and its growth and development. So, because *in vitro* conditions, environmental conditions are controlled and you can study each factor at a time, while in whole plants under natural conditions the soil conditions or your environmental parameters are not under control.

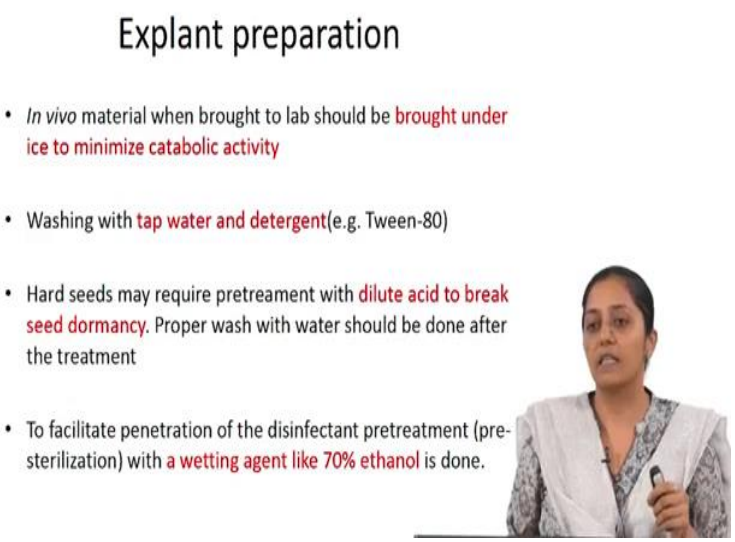
(Refer Slide Time: 01:23)



So, field grown plants can undergo photosynthesis. So, you do not have to provide carbon sources or nutrients to these plants exogenously as they can themselves procure nutrients from the soil and use sunlight to produce the food source. But in case of *in vitro* cultures this photosynthetic capacity is impaired and they do not carry out as large amount of photosynthesis as the whole plant does. So, they need to be supplemented with exogenous carbon source and generally sucrose is used as carbon source. Now what all different parts of the plant which can be used as explants?

They can be nodal or they can be internodal segments, they can be apical or axillary meristematic regions, petioles and even your petals, ovules, ovaries and so any part of the plant can be used effectively as an explant.

(Refer Slide Time: 02:19)



Now, how to decide which kind of an explant to be used? It depends on what kind of *in vitro* culture you would like to carry out. So, we will see when we will be discussing different forms of *in vitro* cultures what are the explants used which can lead to different forms of *in vitro* cultures.

So, what should be taken care of when you are starting to initiate these *in vitro* cultures? If suppose you are bringing an outside plant material to inside to be used as an explant , the first thing is you need to make sure that you bring it in an ice box . As soon as you excise it from the plant the breakdown begins. So, you need to minimize that catabolic activity and hence you should bring it in an icebox as quickly as possible. Now the second thing is there will be contaminants if you are dependent on the outside plants as explants. So, then you need to do surface sterilization which is one of the key and the initial steps in initiating any kind of *in vitro* cultures.

So, how to carry out surface sterilization? When I say surface sterilization which means that removing these surface contaminants. So, first thing is that the material which you bring to the lab from outside should get rid of the soil. So, continuous water wash is done with the help of a surfactant in it or any detergent in it, for example savlon is used sometimes or you can use any detergent.

Then subsequent washing so that the detergent is lost from the surface. Now then you treat it with a wetting agent. Generally used wetting agents is up to 70 percent of ethanol volume

by volume and this is what is used in plant tissue culture labs. So, when I say wetting agents like for example, the 70 percent ethanol is used as wetting agent, what does it do? From the word itself you can make out on wetting agent.

Student: It just increases the time period for the detergent or any disinfectant. We are using this to wet for the longer time. Since if we use 100 percent ethanol, it can evaporate easily as compared to 70 percent ethanol.

Then why at all to use it through?

Closer to what she said wetting agent is not to wet, but wet in a sense it would facilitate the penetration of the disinfectant inside the explant. So, as to increase the probability of removing all the contaminants or killing the contaminants. Now when I say it increases the probability or the penetration of the disinfectant, how do you think it is increasing the penetration?

Student: Increasing the surface tension

Very good, but what does it do to surface tension and of what the liquid in which the wetting agent is there? So, suppose you provide the disinfectant, what it would do is it reduces the surface tension of the liquid and when you say reduces the surface tension of the liquid, it means that the attraction between the molecules of the liquid. So, that they can get dispersed and get inside the gaps available on the explant surface and thereby increasing the probability of penetration and disinfection.

