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

Lecture - 11
Synthetic seeds, Cryopreservation and Freezing methods

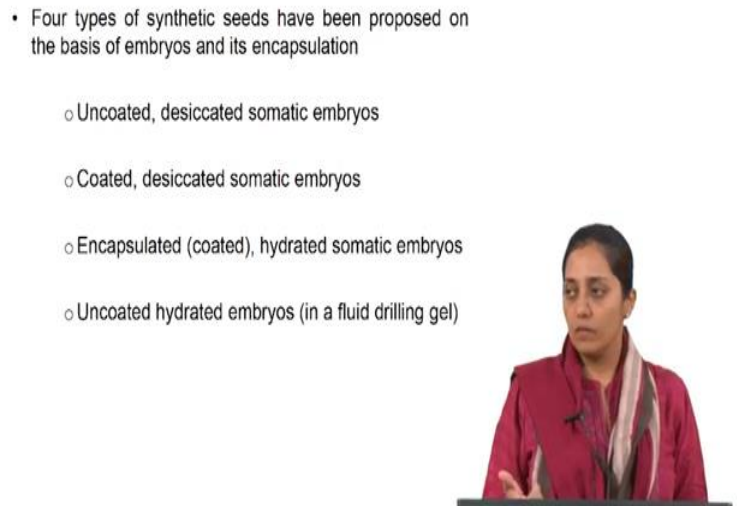
(Refer Slide Time: 00:16)

So, an extension of Somatic Embryogenesis, we will be talking about Synthetic Seed Technology. Now synthetic seed technology involves somatic embryogenesis, which is needed for large-scale propagation of plant material. Now, even somatic embryos can be used for clonal propagation of plants and they can be genetically stable, depending on whether they are indirectly produced or directly produced from the explant. Now large-scale propagation requires what? Planting material of suitable age, and of high germination rate.

The advantage of somatic embryogenesis is that, with the same genetic composition you can produce a large number of plantlets *in vitro* by scaling up somatic embryos. So, either you can have embryogenic cell lines; and then you scale up the embryonic cell culture; and then in a two stage cultivation, convert it into a somatic embryo form.

Now in synthetic seeds what you cannot directly use? *In vitro* conditions you can directly use the somatic embryos, then regenerate into plantlets but if you want to use it outside as seeds for planting material, then generally it is encapsulated.

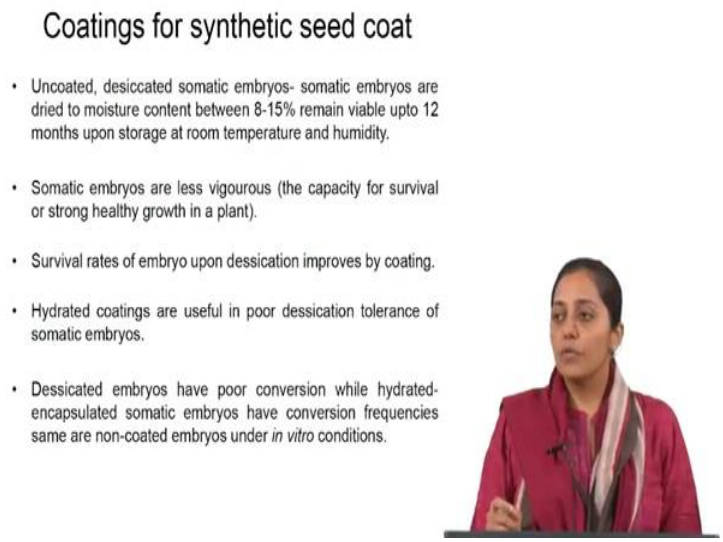
(Refer Slide Time: 01:50)



There are four ways in which synthetic seeds are produced; one is uncoated, desiccated somatic embryos . Then coated, desiccated somatic embryos. Now desiccated means what? Dried. Then encapsulated, hydrated somatic embryos which means, the encapsulation is a hydrogel or which can provide water content. Then uncoated hydrated embryos. Now, there are merits and demerits associated with all these four types depending on the hydration, dehydration, tolerance of the species of the somatic embryo; it can be decided whether to encapsulate it, not encapsulate it

So, you need to make sure whether the encapsulation will be a balance out between its vigorousness to convert, the time it takes to regenerate into plantlets and the protection from the environment, such that it does not become non viable.

