Plant Cell Bioprocessing
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Lecture - 07
Nutritional requirements of plant cells

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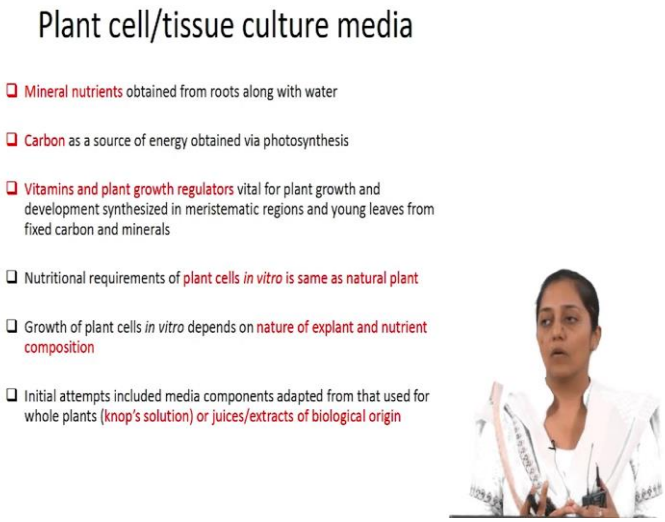

We all know that any kind of living cells would be needing a carbon source and mineral nutrients. The whole plants take their mineral nutrients and water from the soil, but how is it different in plant cell and tissue cultures? Generally, in whole plants, vitamins and plant growth regulators are synthesized at the meristematic regions or in young plants. Probably the reason is because those are the sides where they are most needed and then after the production they can be transported for various other developmental reasons to matured parts of the plant. So, vitamins and plant growth regulators as I said are synthesized in the meristematic region.

Apart from mineral nutrients and carbon, plant growth regulators are of two kinds. One are endogenous which means the cells themselves produce these plant growth regulators. So, there they are called as plant hormones and if they are synthetically produced or they are exogenously being present as signaling molecules for example, which are then inducing the endogenous plant hormones, they are called as plant growth regulators. The initial attempts which were made in media formulations in plant cell and tissue culture

included application of knop solution. Now, knop solution is a very old media which is majorly composed of major salts. Have you heard about, what kind of salts come under major class of salts? Have you heard of micro nutrients?

And then major nutrients. So, any examples for major nutrients?

Nitrogen, carbon, phosphorous, sulfur, magnesium, sodium, calcium and micro nutrients for example, boron and ?

Student: Manganese.

Manganese.

Student: Cobalt.

Iron.

Student: Copper.

Copper. So, knop solution was initially composed only of major salt solutions. When the initial preparations of plant tissue culture media were being discovered or made, knop solution was used or whole plant extracts or coconut water or juices from the plants or any other biological origin was used.

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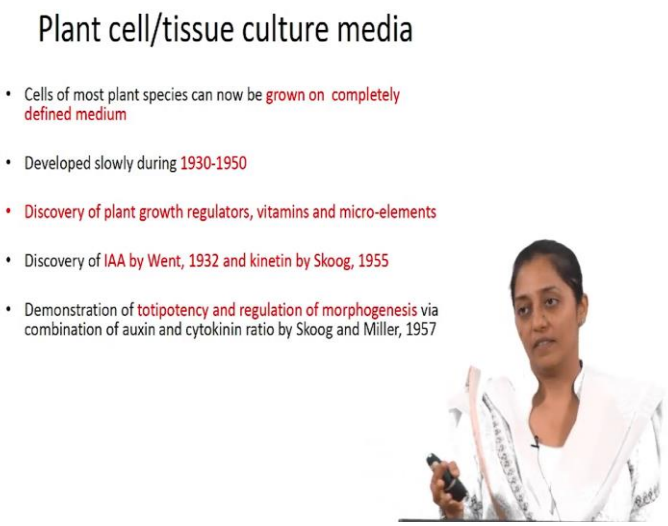

The major formulations of these synthetic media started with non-defined media and now we are at a stage where media is completely defined. Why do you think there is a need? Anyways you see that living organisms grow so well in complex media and coconut milk was being used very nicely. Juices and extracts were found to be working well, but in 1930s to 50s there was a shift and now we are at a stage where you can find readymade media, but they are completely defined media.