Yeah one more point, now when you are using seeds as explants to generate either *in vitro* seedlings or any forms of cultures, sometimes you observe the following. We also observed in lab for *Viola odorata* which is one of the medicinal plants. The seeds were not able to give rise to the plants, despite the fact that whatever was given in literature was being provided as the medium for germination. So, that to avoid seed dormancy or to overcome the seed dormancy, these seeds should be treated with either dilute acids or even heating treatment is given.

So, now why do you think and what does the term seed dormancy means to you and why do you think heating or dilute acid exposure is helping in overcoming the seed dormancy

? What it must be doing ? After the treatment with dilute acid , it has to be water washed properly so that the acid is lost.

Think in nature, till the environmental conditions are conducive for the plant to grow or arise, the metabolic activity has to be kept viable. If the metabolic activity has to be arrested, the seed dormancy is used by nature. Now, it becomes so tough that even when the environment is conducive, the seed is not able to come out.

There is a covering around the seed which is called as seed coat. So, when the seed coat becomes very hard the penetration by water is very difficult and it is difficult to rupture the seed coat for the seedling to come out. The acid treatment or your heat treatment can help in removing this coat. Hence, the seed can sprout into the seedling by the exposure of conducive environmental conditions.

(Refer Slide Time: 08:11)

So, how the explants sterilization is done? This is from a Google book, where simple protocol is given. So, generally what is done is you bring the plant material from the outside and you give a continuous water wash . Then, upto 2 percent savlon wash can also be given along with water and then you treat it with distilled water. This is to remove the detergent and then after treatment with distilled water you take it in the laminar hood, now the laminar hood is what have you used a LAF?

So, what is LAF? Laminar Air Flow.

So, why do we use it? Either there is a horizontal flow or there is a vertical flow. So, how is it maintaining the aseptic conditions?

So, how is that positive pressure being created?

Student: HEPA filter.

There is a HEPA filter through which sterile air can be pushed in through the mesh with high pressure, so that there is a positive air flow outside the LAF and so there is sterile environment inside. You must have heard while working in the LAF it is always preferred to work behind the flame, you lighten the flame and then it is preferred to work behind the flame or close to the flame. Why ? Because around the flame , the sterile environment probability is higher because of the heat generated.

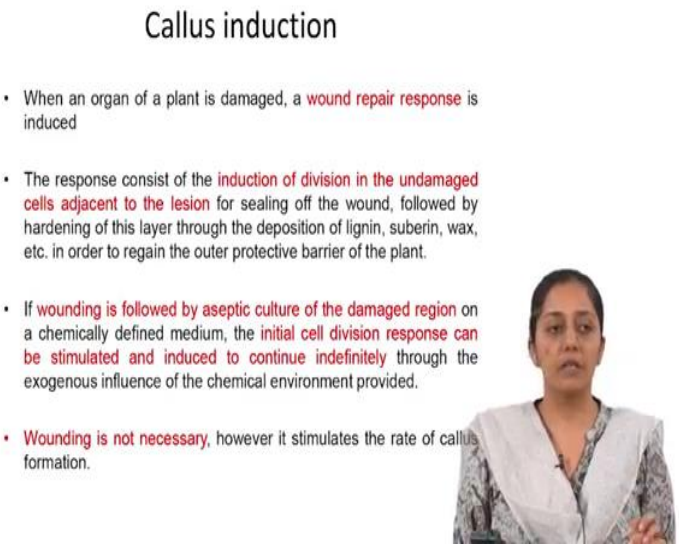
So, then when the explants once have the surface contaminants removed by water wash now that before you treat it with the disinfectant there is a treatment given by the wetting agent for 15 to 30 seconds. Then after treating it with the wetting agent again the ethanol is removed by water wash intermittent water washes is given for every treatment and then after you treat after that you treat it with disinfectants. Now disinfectants generally used in plant tissue culture technology is either you use sodium hypochlorite which is up to 20 percent or you use your mercuric chloride 0.01 to 0.1 percent.

Now, mercuric chloride or sodium hypochlorite are your disinfecting agent. So, the exposure time the concentration of the disinfectant has to be optimized depending on the kind of species or whether you are working with the tree species, whether you are working with an old explant whether you are working with a young explant all that would have to be optimized.

So, generally for *in vitro* cultures young explants are preferred, why do you think young explants are preferred? Young explants means parts of the plant are younger meristematic regions or younger plant parts. So, why do you think younger plant parts are preferred, I can agree with the meristem but why do you think then another question why less contamination? Because the cells are still rapidly dividing and have not differentiated the organogenesis and specially the xylem and the phloem bundles have not become the throughout connections as in the whole plant. So, when it is not there so the chances of the contaminant which can be a virus or a bacteria living inside the plant to get transferred is

less. So, that is one of the reasons meristematic and why younger plants? Because as you people said younger plants the cells are still rapidly dividing it is still a growing region of the plant not yet matured. So, because of the rapid division the ability of differentiating and redifferentiating is much higher than a matured plant part.