(Refer Slide Time: 02:52)



So, let us talk about the coatings. Uncoated, desiccated somatic embryos what are they, somatic embryos are dried to reduce the moisture content up to 8 to 15 percent. So, the moisture content is reduced and it is observed that they can remain viable up to a period of 12 months; which means, they can be stored for a year long period. Now somatic embryos in comparison to seeds are less vigorous. So, the vigorousness means what ? The capacity of survival or remaining healthy in order to regenerate into a new plantlet.

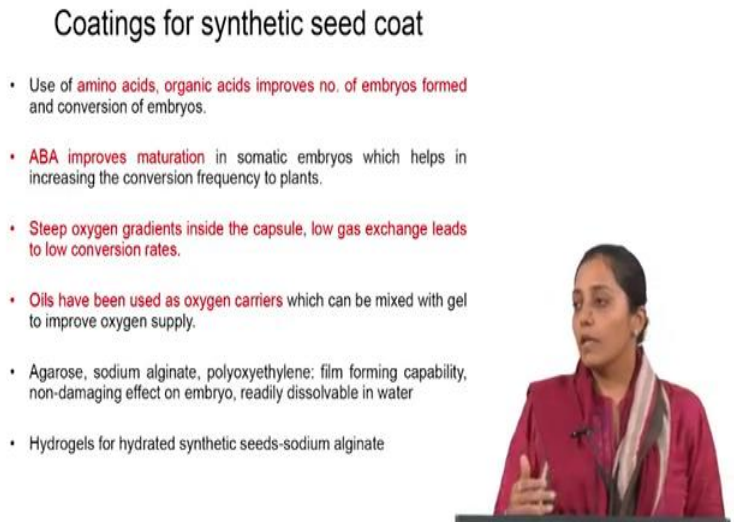
The survival rates of the embryos also depends upon the desiccation tolerance; which means, how much that species is tolerant towards dehydration or loss of water. Now in that case, if suppose a species or the somatic embryos is sensitive, then hydrated coatings are used. Hydrated coatings are generally for example, made of hydrogels; what are hydrogels ? For example, very commonly used, when you make your beads. What kind of beads do you make?

Student: Calcium alginate.

You use sodium alginate mixed with calcium chloride to make calcium alginate and which is a gel bead. So, hydrogel means what, hydrophilic. So, which means with a lot of water they can swell, because of the absorption of water. Now this water is nothing, but a dispersed phase in the cross linking matrix of the polymer and the cation which you are using. So, desiccated embryos have, please remember they have poor conversion while

hydrated encapsulated somatic embryos have higher conversion frequencies, sometimes similar to that of the uncoated somatic embryos.

(Refer Slide Time: 04:42)



So, what all can be used to generate more number of somatic embryos or to increase the vigorousness of the somatic embryos? People have used amino acids, organic acids. Organic acids like aspartic acid, malic acid; so in the form of salts potassium malate or potassium aspartate or succinate, so these are called as organic acids. So, supplementing the media with these organic acids has also helped in increasing the number of embryo generation and even the conversion of embryos.

Now abscisic acid, which is ABA; abscisic acid has been found to improve the development of embryos. There are four different stages, we learnt in somatic embryos. So, the rapidness through which the somatic embryo converts it into a cotyledonary stage; where root and shoot primordium is formed and which leads to regeneration of plantlets. Now what all other factors which can affect is, oxygen concentration; because if the seed becomes encapsulated, there is high chance that there will be mass transfer limitations; and which factor becomes mass transfer limited is oxygen, because oxygen is sparingly soluble.