Why do you think there was a need to shift from a complex to a well defined media? Batch to batch variation can be there, what else? Any other logical reason? Depending on how much impurities you get, but what other logical reason do you think people might have? Do you think downstream processing time would have been the reason? What did I say in the earlier classes that even though these complex media was found to be working well, but everything is in turn is dependent on so many different factors such as the species, the type of explant, the genetic makeup and nothing is full proof.

So, depending on what culture do you want to induce , the media composition has to be varied and depending on the objective of the study there could be a better formulation of the media. So, until and unless you have the clarity of the medium composition and how are these each factors affecting your objective, it will always be a hit and trail. So, gradually depending on the purpose of the study these different media formulations came into the picture. So, starting from knops, then you got Miller's media. Then what else? Murashige and Skoog, B5, but there has been a difference between them. For example, in Murashige and Skoog and between Gamborg's medium which is also called B5 medium there is one major difference.

The ratio between the ammonia to nitrate, in B5 medium is lower than that of MS medium. Now, why this difference is important for example, as I said it is species specific and the kind of culture you want to induce. For example, when we were working with Azadirachtin from neem which is a woody plant species, we were finding it very difficult to induce *in vitro* cultures. MS media is generally used when you want to do shoot bud formation or shoot multiplication on *in vitro* cultures. MS was not working but B5 started working. So, when we started doing and that I will come back to when we will talk about media optimization.

When we did statistical optimization which has the advantage of studying the interactive effects and even studying the single effects, we could find that if you remove ammonia from the medium and have only nitrate as nitrogen source it is very useful or 100 percent efficiency for callus induction in *Azadirachta*. So, it is sometimes explants specific, species specific. So, B5 was then used rather than MS media because, it has lower ammonia to nitrate ratio.

Then the initial discovery which was remarkable and which was also very successful was the discovery of auxins. The first auxin which was discovered was IAA. IAA in plant is synthesized through tryptophan biosynthesis pathway. So, then synthetically also IAA was subsequently produced. Then the discovery came with cytokinins, now again in cytokinins also there are synthetic cytokinins and which are endogenously present, naturally present cytokinins. Also, please note that exogenous additions of these plant hormones, even if they are present endogenously or not present endogenously they also effect the sequestration, the production and the conjugation of these endogenously present hormones or the production within the cell. So, it has to be optimized, then came the discovery of totipotency and regulation of morphogenesis.

Now, first came the callus induction, then there was a need to find a media which could induce root, then the objective became regeneration or shoot multiplication and micropropagation.

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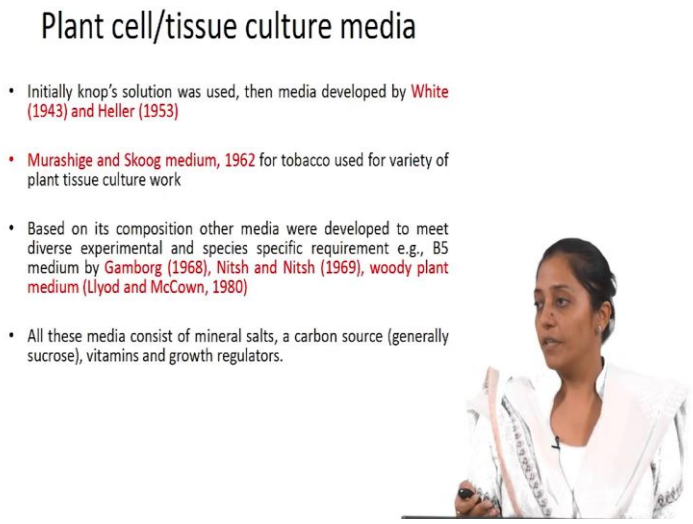

So, gradually with different objectives in mind different media formulations came into the picture, but I must say that Murashige and Skoog medium which came in 1962 till date is one of the most successful media. Its formulations are able to give results in almost all the species. But, still that does not mean it will always work, one has to optimize the composition of the media then as well as the growth hormones. So, rest of the factors have to be optimized and even the environmental conditions play a role.

