Plant Cell Bioprocessing
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Lecture - 18
Optimization Strategies-Part 3

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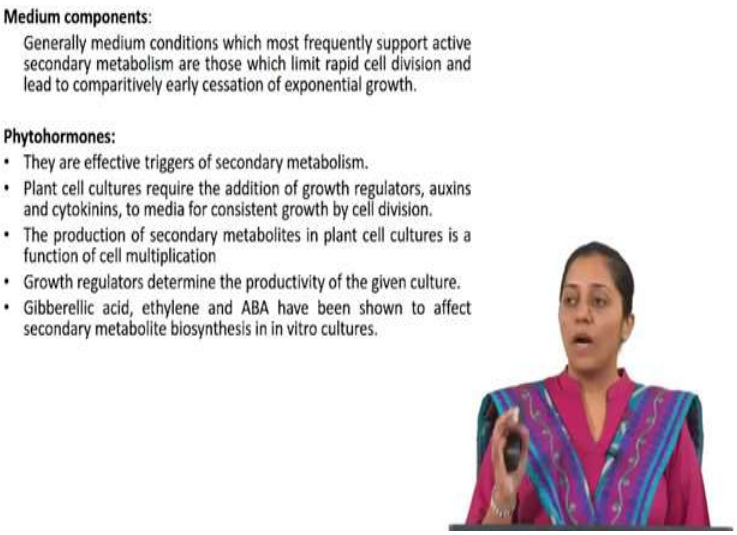

We were talking about pH, effect of inoculum, then we spoke about medium composition. Now, we all know what are the medium nutrients which are required for plant cells, we have done it in the earlier classes. Then similarly we have learnt about different phytohormones which are required for *in vitro* cultures. Now for example, your cytokinins and your auxins - they are generally used for plant cell cultures because they help in cell division and cell elongation.

So, this in turn is related to secondary metabolite biosynthesis. So, therefore its optimization might be necessary under *in vitro* conditions. Other hormones or regulators like ethylene or gibberellic acid or abscisic acid; these are other growth regulators which may play a role in signal transduction; which is related to in turn to secondary metabolism induction or they are directly being inducing secondary metabolite biosynthesis.

So, therefore, exogenous addition of such hormones can be connected to yield enhancements of secondary metabolites in plant cells. When I say yield enhancement, I mean $Y_{P/X}$.

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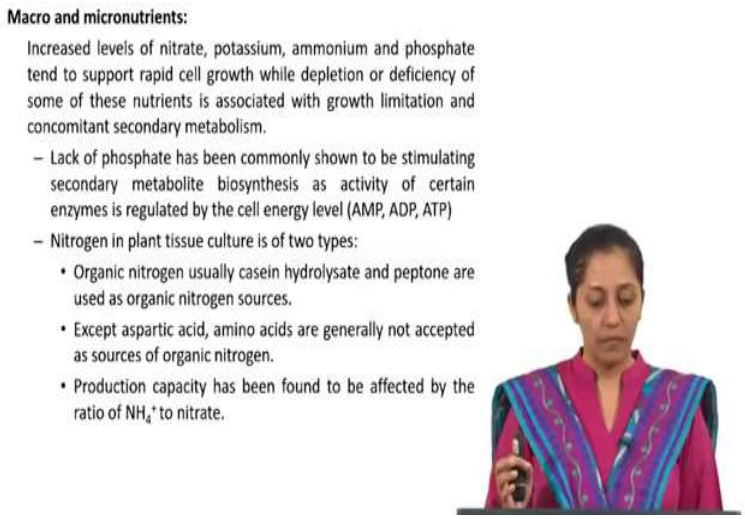

So, macro and micronutrients - you people already know. Nitrogen generally in the form of organic sources - casein hydrolysate or peptone can be used to reduce the production cost. But there one has to judiciously see or select that batch to batch variation should be avoided in the media or in the result because of the variation in the concentrations. Further, amino acids generally have nitrogen source. Only aspartic acid is not used. Moreover it enhances the cost of production; so, that has to be taken into account.

Then ammonium to nitrate ratio is found to be crucial in plant cells. If you remember I gave you an example, in *in vitro* cells like for example, azadirachtin production. If you see literature when using *Azadirachta indica* plant cells, it was observed that when ammonium to nitrate ratio is minimized, where even completely taking away ammonium as nitrogen source and only providing nitrate as nitrogen source, could enhance the azadirachtin biosynthesis.

