Plant Cell Bioprocessing
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Lecture - 10
Somatic embryogenesis and Protoplast culture

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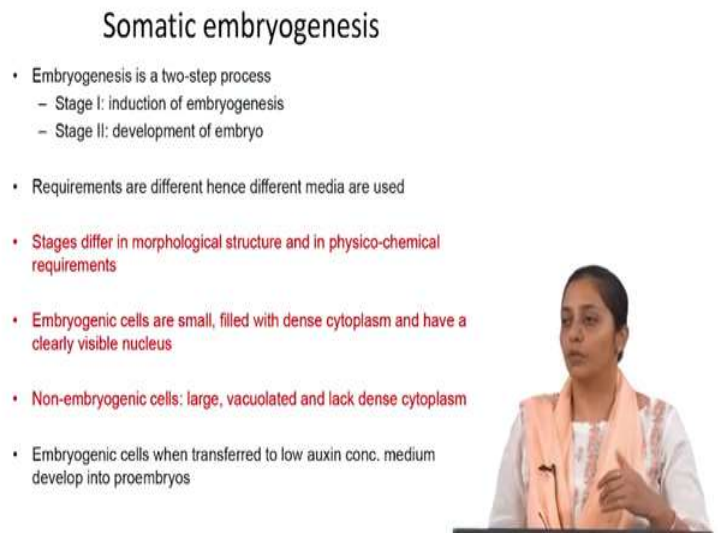

If you need to produce embryos from somatic cells, which is called as somatic embryogenesis, there are two stages. Stage one: Induction of embryogenesis which means that the cells which are meant to form embryos or which are prone to become embryogenic in nature - you induce them to undergo embryogenesis or the cells which are not determined to make embryos are determined and then through reprogramming to make embryos.

So, stage one is induction of embryogenesis and stage two is that once the zygote is formed, the different stages it will undergo to produce the *in vitro* plantlet. So, requirements for these two stages are different in terms of media. It is generally not necessary that whatever media composition is working well with callus induction to induce embryos, it may work well. But for the development of the *in vitro* plantlet after induction of the embryos revision of the media might be required.

So, the different stages in the development of embryos differ morphologically. So, we will be looking at a picture. There are four different stages before the hypocotyl region appears. Embryogenic cells are those which have competence and embryogenic in nature. They are

generally found to be small, rich in cytoplasm and their vacuole size is less and those cells which are non-embryogenic, they will be large vacuolated and have less dense cytoplasm. So, embryogenic cells will have more dense cytoplasm in comparison to non-embryogenic cells. And generally, it is observed that once they are transferred into low auxin containing medium, proembryos are induced.

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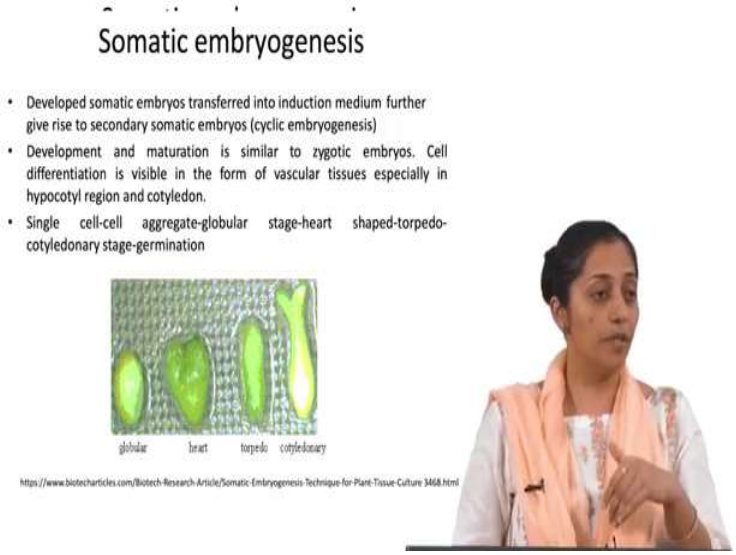

Now, once the somatic embryos are transferred from induction medium to developmental medium, during the induction, the single cell will divide into a number of cells say 4 to 5, then forming a globular structure. Once the globular structure is formed, it undergoes further development which means differentiation to become a torpedo shape, and then after torpedo shape it undergoes further vascular bundle formation.

Generally, you can see in the hypocotyl regions where the xylem elements start becoming visible and in the cotyledonous region the shoot and the root promidium appear.

So, development and maturation, it is similar to the zygotic embryo. Also, there are four stages known: globular, heart shaped, torpedo and cotyledonary stage. Now, all these are morphologically distinct. You can clearly see once the embryo starts developing - the somatic embryos. And moreover, biochemically and molecularly, they are different because differentiation is taking place, vascular bundle formation is taking place and thereby leading to from the meristem region - there will be a root meristem, shoot meristem region appearing.