(Refer Slide Time: 12:04)



So, now what is Callus induction? Generally, in nature, callus is a wound response whenever there is a wound created and it is a repair mechanism in nature. So, suppose there is a lesion which is made in the plant, now the cells near that damaged region start rapidly dividing till they cover that damaged region. Why do they need to cover it ? Because now the inside of the plant has been exposed and it has to be now again protected from the outside environment which has so many other microbes and may be pathogenic environment.

So, then the cells keep on rapidly dividing which are around that lesion till that portion gets covered and then once that covering has happened, further on top of it is covered by deposition of lignin wax. Suberin is one form of wax which gets secreted by these cells to create a hard coat or a layer. So, that is how callus is formed in nature as a wound response. Now when you need to exploit this in *in vitro* cultures what would you like to have, you would like to have a continuous repair mechanism rather than closing it at any time. So, you would like these cells which have now started rapidly dividing to continue to divide forever till you subculture it and provide medium.

(Refer Slide Time: 13:33)

Generally, you may see that even you take small leaf explants and if you provide the right kind of medium you can reprogram the cells. So, the question was what do you think will become critical in that right kind of medium to reprogram the cells or to bring them to dedifferentiated states it is your?

Student: Hormones.

Hormones. So, generally auxins and cytokinins we will come to that.

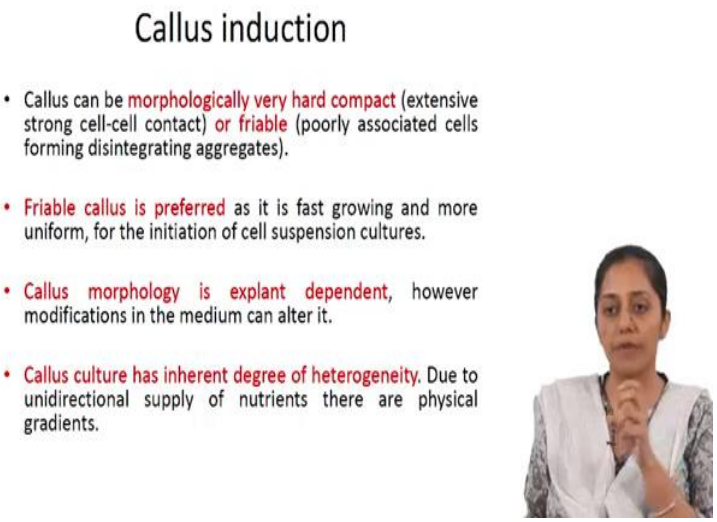
So, if you can give the right atmosphere and even the environmental conditions not all species will give a callus induction response if you provide light conditions. Some callus induction responses from some plants also happen when you put them under complete dark conditions. So, all the conditions have to be optimized depending on species, depending on age of the explant, depending on type of the explant and the genotype.

So, how is it induced *in vitro ?* The cells from any plant species can be cultured aseptically on in a nutrient medium. Now any plant part tissue with living cells can be used as an explant as I said, then under the influence of plant growth regulators generally when we say plant hormones they are endogenously produced plant growth compounds or regulators. But if they are synthetically produced or exogenously provided right, then we call them as growth regulators. So, what happens when there is an excision and there is

rapid division it will force the cells to divide around that region because you are using a tissue which is an organized culture.

So, the epidermis it is a tissue so there will be a dermal tissue also in that it is a complete organ which you are using. So, once the cells continue to divide they will rupture the epidermis the topmost dermal tissue and the cells will come out and after few days you will see that there is a blob of tissue which looks like a callus. Now generally it takes 1 to 5 weeks for complete callus induction to take place. If you see that even after 2 months or so you are not observing complete callus induction or it has become impaired, then it means that either nutrient conditions or growth regulators have to be relooked and then you should revise the media.

(Refer Slide Time: 16:01)



Now, there can be different forms of callus depending on the species on which you are working. Now this hardness or friability of the callus depends on the cell to cell contact, how closely or strongly they are connected with each other and the water content in the callus. So, which means the fresh weight to dry weight ratio of the callus.