So, similarly this gives an indication that the kind of salts or sources which you are using as nitrogen source will also impact your secondary metabolite biosynthesis; apart from the concentration optimization.

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Now, talking about carbon. If it is a photoautotrophic culture, then you need to provide light and CO_2 is used as carbon source. But if it is a heterotrophic culture, then we utilize carbon sources like sucrose, glucose.Generally, sucrose is preferred because sucrose is an economical carbon source.

So, now if suppose your secondary metabolite is found to be connected with chlorophyll biosynthesis, then you would like to have green cultures. So, for more chloroplast production you might be needing to incident light on the cultures. So, then gradually the cultures, they will become mixotrophic because you are also providing a carbon source and there is light in the culture; so, these cultures are called as mixotrophic cultures.

So, the possible mode of actions of sucrose which are known in literature on cell cultures - includes inhibition of endogenous auxin biosynthesis. By manipulating sucrose concentrations, this may indirectly impact the endogenous auxin levels also. The influence on differentiation characterized by increased activity of the enzymes in the pentose phosphate pathway.

So, when I say these as examples to you people; you one cannot generalize; it depends on the species and the kind of secondary metabolite you are working with that is why one has to take cues from literature and then optimize according to the type of culture, type of plant species and the class of secondary metabolites which you are interested in.

So, then we spoke about statistical media optimization; I will just quickly go through.

So, in conventional fermentations we optimize using single factor. The rest of the components are kept constant and one component is varied in a range and you see the effect on the response. Now, this approach is time consuming. It assumes that the process variables do not interact and the process response is a function of single parameter which is varying in a range.

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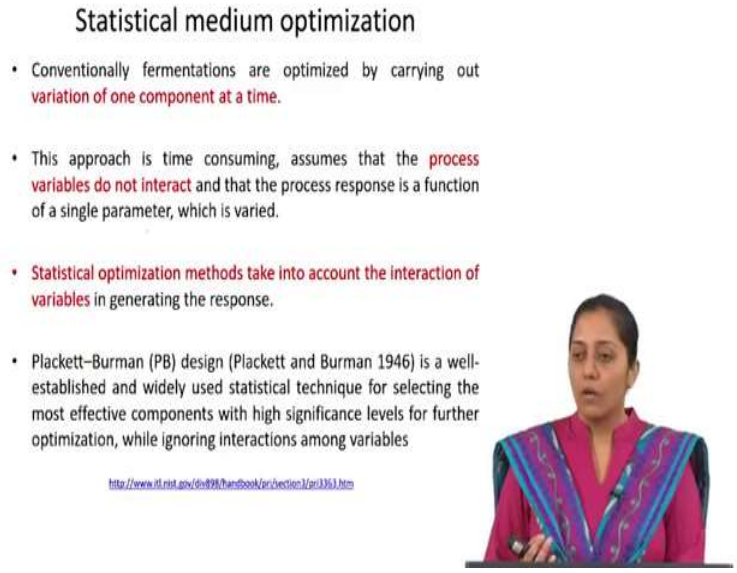

Now, statistical optimization methods, they take into account the interaction of the variables as we know in generating the response. Now Plackett Burman design is a well-established and widely used statistical technique. It is called as a screening design; which is a two level design in which every factor is varied at a at a minimum level and at a higher range which is called as - and + range and we ignore however, the interaction among the variables in this design.

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Now, as I said it is two factorial design. What do you mean by two factorial design? A series of runs in which combination of two factor levels are included and offers the screening of a large number.

So, the advantage of Plackett Burman is that if you have a large set of parameters; suppose 10, 12, 15, 16; then if you have N parameters you can carry out the design and the analysis with N + 1 experiments only.

The limitation - it does not describe the interaction among factors and is used only to screen and evaluate important factors influencing the response. So, which means it will help you in ranking the parameters. It is a resolution III design. What does it mean? It confounds main effects with two factor interactions.

So, which means that as I was talking yesterday that it may confound the effect of two factor interactions. If you are taking two factors, then the interactive effect might be confounded by the main effect of any one of those. So, Plackett Burman, works on two assumptions: one is called as factor sparsity and the other is called as effect heredity.