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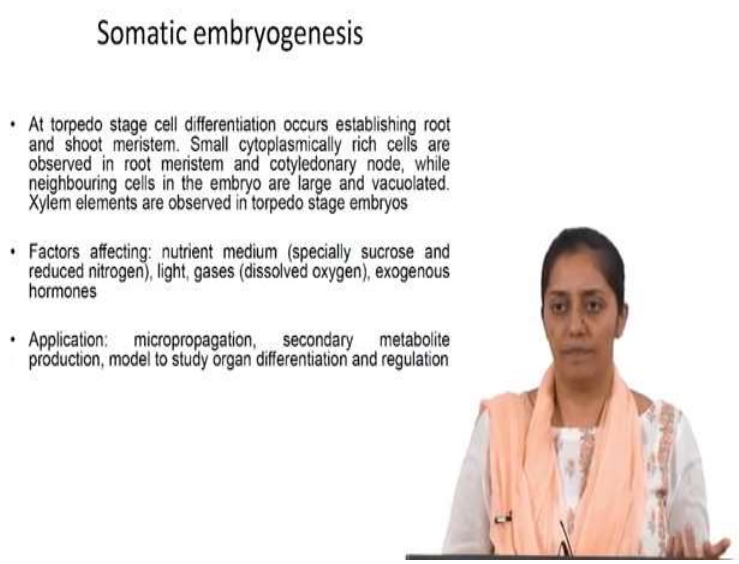

So, what happens? At torpedo stage, cell differentiation occurs establishing the root and shoot meristem. Now, what is the torpedo stage? This third-one which looks like a torpedo. Now, small cytoplasmically rich cells which are embryogenic are observed in these meristems and the rest of the cells, are vacuolated and matured cells will form the bulk. Now, what are the factors which can affect embryogenesis? It is nutrient medium specially sucrose and reduced nitrogen. Then light, gases; gases means dissolved oxygen here, then exogenous hormones - your growth regulators.

So, what can be the different applications of somatic embryos? You can use them for micropropagation. They are also used as organ cultures for secondary metabolite production. So, when I say secondary metabolite production, how do you think somatic embryos can be used for large scale secondary metabolite production? Micropropagation, last class itself we know. Large number of small plantlets can arise. Third application is given as they can be used as model system to study growth of the plants. So, that is also quite obvious. How does an embryo develop inside? So, they can act as model systems to embryos - the real zygotic embryos.

But how come they can be used for secondary metabolites? How and why do you think? Somatic embryos are more organized than the cell cultures?

Student: Ma'am, is it because all of them are being formed from the somatic cells they are more consistent in the production of the secondary metabolite, and no large amount of variation in the quality of secondary metabolite cannot be seen. So, that is the reason.

One of the reasons can be that. Being an organized structure, it is less prone to somaclonal variation. Moreover secondary metabolism is also linked in plant cells with organogenesis and differentiation. It is a higher order function. Secondary metabolism comes later, primary metabolism is for growth and development. So, secondary metabolism is a part of higher functions which means much more organized for survival.

So, that needs cell to cell contacts, organogenesis to happen, and differentiation into organ formation is nothing but getting into those organized functions. Different cells come together to perform a specific function, so it is more defined now. So, these kinds of cells when come together, they become organized structures, there the secondary metabolism is found to be more visible in terms of biosynthetic capacity. So, secondary metabolites yields are found to be higher in organized structures.

Now, and the other is they are more stable than the callus or cell cultures. In the last class also, they were showing for micropropagation, somatic embryos can be cultivated in reactors. So, one somatic will give rise to another somatic embryo - multiplication should happen. Large scale cultivation means what? That in the reactors, multiplication is happening. So, will one somatic embryo form another somatic embryo? No, it has to start from the cells.

So, logically can you propose a solution to it? What should be the starting material? What kind of cells? All cells, meristematic or much closer which will have higher probability. You remember we had talked about cells with embryogenic potential which are determined to produce embryos, and those which are induced to produce embryos. Pre-determined embryogenic potential. So, if you can find those cells which have embryogenic potential, you will use those cells, bring them in suspension, do large scale cultivation and then give them conditions to form organized structures called somatic embryos.

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Now, meristem culture we were discussing. Meristem culture is nothing but you make use of the meristematic regions of the plant which are lateral bud regions, axillary bud regions or apical meristems and which will be useful in making virus free plants.

We also discussed about the reasons. Now, virus distribution is uneven throughout the plant and is much less in a meristem. The reasons were, virus cannot travel quickly enough through the plasmodesmata. These are connections. So, as these meristematic region - the cells are rapidly dividing. So, the rate at which they are dividing and forming new cells, the virus to travel from through these connections is the rate is lesser than that.