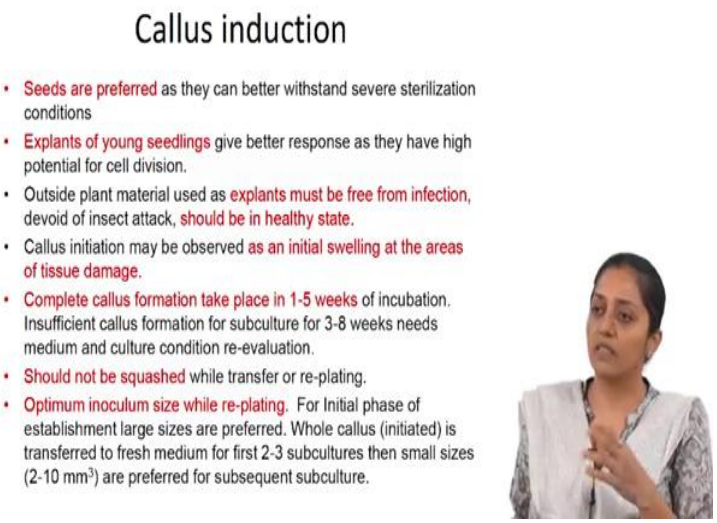
So, generally for suspension cultures or *in vitro* cultures , friable callus is preferred. Friable callus means that the cells of the callus should be able to get dispersed easily when given suitable conditions. Because hard and compact callus will become limited of what? What limitation do you think you can face with plant cell cultivation, if your beginning material which is the callus is very hard and compact ?

If you want to exploit plant cells *in vitro* you need to mass produce isn't it ? Now, mass producing the callus you can do in 2 stages : you continue to derive the callus in the solidified medium and then you can disperse the cells and bring them in suspension where cells can continue to divide and scale it up.

So, but what is the problem if I continue to do it in the solidified medium? There the entire tissue is not exposed to the medium. So, how does the callus afford to multiply or grow it is because of that compactness on the solid medium. The cells which are in touch with the medium they get the nutrients and then from it is subsequently transferred to the other cells which are not very close to the media. Now what is a demerit here ? This also gives heterogeneity to the callus nature because there are physical gradients, nutrient gradients thereby leading to biochemical gradients and maybe their bio synthetic capacity.

So, that is why callus can be heterogeneous in nature. Now if you need a synchronous culture and all cells giving you reproducible results in terms of productivities then all cells have to be given the same environment. So, if the cells get dispersed in the liquid medium, all cells are getting exposed to the same environment. So, the chances are high that the culture would be synchronous in nature and then the mass transfer limitations which you people were referring to will also be overcome to some extent.

(Refer Slide Time: 18:33)



Now, because they are on the solidified medium, the callus growth in comparison to cells in suspension is also slower. If you compare it with bacterial cells, the doubling time is still 24 to 72 hours here and there in bacterial cells is between 10 to 12 hours or 24 hours.

Now, callus inductions seeds are preferred as they can withstand severe sterilization conditions. So, I would still say it depends on the kind of culture, you would like to and even if you see the books, different books say different things, but it is very species specific. So, generally if you want to end up depending on the objective of your study. If the objective is to produce a particular phytochemical from that cell using plant cell cultivation, then in the end what do you need? You need a very high yielding cell line.

So, for generating a very high product yielding cell line , your starting material if it is high yielding, the chances are all the cells there are high yielding. So, if you can get a callus from that explant which is high yielding, the probability is high that you will end up in plant cell lines which are high yielding. So, ideally what should be done is, you should pick up a plant variety which is high yielding. For example, I will give you an example I work with Azadirachtin production.

So, there are neem as ubiquitous in our country. It can be found in many regions of the country, but because of the geographic and climatic variation even in the neem variety which is present in different parts of the country including north and south India the yields are variable. So, you must pick up a variety which is higher yielding with average high yields and then you must pick up that explant or that region of the plant, which is highest yielding in that plant. So, for example, in Azadirachtin, seeds generally have the highest yield of Azadirachtin. So, we picked up a plant which is a Trivandrum variety and it was very high yielding in comparison to the plants in IIT Delhi.