So, these are some of the examples of different media which are now available in the market Gamborg's I said is B5, it contains B vitamins. So, then that was another discovery, when vitamin addition in the formulated media was also discovered where 3 major vitamins : pyridoxine, thiamine were added along with your vitamin B5.

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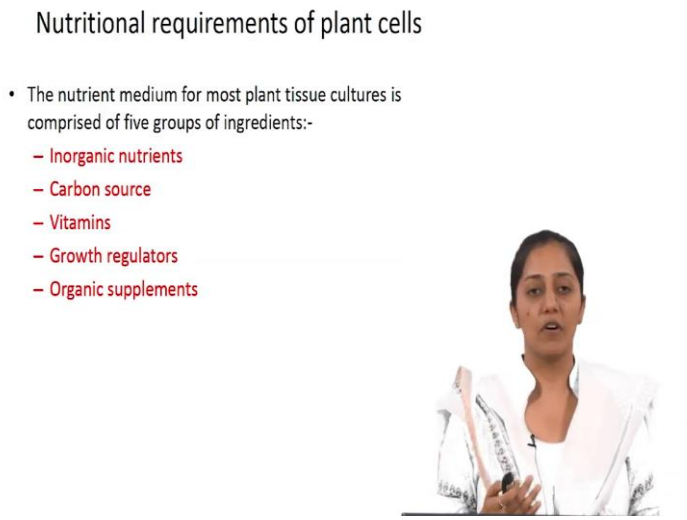

So, the nutrient media is composed of these different things, one is inorganic nutrients, then carbon sources, vitamins, growth regulators and organic supplements. Organic supplements here any idea? Complex nitrogen sources or like for example, organic nitrogen sources, any organic nitrogen source which is well known and well used in microbial fermentations? Corn steep liquor. What else? Amino acids are also added; yeast extract, peptone, malt extract.

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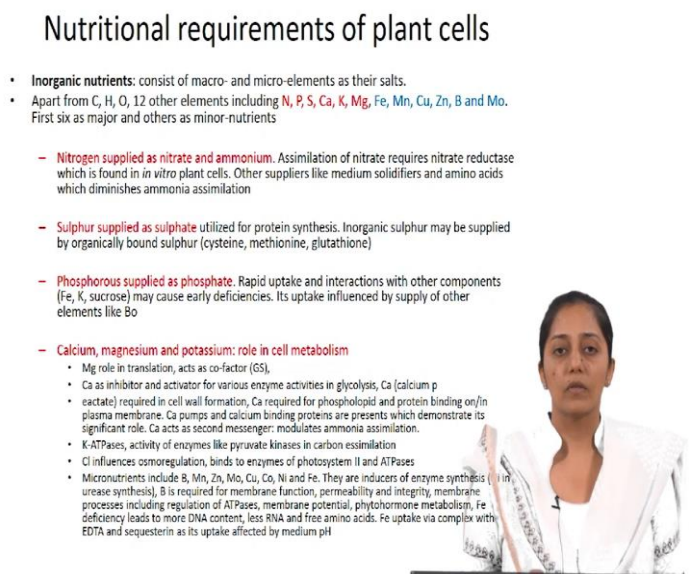

So, coming on to the inorganic nutrients, now inorganic nutrients as I said apart from carbon, hydrogen, oxygen there are 12 other elements. Now, what are those elements? The major elements include nitrogen, phosphorous, sulfur, calcium, potassium and magnesium. The micronutrients generally added include iron, manganese, copper, zinc, boron and molybdenum. So now, let us talk about the nitrogen which is supplied. In whole plants in nature, nitrogen is assimilated in the form of nitrate or ammonium ions. There are ammonia transporters and for nitrate there are many nitrate transporters.