So, one is this, the probability of virus transfer is reduced. The second is as we were talking about the presence of vasculature is missing, vascular bundles in the meristematic regions is missing. They are still to form vascular elements. So, the transfer of virus is difficult to these regions. So, therefore, meristem culture is preferred to generate virus free plants.

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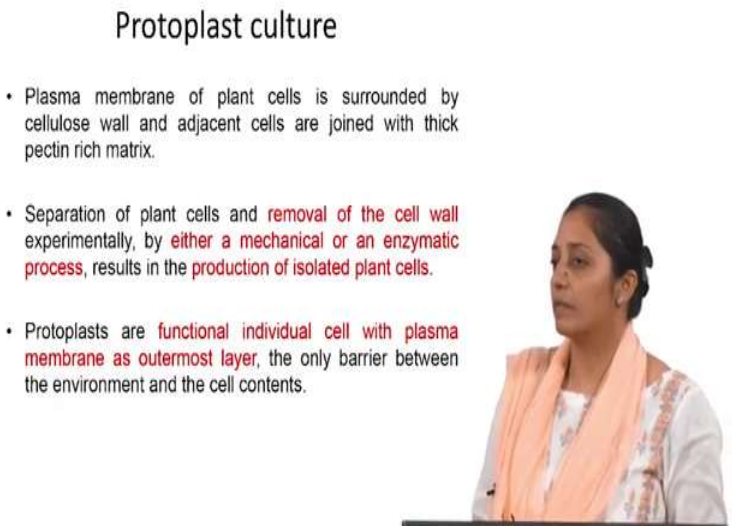

Coming onto protoplast culture. Now, what is protoplast? Cells devoid of?

Cell wall. So, where do you think or why do you think protoplast culture is used?

Whole cells cannot be used for hybridization?

Even with whole plants we see in agriculture - hybrids are produced.

But it is about what is much easier, making the process much easier. So, when we say somatic hybridization, it is because somatic cells can easily be combined together.

What are hybrids? When two cells can be combined such that the two nuclei combine to make one nuclei, all their cellular content are combined into one cytoplasm and one nucleus.

So, the nuclei have to combine together and the cytoplasm gets mixed. How is it done? We know that the plant cells are attached to each other. They are attached through the pectin rich layer. What is that layer called?

Student: lamella?

Middle lamella. So, that layer has to be removed before the cell wall is removed, so now, the attachment is off. Now, the cell wall is removed for the protoplasm to come together. There are two ways to remove the cell wall, one is mechanical, the other is enzymatic. So, in mechanical we use a very sharp blade to cut the cell wall.

Now, if the plasma membrane in general will be closer to the cell wall, so there are chances that the membrane can get ruptured. So, what should be done to avoid that? To ease out this process, mechanical separation of cells - what should be done? Can you give a solution?

Everything is done under the microscope. First prerequisite is what? To disintegrate, to separate the cells. So, now, that separation requires pectinase treatment or, suppose the cells have been separated, you have a single cell. Now, there is a cell wall and the plasma membrane, I need to cut the cell wall. Knowing that the plasma membrane is very close, how to avoid that plasma membrane to be ruptured?

Student: Plasmolysis

Very nice, very good. So, now, can you just elaborate on this? Plasmolysis will help how?

Student: Putting them in a hypertonic solution and they will shrink.

Do you agree? So, that is what is done. So, you change the osmotic pressure by adding osmoticums such that, the cell membrane contracts; such that the cell wall can be easily cut off. Now, what care should be taken in this?

The viability should not be lost.

So, as it is cut, the balance has to be maintained but now it is becoming more fragile with only plasma membrane. So, you have to bring back the osmotic pressure such that it is plasmolysed and once the cell wall is out and the plasma membrane is the only barrier between the outside and the inside, now it was plasmolysed. It was plasmolysed because of?

Higher or lower concentration outside of the solutes of the salts? Hyper means?

Student: Higher concentration.

Agreed.

So, if it is higher concentration and the plasma membrane is the only barrier left. It is very high. So, now, what will happen? The viability will reduce. So, now, you would like to balance it. Now, while balancing you would like to reduce the concentration. It is recommended not to suddenly reduce the osmotic pressure. Why it is recommended so?

Student: If we suddenly reduce the osmotic pressure water may rapidly get into the cell and burst.

Yes, so, this care has to be taken. The balance of the osmotic pressure when you are creating protoplasts.