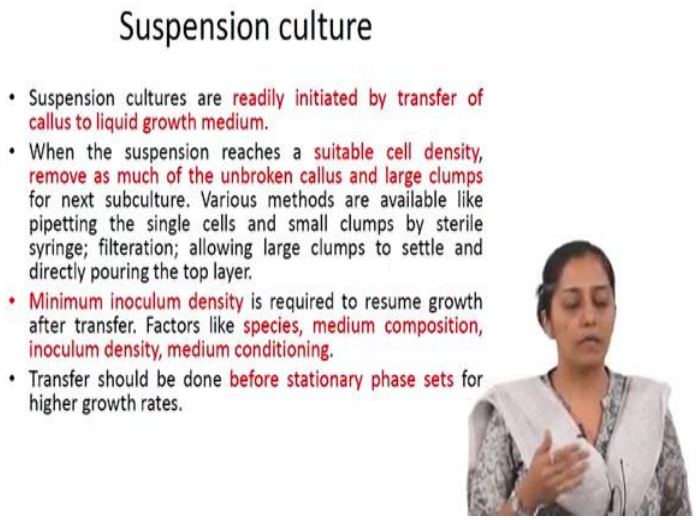
But then we made a comparison we picked up the plant from here in South India which was showing high yield and from that plant we picked up the seed of that plant because the seed is known to have highest yield and that seed was used to generate the callus. So, what I mean to say here is, it is not necessary that you need to pick up the seed only you must pick up that explant a part of the plant which shows highest yield of that particular metabolite.

So, once you have a successful callus induction after 2 to 5 weeks then how do you subculture? Let it grow for some time, the initial 2 to 3 subcultures, the entire callus which

has been induced should be transferred to the fresh medium. You should not disintegrate it because factors like minimum inoculum density which you must have heard in your microbial fermentation or fermentation technology that critical inoculum density is crucial for the subsequent growth of the culture.

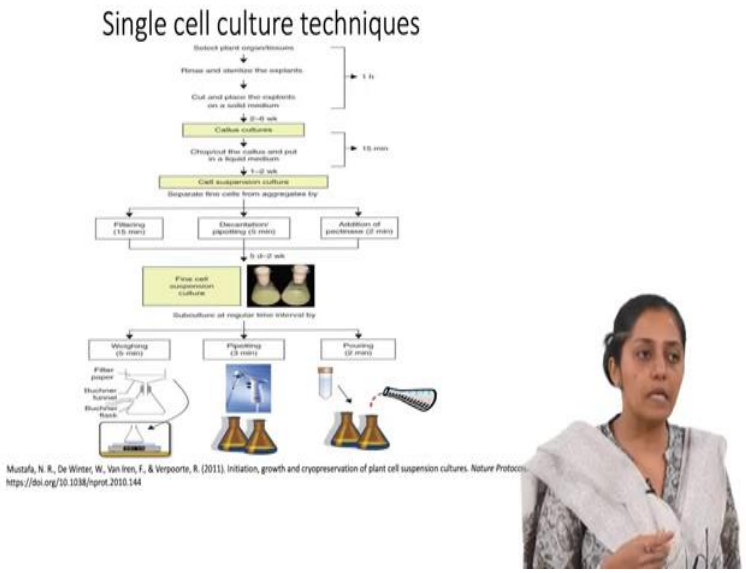
So, the minute the callus has come out that does not mean that you rupture it and disintegrate it into fewer smaller sizes as any size or any small amount of cells would lead to multiplication. There has to be a critical size a minimum inoculum size which is needed for multiplication further. So, once it has grown to some 10 millimeter cubes or so, then you take that callus after 3 subcultures of the entire callus blob tissue and then you can divide it into two and further subculture it for propagation.

(Refer Slide Time: 22:18)



Now, coming to the suspension culture. So, now, how is suspension culture induced from callus? Once you have got the callus then you bring it in suspension there are different ways in which suspension cultures are created either you can use Buchner funnels, I will show you a picture.

(Refer Slide Time: 22:35)



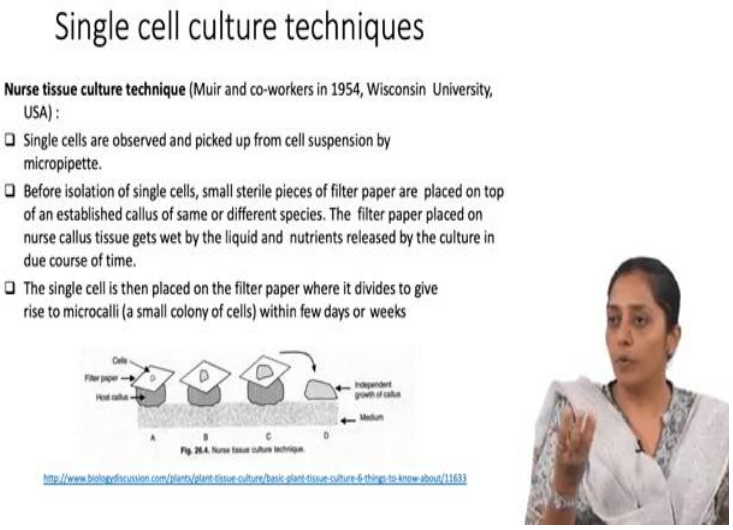
So, either you can use Buchner funnels and if you want a very synchronous culture or very fine suspension, then you can even in pipette out few cells. People have done callus induction with single cells and then subsequent cell suspension also with single cells or small amount of cells. So, you can pipette out the cells and then use that as inoculum or you can directly filter and remove the settled aggregates and use rest of the cells in the liquid part , the filtrate to be used for suspension culture initiation.