So, inorganic salts of nitrates are assimilated by the plant mixed in the xylem and the other through minerals. They come to the plant either in the root or in the shoot itself. It is then converted into nitrate and then from nitrate it is converted into ammonium and assimilated in the form of amino acids and other nitrogen compounds. So, which pathway have you heard which is known in plants for assimilation of nitrates, where nitrate reductase is involved? What is that pathway called? Gogad pathway, have you not heard; so, please go back and read. It is about how nitrogen is fixed for by the plant and majorly nitrogen is in the form of nitrate.

So, it is an energy driving process and so it requires energy. So, active transport of nitrate happens and sulfur is supplied as sulphates. Then it is utilized where all? In protein synthesis, inorganic sulfur maybe supplied by organically bound sulfur. The organically bound sulfur is present in the form of cysteine.

So, phosphorous is a major nutrient. Then calcium, magnesium and potassium play a role in cell metabolism. Let us see for example, magnesium; magnesium plays a role in translation, it acts as a cofactor. Then calcium, calcium it acts as an activator and inhibitor in various enzyme activities in glycolysis. Then it is required in cell wall formation as calcium pectate. So, calcium is an important nutrient, then calcium pumps and calcium binding proteins which are present they demonstrate that it has a significant role as in the cell wall formation as well. Now, calcium is also used as a secondary messenger.

So, primary messengers are the signal molecules outside the cells and secondary messengers are in response to the primary messengers and there is a signal cascade inside the cell which then regulates the metabolism of the cell. So, calcium is one of the secondary messengers in the cells. Then micronutrients for example, your boron, manganese or copper or iron they are inducers of enzyme synthesis. Now, boron is used for membrane function and it also affects the sequestration of other elements as well. So therefore, this is also one of the crucial elements which might have come into the picture while formulating media.

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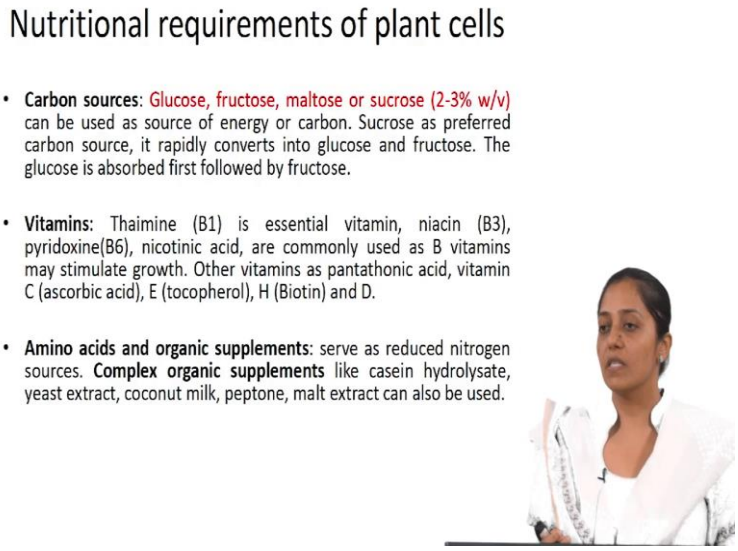

Now, carbon sources, it is again very species specific; as I said generally sucrose is used. Now coming to the question, why do you think generally sucrose is used? Ultimately, it has to be broken down to the most easily metabolizable sugar which is glucose. So,

sucrose is less costly, there are sucrose transporters present in the cell wall and also there is invertase enzyme. So, it is not that all the sucrose in the media initially at one time itself gets broken down, this is a simultaneous phenomena. Sucrose is been taken in and it is also being divided or broken down into glucose and fructose.