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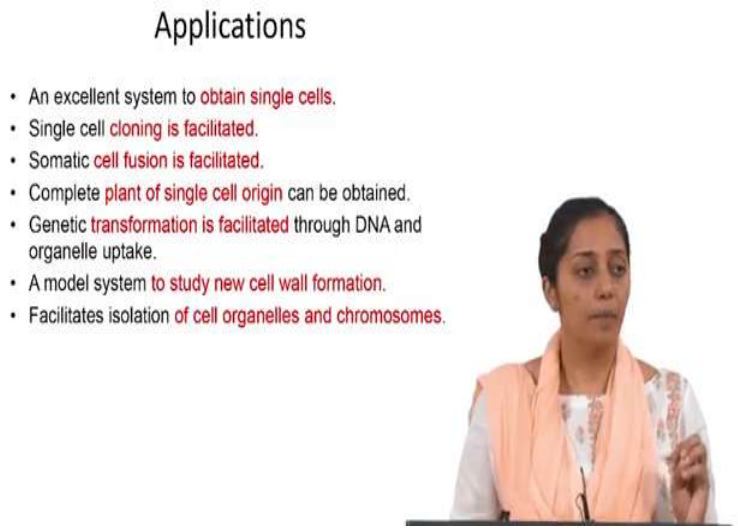

So, these are functional individual cells with plasma membrane as the only outermost layer. What can be the different applications of making protoplast or doing protoplast cultures? You can obtain single cells. Why single cells? If you want to do clonal propagation from single cells, you want to then divide, form a callus and then regenerate it into plantlets.

So, this is a clonal propagation. For even desirable genetic modification you need to do some uptakes from extracellular to intracellular environment. What facilitates that uptake? In plant cell, if you want to do transformation what is the barrier, first barrier?

Student: Cell wall

Cell wall. So, protoplast is devoid of cell wall. So, now transformation is much easier because the only barrier is plasma membrane. Complete plant of single cell origin can be produced, then transformation is facilitated. Once you have protoplast, depending on the media composition, once the protoplast starts dividing, the nutrient requirements of these divided cells may be different from the original protoplast.

Now, the new cells need not be protoplast because they have the genetic machinery to produce cell walls, so the newer cells which will get produced will eventually develop cell wall. So, their nutritional requirements would be different and they can also be used for isolation of different cell organelles if the studies have to be done.

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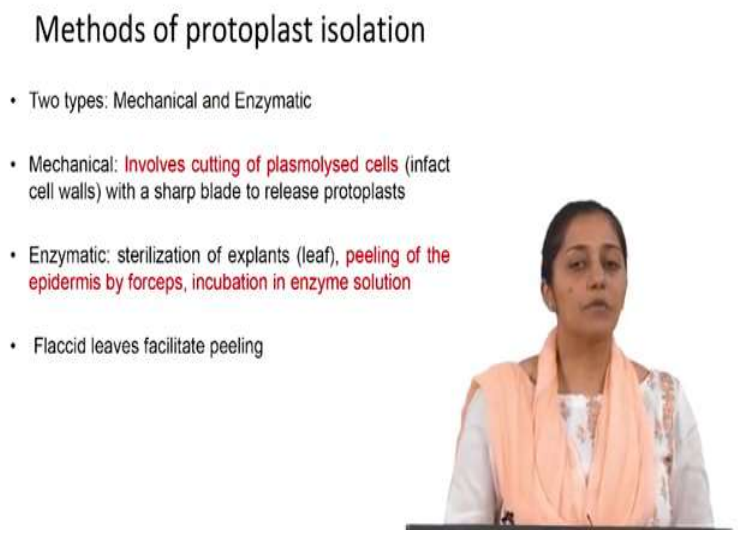


So, then I was talking about what are the different ways of making protoplast, one is mechanical the other is enzymatic. Now, mechanical involves cutting of plasmolysed cell with a sharp blade to release the protoplast. Now, enzymatic would involve exposing the cells to various kinds of enzymes we know. What kind of enzymes? We need to remove the cell wall. So, all those enzymes which can degrade the cell wall or decompose the cell wall.

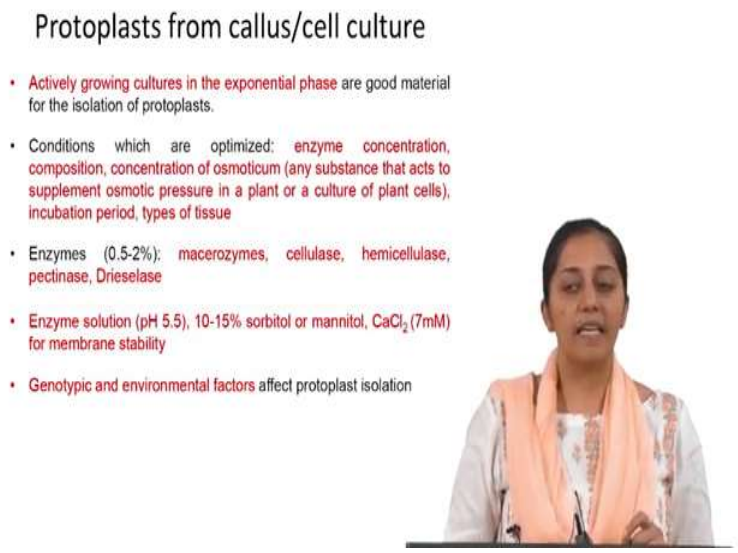
For example, What are they, you should know what is the composition of the plant cell wall - cellulose, hemicellulose, pectinase. Macerozyme, driselase, these are names given by some companies for mixture of these enzymes. Their origin of organism is generally fungus. So, these class of enzymes together as a mix are sold for plant cell wall degradation.