So, steep oxygen gradients have been found to be limiting the somatic embryo regeneration. So, in that case as you use in fermentation technology, oxygen vectors are used; these are nothing but organic compounds like your, for example, you will hear

dodecane or these are nothing but hydrofluorocarbons. So, why are they used as oxygen vectors, even in fermentations you will find that oxygen vectors are added in the medium to improve the oxygen transfer capacity. Now how does it improve? Because these have higher solubility of oxygen in comparison to water; like in water how much is soluble at ambient temperature and pressure? Around 8 mg per liter.But for example, in dodecane it is around 55 mg per liter; then in hydrofluorocarbons it can go even up to 100 more than 100 mg per liter. So, depending on the size of these hydro fluorocarbons or hydrocarbons you disperse them into the aqueous solution, they will form a film around the gas bubble. So, what are all different phases are now involved; the gas bubble to this oxygen vector film, from the oxygen vector film to the aqueous layer, from the aqueous layer to the cell.

So, now what will improve, the driving force improves, isn't it; because there is higher oxygen transferred from the gas to the oxygen vector film, so thereby leading to better oxygen transfer rates. So, these are also sometimes preferred, but depending on what is the droplet size, how it is dispersed in the medium is also a critical thing. So, agarose, and other hydrogels materials are agarose, sodium alginate, polyoxyethylene. They form a film which has a non damaging effect on the embryo.

What are hydrogels? Water loving polymeric matrix. So, which absorb water, so water is the dispersed phase and these matrix, polymeric matrix which then swells up.

(Refer Slide Time: 08:30)

Now, let us talk about germplasm conservation; somatic embryogenesis or synthetic seeds can also be used for germplasm conservation. Generally, seeds are used for germplasm conservation, but there are demerits associated with it, we will talk about it. So, first thing what are the different ways in germplasm conservation is done; one is called as in-situ conservation, the other is ex-situ conservation. Now, in situ preservation of the plant material is using your biological national parks, or reserves, biosphere reserves you will find, then gene sanctuaries are where the original plant material in its natural habitat is maintained. Now there are limitations to this kind of conservation process, the limitations logically what is it?

Student: Space.

One space, but more than that, if we are talking about conservation what will be the impacting factor on this strategy.

Any geographical or an environmental calamity, so you cannot save it. Then ex situ preservation is what, that you provide suitable conditions in the gene bank to preserve this material; for example, you preserve them either in the form of seeds, synthetic seeds, or different forms of *in vitro* cultures like even shoots, meristems, or somatic embryos. But then conditions have to be provided; which means, the media, how do you cryopreserve it, or how do you preserve it matters. So, that the viability is not lost during the prolonged storage period, we will talk about that.

(Refer Slide Time: 10:05)

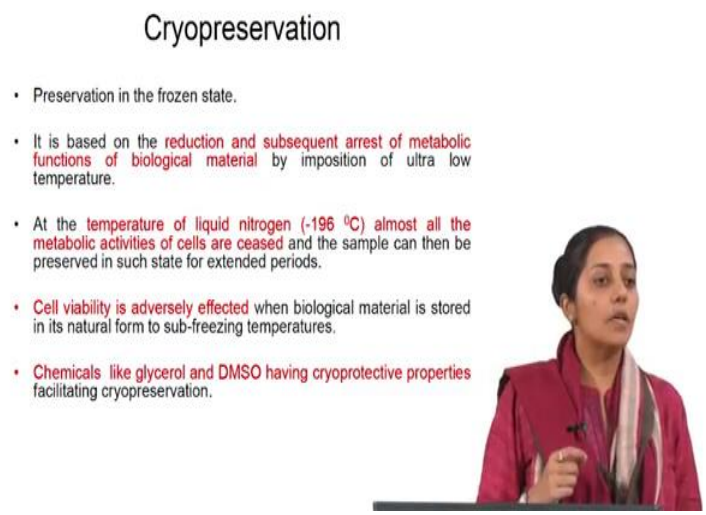
So, I said there is a demerit associated if you are conserving the genetic material in the form of seeds. The demerits are, it can lead to seed dormancy. For example, we are working with *Viola odorata* that Banafsha plant, if you remember for cyclotides production I was talking about and we found it very hard. We got the seeds from one batch, we got from UK and the other we were trying to procure from Australia; but we did our level best, but those seeds could not regenerate into plants and it was known that *Viola* suffers from this demerit, propagation of *Viola odorata* is not preferred through seeds, because of the seed dormancy problem.