And, separately glucose and fructose are also being taken in and it is then diverted towards glycolysis . Sucrose also has sucrose metabolism in the cell. So, sucrose is also taken up by the cells. So, if there is only glucose present, the growth can be faster because it is an easily metabolizable sugar readily available to the cell and can be taken up. But, one is the cost and the second is the not all glucose is needed at one given time. So, slow assimilation is also one of the reasons. Now, vitamins; vitamins for example, thiamine is essential vitamin, then vitamin B3, pyridoxine.

So, as I said your vitamins which are included in the media they include pyridoxine, nicotinic acid, niacin and your thiamine which is present in these media which has called as B vitamins to induce the growth. Other vitamins which might be added depending on the species or the objective are vitamin C, D and E. So, amino acids and organic supplements serve as reduced nitrogen and complex organic supplements like casein hydrolysate, then yeast extract, coconut milk are till date being used as complex nitrogen sources to reduce; the why are they still being preferred despite the fact we were saying that it can lead to batch to batch variation this and that?

To see if you can control the batch to batch variation, these things can bring down the cost of the production media because, many of these are obtained as?

Student: Waste by products.

Waste by products, by products which are in the waste of the industries.

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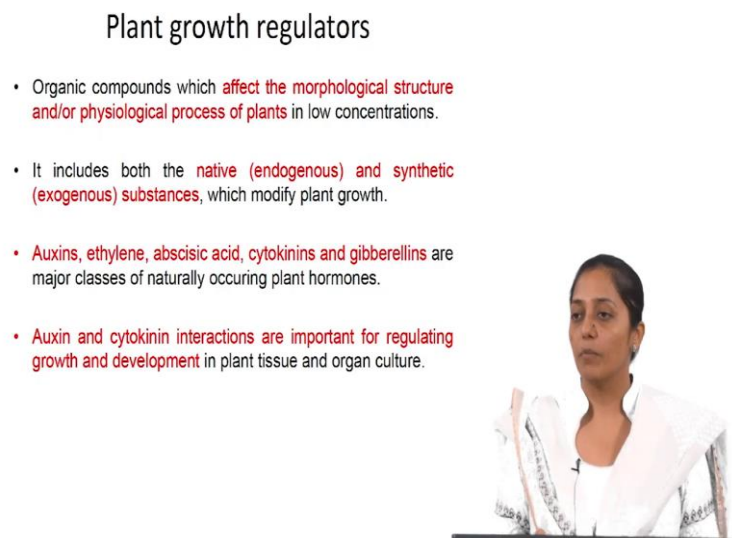

So, now let us talk about plant growth regulators, organic compounds which affect the morphological structure and physiological process in plants in very low concentrations; they form the plant growth regulators. So, as I said these plant growth regulators can be native, can be synthetic which means exogenously added and which can modify the plant growth. When they are native or endogenous they are called as plant hormones and rest are also called as growth regulators. Now, as plant growth regulators some of these signaling molecules are also termed as plant growth regulators. Which is one of the hormones which is volatile agent ? Any hormones which you know is a volatile agent and forms a part of plant signaling?

Student: Ethylene.

Ethylene, very nice even these jasmonates and when salts of jasmonate are exogenously added like methyl jasmonate or jasmonic acid, they are a part of the signal cascade inside the cell as well. So, auxins, ethylene, abscisic acid, cytokinins and gibberellins these are major class of plant growth regulators or hormones. Then because, these are naturally present, the auxins and cytokinins interact and they are important for regulating growth and development of the plant.