So, how it is done? You sterilize the explant. So, generally you sterilize the leaf explants. The epidermis is removed, and then and the explant is incubated with these enzyme mixtures for some time and it is said that flaccid leaves facilitate peeling. Flaccid leaves means? Think logically, what can it mean?

Student: Is it referring to the dry leaves or something?

Right. Drooping leaves. Drooping leaves means what?

Lesser turgor pressure.



So, when we are using cells, actively growing cells are used which are from the exponential phase for isolation of protoplast. Now, conditions which should be optimized if you want to isolate protoplast are enzyme concentration, exposure time, enzyme composition, which is the different kinds of enzymes we are using. Then osmotic pressure, the presence of osmoticums, what kind of solutes, what concentrations we are using for the osmoticums.

Now, the enzymes are generally used in the concentration range of 0.5 to 2 percent, where macerozyme which is a mixture of these kinds of enzymes or separately you can use cellulose, pectinase, hemicellulose. So, enzyme solution and sorbitol, mannitol, is added. what is the purpose?

Student: Osmotic balance.

Very nice. So, they are used as osmoticums to balance out the osmotic pressure. Then calcium chloride, for membrane stability. This, tries to neutralizes the surface charge.

So, generally, if they have to be brought together for hybridization, the surface charge should be neutralized, so that they do not repel, the membranes have to fused, isn't it? So, therefore, it is treated with calcium chloride and it is also said that the genotype and the environmental factors can also impact protoplast isolation.

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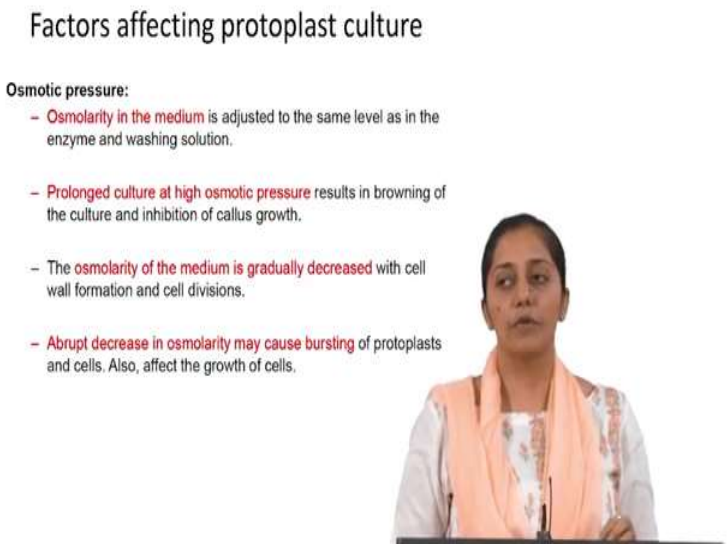

So, the factors which will affect protoplast culture: if you only want to do culture of protoplast, the only barrier is plasma membrane. So, osmotic pressure has to be balanced out carefully. So, osmotic pressure, osmolarity of the medium is adjusted to the same level as the enzyme or the washing solution to which you are exposing it, otherwise the cell can rupture or get non-viable.

Then, prolonged culture at high osmotic pressure can result in the browning of the cells. Browning means necrosis or viability loss. Now, the osmolarity of the medium, is gradually decreased with cell wall formation and cell division. Gradually decreased? We have discussed, the reason being suddenly if you decrease it the water may gush inside the cell and the cell may burst. So, as the cell wall is getting formed, with the barrier is getting formed, the osmotic pressure is reduced. Abrupt decrease therefore, should be avoided in the osmotic pressure.

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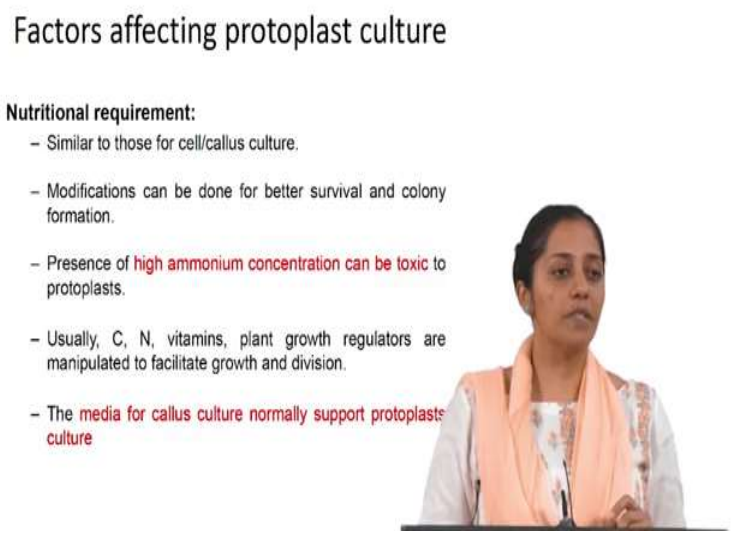