Now, what is key here is that, as you do in fermentation of microbes inoculum age, inoculum size has a role to play in your growth and production kinetics same is true here. Now it is not that if you directly take the cells they will start dividing , it depends on what all conditions? It depends on the production medium. Now if you suddenly bring the cells from your inoculum preparation medium to production medium which is completely different, the cells may not divide or there may be a long lag depending on the size of the inoculum which you have used. So, the production media , the environmental conditions which you are using , the media composition which you are using and also your minimum size of the cells is critical.

Now, there are techniques how you can reach to synchronous cultures. Now for doing synchronous cultures people use sieve cell methods. Now this can lead to different filtrates which will be obtained from different mesh sizes which can then be used for generation of suspension cultures as with a high probability that they would be all synchronous in nature.

Now why do you think these all filtrate? Suppose I use 80 mesh size or 150 mesh size and I use that filtrate to generate a cell suspension why do you think that this increases the probability of getting a synchronous culture? So, because as she said with the same size and same shape of the cells coming in , the probability is higher that their metabolic activity or their age would also be same thereby leading to synchronous cultures and when I say nurse tissue culture there is a nursing tissue involved.

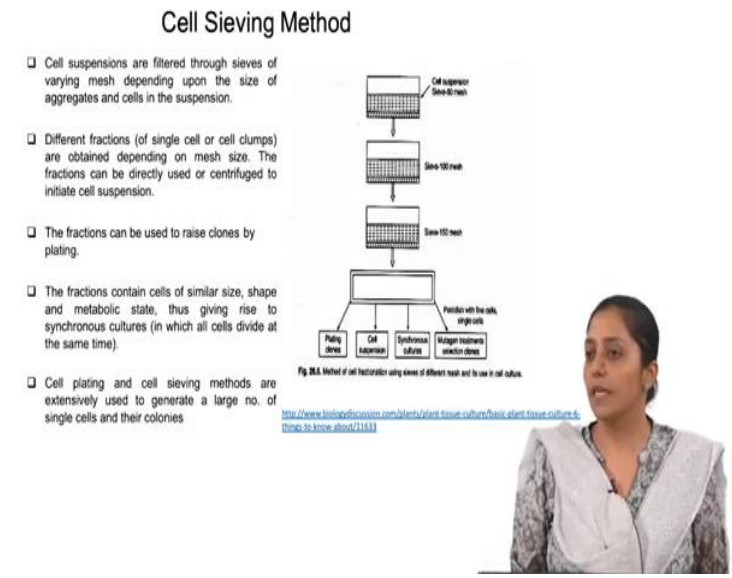
(Refer Slide Time: 25:06)



So, in this technique what is done is, you take a small piece of a callus of the same variety or of a different variety and put it on a sterile environment in a petri dish and put a small sterile filter paper on top of it and the metabolites or the liquid which gets released by this fresh callus which is nursing will wet the tissue on top of it. Now on this wet tissue which is now supposedly having all the nutrients and other metabolites which have been released onto that tissue, you then inoculate your single cell from which you need to generate the callus using the pipette.

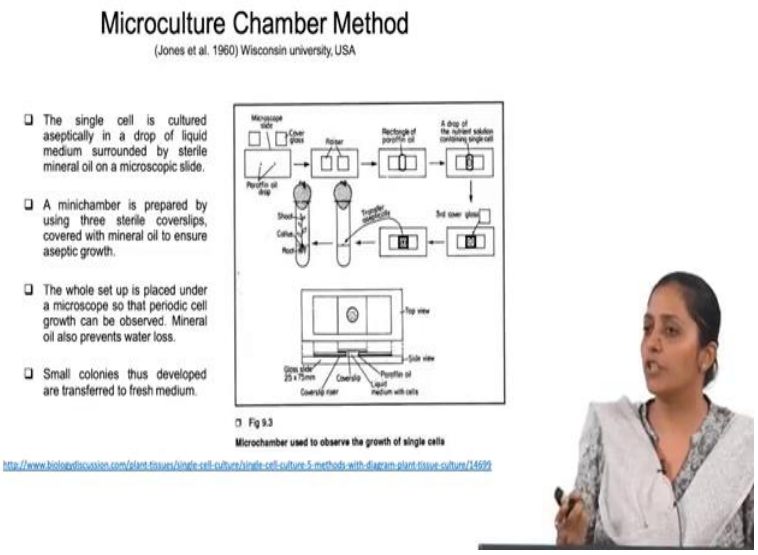
Once it gets the nursing from this parent callus tissue underneath the filter paper which is wet filter paper, you will see that the cells will begin to divide. The cells as they divide after some amount of division you will find the callus become visible and then using that callus for subsequent sub culturing can lead to a real synchronous callus or cell culture because you have started with single cell leading to it's subsequent clones. So, this is what is called a single cell culture technique.