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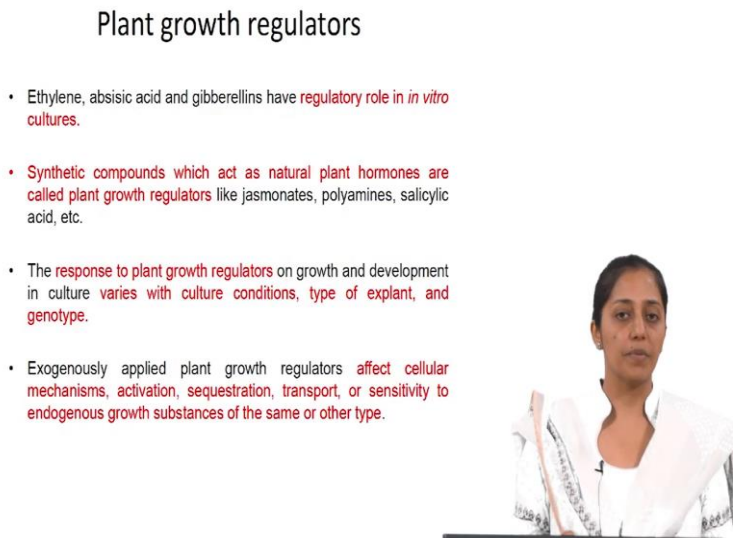

Now, ethylene, abscisic acid and gibberellins , generally form the regulatory role in plant metabolism or in the *in vitro* cultures. Now, synthetic compounds which are not natural plant hormones as I said they are called as plant growth regulators. Although, it is mentioned here jasmonates, polyamines and salicylic acid, now salicylic acid is also produced as a secondary metabolite in many plants. But, when exogenously added it forms a part of the signal cascade agent and also jasmonate salts they then are termed as plant growth regulators because they are being exogenously added. Otherwise jasmonic acid is a part of endogenous signal cascade mechanism in secondary metabolism.

So, the response to plant growth regulators on growth and development in culture varies with culture conditions, type of explants and genotype. Now, exogenously applied plant growth regulators affect cellular mechanism and as I said also the sequestration, activation, transport and sensitivity even towards the endogenous growth substance of the same kind or even of the different kind.

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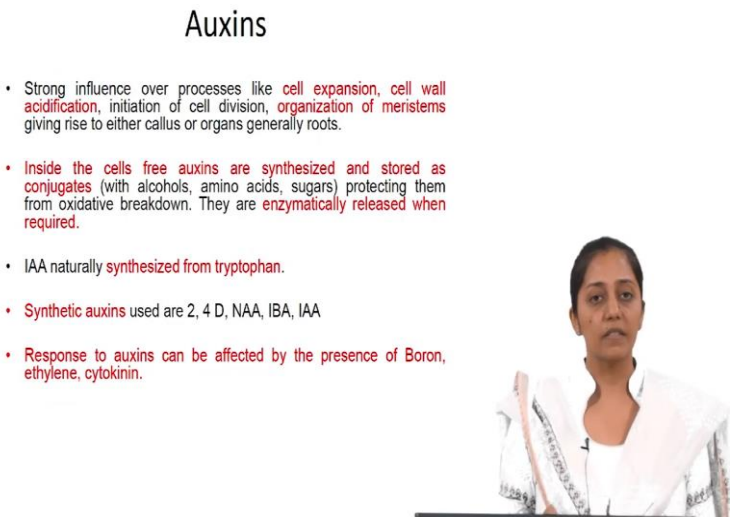

So, auxins they strongly influence processes like cell expansion and cell wall acidification. Now, it is said that auxins are useful in the growth or extension of the plant roots and shoots length by the mechanism of cell elongation and cell wall acidification. So, now how does it happen? Auxins they not only help in expression or controlling the expression of certain proteins in the cell wall which are called as extensins which loosen the cell wall but also prepare extra cell wall material. The cell wall acidification is another mechanism where auxins are involved in which they induce H+ ion concentration in the cell wall region. Because, of this what happens is that the H+ ion concentration has increased, the water gushes inside causing a turgor pressure. The vacuole gets extended, forces itself against the cell wall. The cell wall has been loosened by the effect of auxin by having the expression of extensins which then loosens the cell wall and therefore, the cell wall is now extended and expanded. So, this is how the cell increases in size, in length , elongates and cell wall material is formed and the length of the plant or that tissue is extended.