What are the nutritional requirements? As I said generally to begin with, they can be similar to cell or callus culture, but once the cells start dividing or then you need to revise the media composition. Presence of high ammonium concentration can be toxic to protoplast. Why? We were that day discussing that the nitrate and ammonium are the nitrogen source for the plant cells, isn't it? And easy assimilation is ammonium. Nitrate also once it gets assimilated, taken up, it is converted into ammonium ions to be integrated into amino acids or many different other compounds wherever it is needed.

Generally, it is observed that if you give higher concentrations of ammonium, there is viability loss, there is loss in growth of the plant. This is called as ammonium toxicity. Knowing that, isn't it interesting? Ammonium is needed ultimately, nitrate is also getting converted to ammonia only before it gets into the metabolism. So, why not to give ammonium in directly? If you try to give ammonium directly to the plants, it can be toxic to the plant cells. Why so?

Student: pH change

Very nice, very good. So, ammonium assimilation is found to be also related to release of H+ ions. So in order to balance out, there are channels. So, ion transport takes place there is parallel influx and efflux. So, that is to maintain the membrane potential. So, once the hydrogen ions are effluxed, there is acidification in the environment of the cells and the pH of the cell gets disturbed.

So, which is said to be one of the reasons why plants growth is inhibited. You have usually carbon, nitrogen, vitamins and plant growth regulators, they are manipulated to facilitate any form of cultures.

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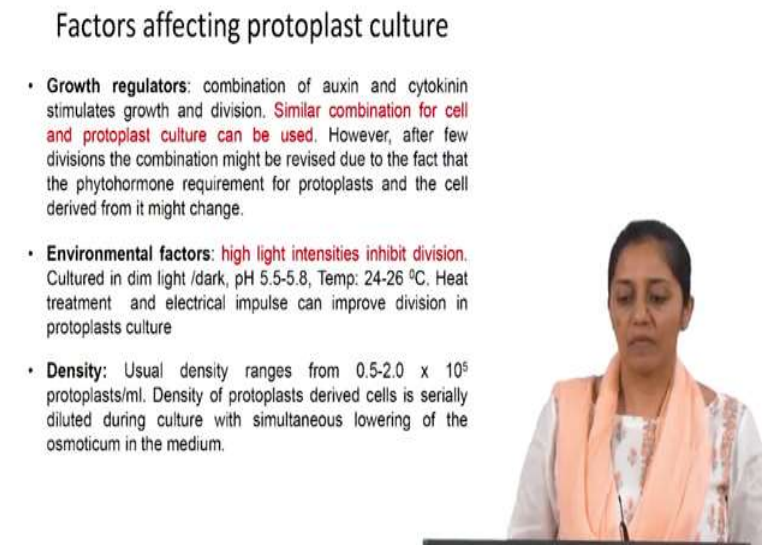

Now, growth regulators. Combination of auxin and cytokinins, it stimulates growth and division we know that. Combination which we are using for callus or cell culture might work initially for the protoplast cultures. But once the protoplast starts dividing into cells then it might need revision. Environmental factors: light, intensity. Very high light intensity can inhibit division in protoplast.

So, generally it is preferred that you use dim light or you carry out in dark. So, and what else, pH, temperature is generally the same what to use for your plant cell cultures. Right. Heat treatment and electric impulse is found to induce division in protoplast.

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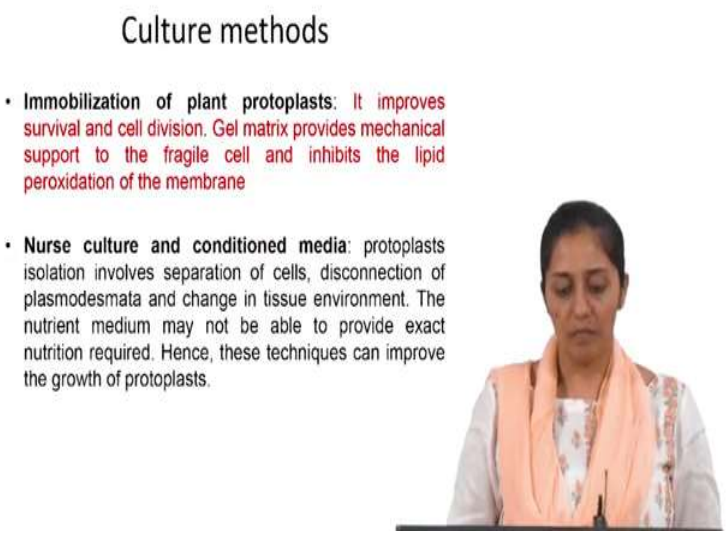

It is said that immobilization sometimes helps in protoplast cultures which is very obvious. Why do you think immobilization would be useful in protoplast cultures?