So, it is generally propagated vegetatively through runners, which is a lower part of the stem. So, that is a problem, seed dormancy can be a problem, seed viability can be a problem; then seed destruction by endogenous pathogens can be a problem, so where you can lose the genetic material. Now, if you are using a seed for conservation, then it is confined only to plants which propagate through seeds.

Plants which do not propagate through seeds which are vegetatively propagated, then there has to be a way for conservation of such plant material. So, vegetatively propagating plants are generally conserved using *in vitro* conservation techniques, where I said you used different parts like shoots, meristem, somatic embryos kind of cultures for preservation.

Now the advantage is, that you can conserve as she was saying one of the demerits is that large space is required; but if you are using *in vitro* cultures in small amount of space you can conserve and store a large amount of material. Then, it overcomes the problems of climatic dependency or environmental dependency and provides large amount of material for culturing and for regeneration.

(Refer Slide Time: 12:14)



So, Cryopreservation; now once we are talking about conservation, for storage you need to store it under right conditions and properly, so that the material remains viable during the storage periods. So, for that cryopreservation techniques are important.

So, how do we cryopreserve plant materials or any kind of cells, cell cultures? Now cryopreservation is what? Preservation under subzero temperatures, freezing temperature, subfreezing I would say. So, generally you will find whether it is animal cells, plant cells, microbes, liquid nitrogen. So, for plant cell cultures, animal cell cultures the biological material is stored under liquid nitrogen or in the vapours of nitrogen.

Why are we storing the material at such low temperatures? Because it has been observed that temperatures in the liquid nitrogen which is so low, subzero, entire metabolic activity of the cell can be brought to stand still, can be arrested.

So, what is preferred, you gradually first reduce the metabolic activity and then bring it to stand still. Now then, what is the right way? You take the biological material and put it in liquid nitrogen and so, it can be stored. But, there is a demerit; This is not how it is stored, specially the cells where viability is important. Now why do you think, it is not stored as it is, bringing it in and storing it in liquid nitrogen; cell viability is adversely affected under subzero temperatures, the first question which arises is why?

Student: Water in the cell freezes

Very nice. So, there will be crystallization happening. So, what will happen, it will form crystals, the crystals will grow an in turn as the crystallization of water might happen, many things happen; one is that they can be of direct damage to the organelles, they can pierce the cell membrane, rupture the cell membranes, or the organelle membranes; thereby reducing the viability, what else ? There can be dehydration. What else dehydration in what different ways? Internal crystallization is more related to internal damage. So, how does the freezing begins? Suddenly everything all together, so outside there is water getting frozen, then the cell. So, what happens?

Student: There is a water.

There is a water yeah, there is a water loss causing dehydration and as you said some effects due to the internal water also freezing, not been available; so then that also causes dehydration and loss in viability. So, what is done? Cryoprotection is done, before cryopreservation. So, what is cryoprotection? We use cryoprotectant agents which can reduce the damage because of cryopreservation. Now what are cryoprotectants? Cryoprotectants, now in nature also plants must manage, because plants cannot move isn't it. So, extreme in cold countries, how do plants manage? What it is called as?