Inside the cells, the free auxins are synthesized and stored as conjugates. There is a purpose why auxins are stored as conjugates. This is because, they can be used later and otherwise free auxins can get broken down. So, in order to prevent their break down they are stored as conjugates with amino acids or alcohols or sugars thereby, protecting them from the oxidative breakdown. And, whenever needed via an enzymatic process these

auxins are set free in the cytoplasm or in the cell. Now, IAA as I said is a naturally occurring auxin which is synthesized from tryptophan bio synthesizes pathway.

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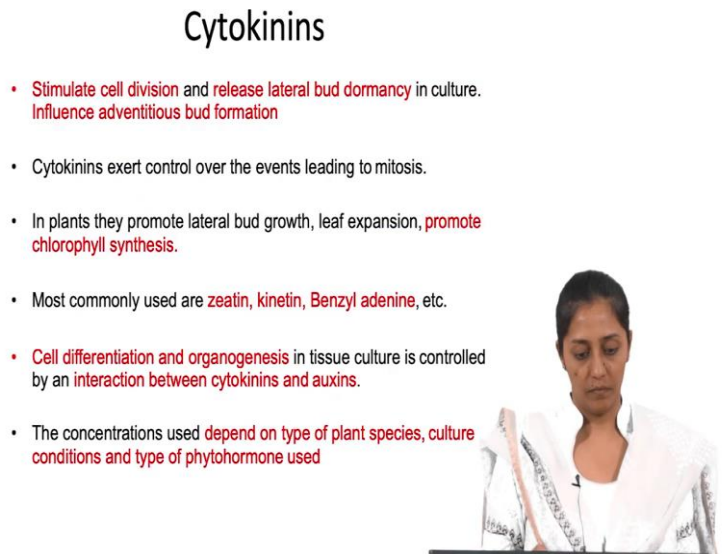

Now, cytokinin; cytokinin induces cell division by accelerating or inducing mitosis. It regulates the activity of a protein which then activates mitosis in the cells thereby enhancing cell division. So therefore, if you think go back and think why a callus induction is an event which happens, because of the combination of cytokinin and auxin it looks logical. Because, there is cell elongation, cell expansion and cell division which is happening and also in *in vitro* plant tissue culture. Higher concentrations of cytokinins can even lead to release of lateral bud dormancies which means branching can happen then.

So, cytokinins exert control over events leading to mitosis and they can also in some species can even promote chlorophyll biosynthesis. So, most commonly used cytokinins are zeatin, kinetin, benzyl adenine. So, you will find majorly benzyl adenine being used, even your thidiazuron is also used. So, cell differentiation and organogenesis in tissue culture is controlled by interaction between cytokinins and auxins.

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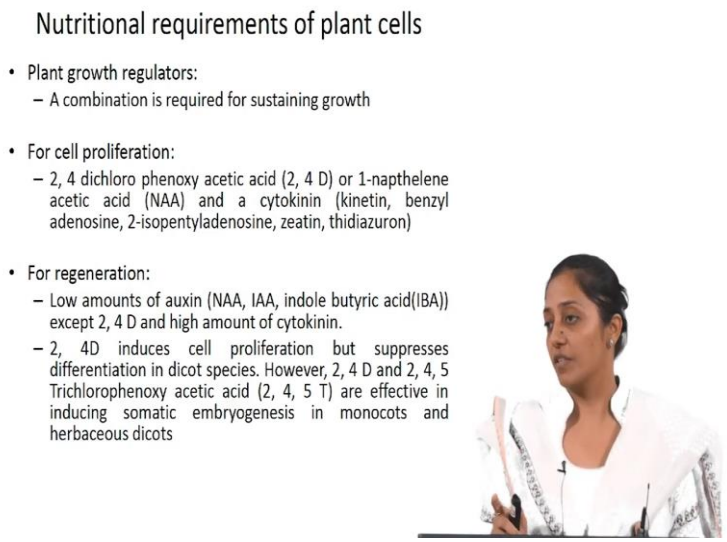