Student: Think it gives support to the cell.

Right.

Student: Protection.

So, because we know that it is very fragile, there is only the cell membrane barrier. So, encapsulating it in a gel form is giving it more protection. Now, nurse culture and conditioned media is sometimes found to be useful to protoplast cultures. Nurse tissue culture technique which we had read earlier what was that?

Student: Using the callus

Using the extracts of the other cells can be useful for the growth of their desired culture.

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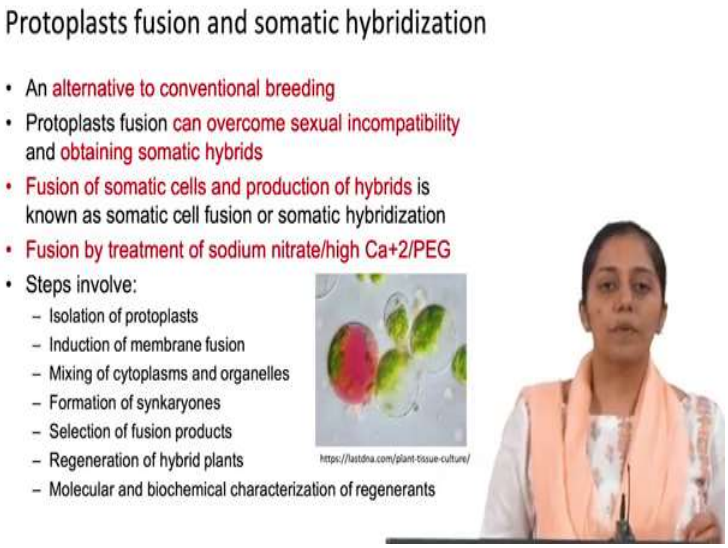

So, protoplast fusion of somatic hybridization how is it done and what is it? It is an alternate to conventional breeding. Protoplast fusion can overcome sexual incompatibility and obtaining somatic hybrids, if you want to use somatic cells to produce hybrid cells. Now, fusion of somatic cells and production of hybrids is known as somatic hybridization. So, what all is needed? You need either sodium ions, so it is treated with sodium nitrate, maybe it will be then calcium chloride, then PEG. PEG is?

Student: Polyethylene glycol.

Polyethylene glycol. So, it is facilitated. It is said that if you need to fuse, you need to bring the cells together. So, therefore, you will treat it with cations like sodium ions or calcium ions and polyethylene glycol. How is polyethylene glycol used in transformation?

Student: Permeability.

So, it is manipulating the permeability of the cell membrane reversibly. So, if it is used, it would facilitate what? The cell membranes have to fuse together, so that there is a transfer of nuclei, organelle, cytoplasm has to become one in hybrids, isn't it? So, then transient pores have to be formed and the membranes have to get fused. So, polyethylene glycol is helping in fusion. So, all this is facilitating coming together of the cells, fusion of the membranes by changing the permeability of the membranes, fluidity of the membranes.

So, what are the steps involved? Isolation of protoplast first, then induction of the membrane fusion, mixing of cytoplasm and organelles would then happen, then formation of synkaryones. Synkaryones means?

Two nuclei coming together to form one nuclei. Then selection of fusion product. So, once you have obtained, you would select the desirable depending on whether proper fusion has happened or not, under the microscope. You will select the fused cells or hybrids. Then you give optimum conditions for those hybrids to multiply, and once they multiply then they would form callus and then from callus you can regenerate into hybrid plants.

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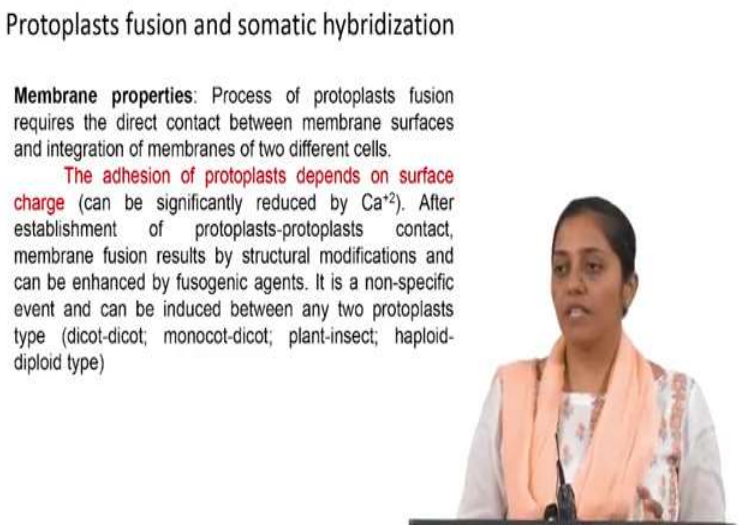