Student: cold acclimation

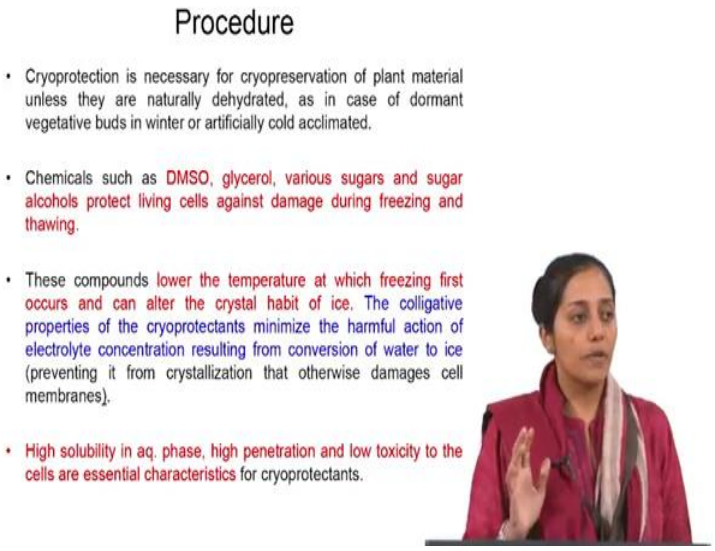
Very good that that process is called as cold acclimation. So, what does it mean? It means that entire genetic program is switched on, which leads to up regulation and /or down regulation of many proteins, and also which in turn leads to change in the metabolism, change in the growth, reducing the growth, balancing out the energy levels, the amount of PR proteins which means, specially antioxidants; then the cell membrane composition in terms of unsaturation of your lipid bilayer changes; then PR proteins antifreeze proteins are produced sugars, polyalcohols, polyols; then proteins which can act as or substances which can act as osmoticums, the cell starts producing.

So, similar thing we try to embibe *in vitro*. So, we add cryoprotectants which include glycerol, then you must have heard about the glycerol stocks; why do we prepare glycerol stocks? That is the very reason; the glycerol stock composition also vary, sometimes you will see in literature people use 45 percent glycerol stock, some people

use 20 percent glycerol stock depending on what species you are working; whether you want to cryopreserved bacterial cells, whether you want to cryopreserve fungal cultures, whether you want to cryopreserve plant cells; the glycerol percentage keeps varying.

So, these are sometimes DMSO. Now these are called as cryoprotectants depending on the different nature in which they are used; some are cell penetrating agents, some are cell dispersing agents which can easily get mixed with water and balance out the osmotic pressure.

(Refer Slide Time: 18:06)



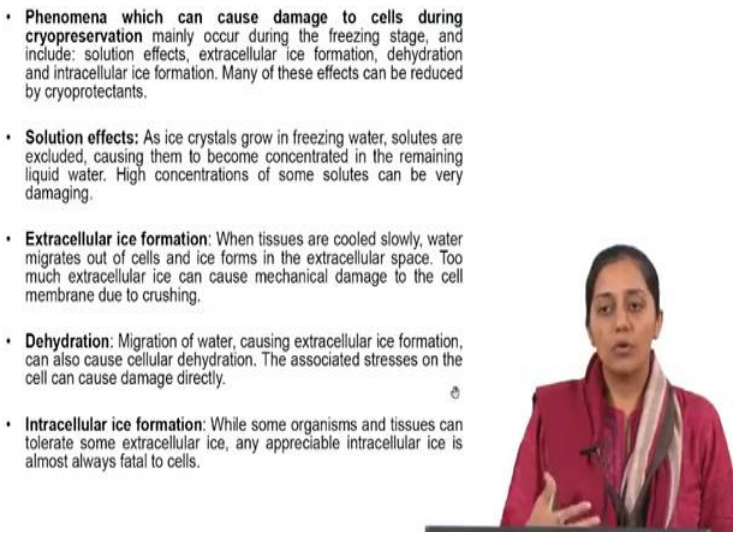
So, what is cryoprotection? So, I will repeat cryoprotection is necessary for cryopreservation of the plant material unless they are naturally dehydrated. So, generally when we say cold acclimation which plants use, it is nothing but a slow dehydration process; you will find that suddenly the spring comes and you will find new buds coming in, new leaves starts coming in.