(Refer Slide Time: 26:30)



And this is to achieve synchronous culture what I was talking as the cell sieving method.

(Refer Slide Time: 26:36)

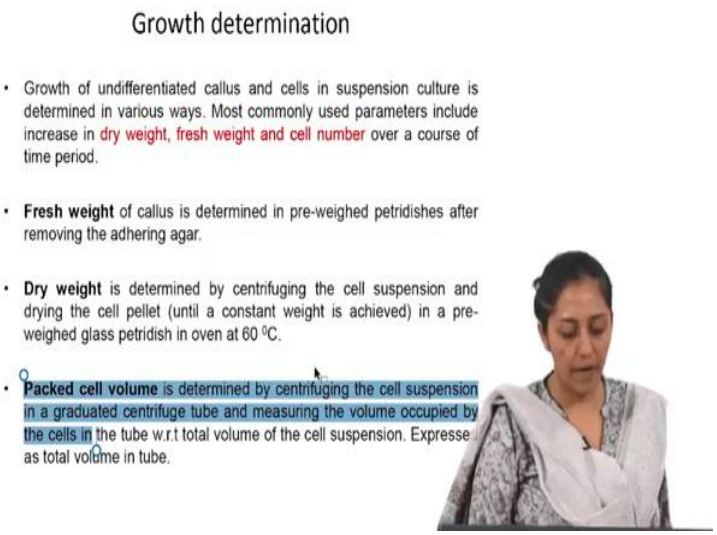


Now, another way of carrying out the single cell culture technique is also micro culture chamber method which can be used even now. Here, you use a sterile petri dish and you put paraffin oil on top of that sterile petri dish . Then, you make use of sterile cover slips, I hope you people know what are cover slips. Now, take three cover slips, two cover slips are put on top of that glass slide with the oil and they will form this gap which is shown here this gap between the two cover slides.

Now, because there is oil underneath it will form a small oil boundary so as to give a sterile environment and so that whatever is inside now in this square would not be in touch with the outside world. Then you inoculate your one drop of liquid nutrient with your cell inside that gap and then you cover this with the third cover slip on top with the paraffin oil. So, now, it becomes like a square small chamber mini chamber or micro chamber where that single cell is in the drop of the liquid nutrient and then you can see under the microscope let it divide.

The cells would divide and then once you have seen it has reached to some groups of cells mass , they can be subsequently transferred and can lead to generation even of the whole plant. So, this is called as micro culture chamber technique.

(Refer Slide Time: 28:26)



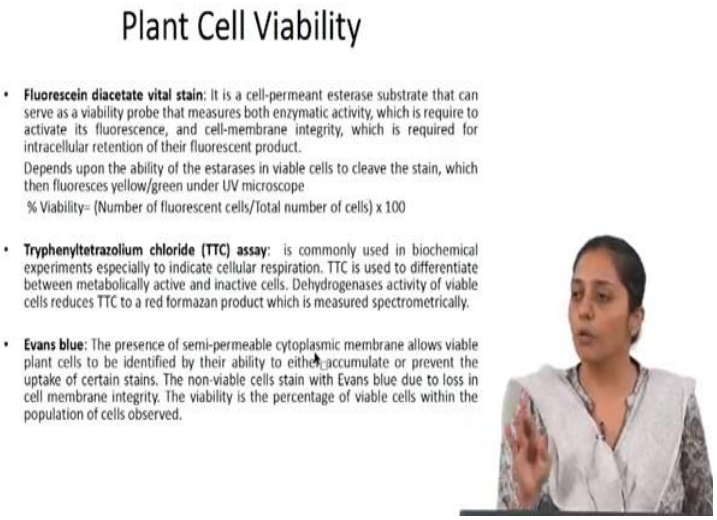
So, before we begin with different forms of culture, it is important for us to know two things; one is how will we find whether the cells are growing and the second is how will you find whether the cells are viable. So, how do we find or what parameters do we use to quantify the plant cell growth or plant tissue growth? There are different ways. One is you do simple dry cell weight estimation in which you dry the plant biomass or the callus or the tissue biomass in oven at 60 °C, and let it dry until constant weight is achieved. So, that is simple you calculate the biomass concentration.