So, protoplast fusion and membrane properties - this is what we were discussing. Now, process of protoplast fusion, it requires what? It would require direct contact of the protoplast. The addition would be facilitated by increasing divalent cations which means calcium or sodium ions. So, we have already discussed this. This would change the surface properties of the cell membranes, and using polyethylene glycol, would change the fluidity of the membranes, reversibly increasing the permeability of the membranes to take up the extracellular material.

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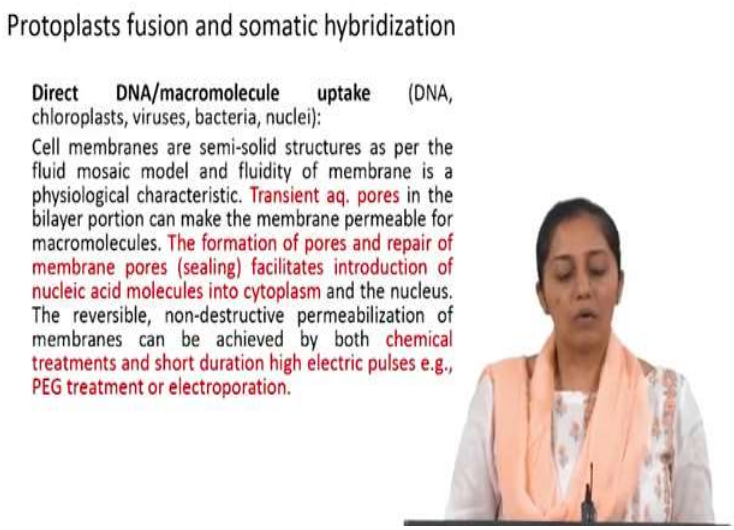

Now, direct DNA or macromolecule uptake. Cell membranes are semi-solid structures. This is what we have learnt in fluid mosaic model. Now, fluidity of this membrane, is a physiological characteristic. So, now, what is required? How it is done? We need to as I said reversibly increase the pore size.

Generally, this is what is happening in the cells also. If the cells want to take up some macromolecules, there are transient pores which are getting formed and then even when it gets damaged, there is processes in the cell to again bring back the membrane to its position and closing the holes.

So, the same process is being facilitated by using your polyethylene glycol or any chemicals for a short duration. You must have heard for transformations, heat shock treatment is done or electroporation is done. So same thing can be used here to improve results of somatic hybridizations.

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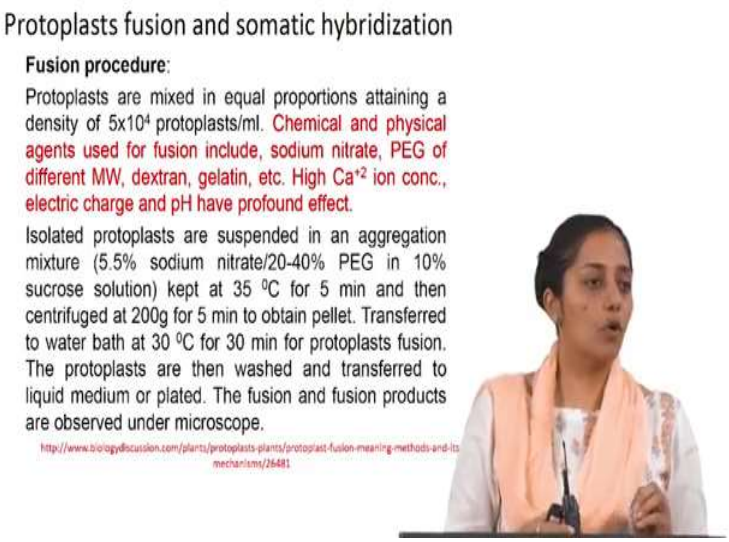

So, this is one of the protocols which is used for protoplast fusion in which you use high calcium ion concentration, electric charge and the pH is also changed because it is said to have a profound effect on the fusion of these protoplast.

So, what is it done? You mix sodium nitrate, PEG and sucrose, sucrose being an osmoticum knowing that once the protoplasts are there. So, you need to balance the osmotic pressure. Then you heat it at 35 ℃ or and then you centrifuge whichever cells you want to produce a somatic hybrid, and those cells are centrifuged and then again it is left at 30 ℃ for some time for the fusion to happen.

So, one is you are facilitating the fusion and then you are giving time for the synkaryones to form. Once the synkaryones are formed then you check it, do the screening and then change the media composition for it to divide and form the cultures.