So, how is it happening? Because these axillary buds or these buds which will regenerate into new plant material are cryopreserved or are cold acclimatized, so through this process which I was talking about. So, chemicals such as DMSO which we use and other chemicals *in vitro* conditions such as glycerols, various sugars, sugar alcohols they help by protecting the living cells against the damage under subfreezing conditions. Now the question arises, how? So, how do they protect?

These compounds they do what; you increase, you must have heard during your 10th, 12th standard, lowering of freezing temperatures. How does it happen? You increase the solute concentration; the freezing point reduces for the solution or the solvent. So, the same thing happens here, now you lower the freezing point of water by adding these certain agents, like sugars. So, it lowers the temperature at which the freezing would first occur and can alter the crystal structure of ice even. So, the colligative properties of the cryoprotectants minimizes the harmful effect by the crystallization of ice.

So, what all properties of cryoprotectants are essential? They have high solubility in aqueous phase; then they should be highly penetrating, so that they can reach inside the cell; then they should be low toxic, should not be very toxic to the cell. So, the toxicity has to be taken into account; which means, the amount of cryoprotectant you are using, the concentration it matters has to be optimized.

(Refer Slide Time: 20:17)



So, what are the different ways through which these cryoprotectants work? The phenomena which can cause damage to the cells during cryopreservation; so what is the phenomena? Mainly occurs during the freezing stage and includes solution effects, extracellular ice formation, dehydration, and intracellular ice formation. So, what do we mean by all these four things, what is solution effect; an ice crystal which grows in freezing water, solutes are exuded, causing them to become concentrated in the liquid

water. Once as you people said the ice is forming, so rest of the cytoplasm is getting concentrated.

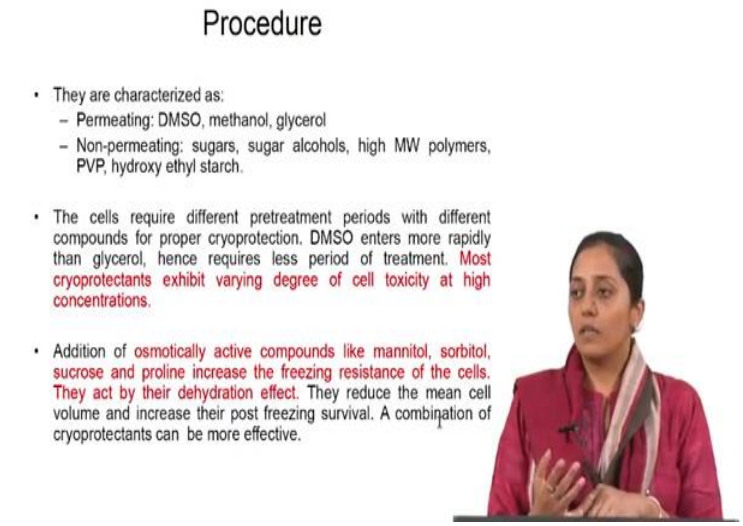
So, this high concentration of some of these solutes can be very damaging. Now extracellular ice formation how is it happening? When tissues are cooled slowly, water migrates out of the cell, we know because of the osmosis; now as the water migrates what it will cause?

Student: Dehydration.

Dehydration. Now when tissues are cold slowly water migrates out of the cell and the ice forms in the extracellular space. So, sometimes the ice which is forming in the extracellular space will also cause damage by pressing the cell wall and the cell membrane. So, generally you will find that when extracellular ice formation starts taking place, the intercellular spaces they get widened; there is cell to cell contact. So, now, you will find there is more gap and the water there gets crystallized and the cell walls and the cell membranes may get damaged because of the crystal formation outside.