So, now some general guidelines, but let me tell you these general guidelines are general because for most of the species they have found to work. But, still it does not mean depending on what species are you are working; you can take it as a guideline, but it is not necessary that the same concentration or the same hormone would work in that particular species. So, optimization is still essential.

So, what are these general guidelines? Plant growth regulators, a combination is required for sustaining the growth of *in vitro* cultures. Now when you need cell proliferation which means cell multiplication to happen auxins like 2, 4 dichloro phenoxy acetic acid which we know as 2, 4 D or 1-napthelene acetic acid which is NAA or a cytokinin like kinetin, benzyl adenosine or thidiazuron is used. Now for regeneration which needs organogenesis to happen. So, for regeneration what is in general found to work is you reduce the concentration of auxins and increase the concentration of cytokinins.

So, generally it is seen that once you reduce the concentration of auxin to cytokinin which is a ratio of auxin and cytokinin , it leads to organogenetic events. 2, 4 D in dicots induces cell proliferation, but suppresses differentiation while, in monocots and in herbaceous species it has been found that high concentrations of 2, 4 D can lead to morphogenesis or organogenesis thereby, leading to even somatic embryo formations.

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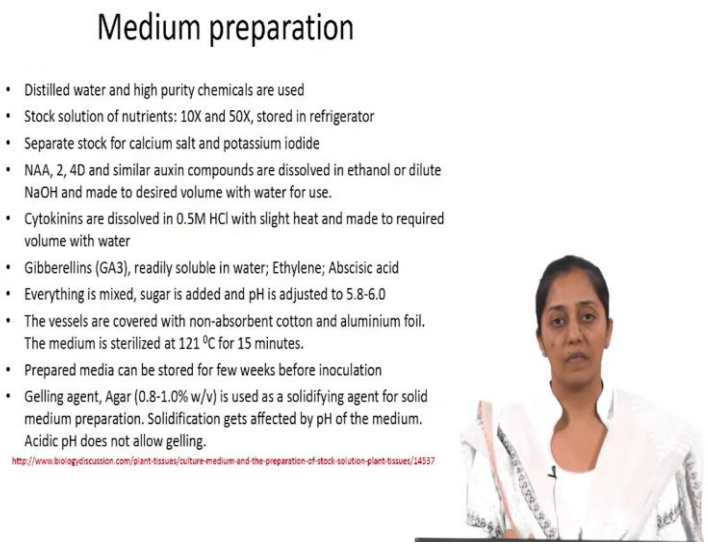

So, how is the media prepared? These are generally recommended guidelines. You use 10X to 50X stock solutions of your major salts, micro salts are autoclaved separately and including iron is in the form of stock autoclaved separately. They are mixed at diluted concentrations depending on your media formulation. Since some of these vitamins are found to be heat sensitive, heat labile. So, depending on what vitamins you are using care should be taken whether you can afford to add them before autoclaving or they should be added and filter sterilized after autoclaving the major and micro nutrients.

Now, in general, the plants grow well in acidic pH which is nearly acidic in the sense slightly acidic pH which is around 5.8 to 6. So, the initial pH of the media is then maintained after autoclaving because, during autoclaving the pH can get disturbed. So, after autoclaving the pH should be ideally maintained to 5.8 to 6 and then gelling agents are added, if you want to work with solidified medium; acidic pH would prevent gelling. So, the concentrations in general preferred are 0.8 to 1 percent weight by volume of agar or any gelatinous substance.