Now, the second is you use packed cell volume because plant cells are large in size this works appreciably well. So, you can use a centrifuge tube and since the plant biomass form

aggregates and you will see that the plant biomass after some time will settle down and then in comparison to the total volume occupied in that centrifuge tube, the percentage which the biomass occupies is represented as packed cell volume. That can also be a rough indication for growth of the biomass.

Now what else? Fresh weight to dry weight, which is called as growth index. Now growth index is another parameter which is used when you measure the growth of the callus which is final fresh weight after some amount of time, you calculate the final fresh weight minus the initial fresh weight which means that the amount of fresh weight which has increased from the initial amount of fresh weight. So, this ratio is called as growth index and is a parameter which gives you an indication of how much growth has happened.

(Refer Slide Time: 30:11)



Now, talking about plant cell viability there are many different ways, but here I have listed on three different ways which are generally used in literature to determine plant cell viability. One is the fluorescein diacetate vital stain. So, this particular stain is based on the activity of esterases in the viable cells. Now these enzymes can reduce this dye to produce a fluorescent product . When the cells are viable, the membrane integrity is in place and so they can hold on to the fluorescent product which you can see under the UV light and percentage viability is given as the number of fluorescent cells to the total number of cells. So, those which are fluorescent would be viable because it shows integrity of the

cell membranes is still in place and it also shows that the esterases are still active in the viable cells. So, this is how percentage viability can be calculated.

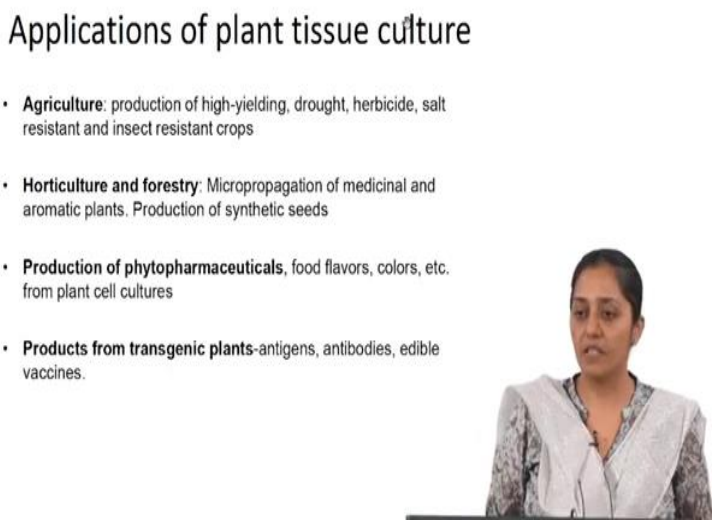
The second way is using tetrazolium salts which you must have heard even in your animal cell cultures , MTT assay. So, TTC is a Tryphenyltetrazolium salt. It is generally dependent on the activity of dehydrogenases enzymes. The dehydrogenases signifies the metabolic activity of the cell and this assay reveals whether the mitochondria is actively functional or not. So, what it does is, because it is dependent on dehydrogenases. So, once it reacts with this tetrazolium salt it forms a red Formazan product.This red Formazan product can be measured spectrophotometrically, which can then be used to calculate percentage viability of the cell.

Now Evans blue; Evans blue or any kind of such stains depends on whether the cell membrane integrity. If the cell is alive, the membrane integrity is in place and if the membrane integrity is in place it is selectively permeable. Now, selectively permeable either you can use not taking up a particular stain as a viable marker or taking up a particular stain and not letting it go as a viable marker. So, in Evans blue dead cells become?

Student: Blue.

Blue which means what are you looking at? Because the cell membrane integrity of the non-viable cells is not there. So, the dye will be able to get in and stain the cell. So, that is why we Evans blue is used to can be used for determining the viability of the plant cells.

(Refer Slide Time: 33:04)



Applications of plant tissue culture : So, in agriculture for production of higher yielding or drought resistant or herbicide resistant or salt resistant species of crops. Then horticulture and forestry if you want to do micro propagation or if you want to produce synthetic seeds. Then in production of phytopharmaceuticals your callus or cell suspension cultures or hairy root cultures which is organ culture comes to play a role. Then products from transgenic plants you must have heard about edible plants or GMOs or plants with specific nutrient quality like your golden rice or plants which can be protected from certain insect pests can be produced. So, for example, your BT cotton.