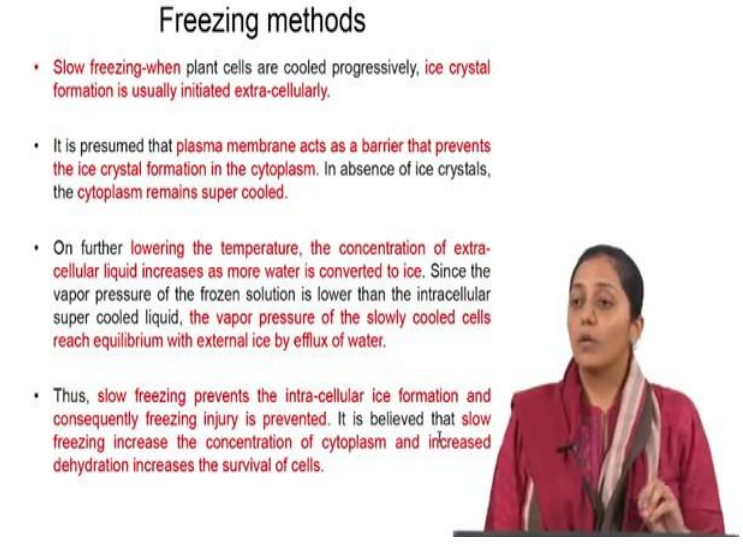
Now, dehydration, now you understand how dehydration would occur; migration of the water causing extracellular ice formation, can also cause cellular damage, because inside now things are more concentrated. So, everything is interrelated even the solution effects, the extracellular ice formation everything is happening simultaneously. So, then intracellular ice formation; if suppose intracellular ice formation happens, then the crystal is growing, it can directly cause damage to the intracellular organelles. So, if I ask you in what different ways the cell damage, or subzero freezing, or cryopreservation may damage the viability of the cell, you should be able to tell.

(Refer Slide Time: 22:50)



So, what is the procedure? The different cryoprotectants which are used are one is called as permeating cryoprotectant; for example, DMSO, methanol, glycerol there are highly soluble in water. Then non-permeating can be: sugars, sugar alcohol, high polymeric substances, molecular weight polymers. Now you can use the best strategy is to use a combination of these cryoprotectants, because the mode of action is different; some are cell permeating, some can cause reduce the dehydration process by reducing the osmotic pressure, so can have osmotic effect. So, better the proposed solution is, you use a mixture of these cryoprotectants.

(Refer Slide Time: 23:38)



So, what is the general protocol for freezing? When you are freezing, it is recommended to do slow freezing not sudden; it is not recommended to bring the biological material dip it in your cryoprotectant and suddenly you put it in -196 °C. So, that adaptation is what, when you are doing a slow freezing versus very fast freezing. If I give you a hint, then when you do slow cooling it will give the cell the time to equilibrate; this equilibrium, vapour pressure equilibrium will in turn facilitate the internal water not to freeze, but remain at subcool temperatures. The idea is, to prevent internal crystallization. So, how does it happen, once you lower the temperature the concentration of the extracellular liquid increases, as more water is converted to ice.

Now, since the vapour pressure of the frozen solution is lower than the intracellular supercooled liquid, the vapour pressure of the slowly cooled cells reaches equilibrium with external ice by effluxing water. So, you are giving time for the cells to adapt. So, thus slow freezing can prevent the intracellular ice formation and consequently the freezing injury by the crystal formation; and then subsequently the crystal growing inside the cell.

So, in order to avoid this crystallization and recrystallization, so we try to thaw it. Which means what are you doing, to improve the heat transfer rate there has to be a bigger driving force. So, you will be suggested to put it in a water bath, 35 to 40 °C or you thaw using your thumb from subzero which is -196 °C to, what is the palm temperature approximately.

Student: 37 °C

So, what is recommended to prevent this recrystallization of ice ? So, generally for plant cells what do we do is at a constant rate of 0.5 to 2 °C it is slowly brought down to some intermediate temperature before it is immersed in the liquid nitrogen, and then rapid thawing is recommended.

(Refer Slide Time: 26:25)

So, the applications of this preservation or conservation is that you can have clonal propagation or same genetic material propagated later and you can conserve the genetic material. Preservation of rare genomes can be done using this method and free storage of cell cultures or even cell lines, for even secondary metabolite production or any other commercial application can be done.