Factor sparsity means the factors which you have chosen, it assumes that they are independent parameters. The second is that for any interaction to be significant on the response at least one of the main factors should be significant; So, based on this it fits the response and the

concentrations of the factor values in a linear model which means linear means taking into account only the main effects.

So, then coming on to optimization tools after screening designs; the most widely used tool is response surface methodology. Now, when I am talking about Plackett Burman and response surface methodology, it does not mean that these are the only tools available in design of experiments. There are many other design of experiments which can be used.

But depending on the assumptions which you can take and what factors you would like to rank, depending on whether the assumptions of effect heredity or factor sparsity can really practically hold through; you must adopt or adapt any kind of such tools. You must look into what assumptions are behind that model or behind that tool and then accordingly adapt that tool.

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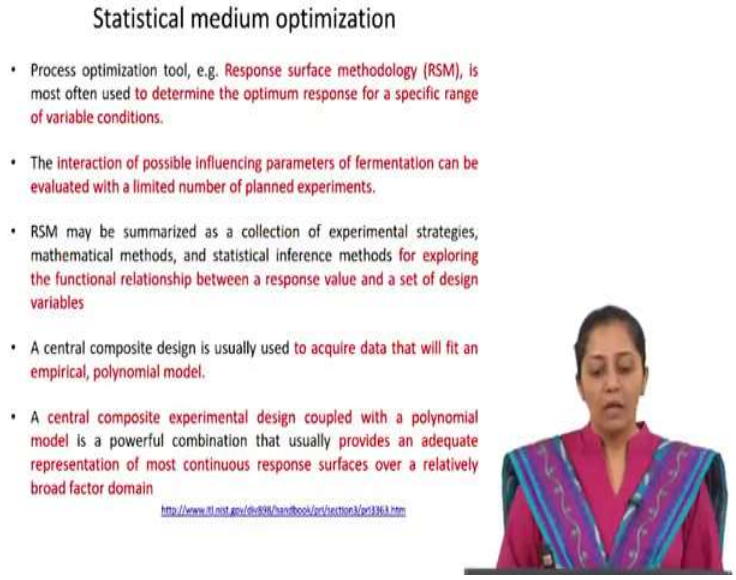

Now, response surface methodology is a process optimization tool; it is most often used to determine the optimum response for a specific range of variables.

Now, the interaction of possible influencing parameters can be evaluated in a limited number of planned experiments. So, yesterday I was talking to you that every factor, if it is varied in a range; there can be N number of permutations and combinations. Even between 1 and 2; there can be 1.1, 1.2, 1.3, 1.12, 1.13 so there can be N number of permutation and combinations. So, these are fraction factorial designs which will pick and choose only a fraction of this design space.

And will give you a recipe of experiments and will be clubbed with statistical analysis in order to make sure that the data fitting which is done in a polynomial equation is statistically valid or not. Now, yesterday I did not talk about this : any model will be confident and you can easily use it for prediction and for extrapolation of that model, apart from the design space which you have created is crucial.

So, tools like response surface methodology; the ANOVA which is done or the confidence which is calculated; you will always see that such design tools will always give you experimental recipes which are lying outside the design space. Any model is said to be robust or applicable only when it is able to predict outside the design space. You cannot prove a model to be nice using the same data which you have used to fit the model. Obviously, it will fit the model because you have used the same data to make the model.

So, the fitting of the model or the confidence of the model is only true when it is able to predict something out of that design space. So, that is what these tools also do; apart from the range which you provide as a user - 1 and + 1; there will be some of the design points which will be lying outside this design space and the experimental recipe you will carry out; the analysis is done to calculate the confidence or the predictability of the model is based on the response which you get in some of the recipes which are outside the design space.

So, RSM may be summarized as a collection of experimental strategies or mathematical methods and statistical inference for exploring the functional relationship between a response value and set of design variables; so therefore, it is a polynomial.

Now a central composite design, which is one of the tools in RSM is usually used to acquire data that will fit an empirical polynomial model; empirical means there is no scientific basis to it. A central composite experimental design coupled with a polynomial model is a powerful combination that usually provides an adequate representation of most continuous surfaces over a relatively broad factor domain.

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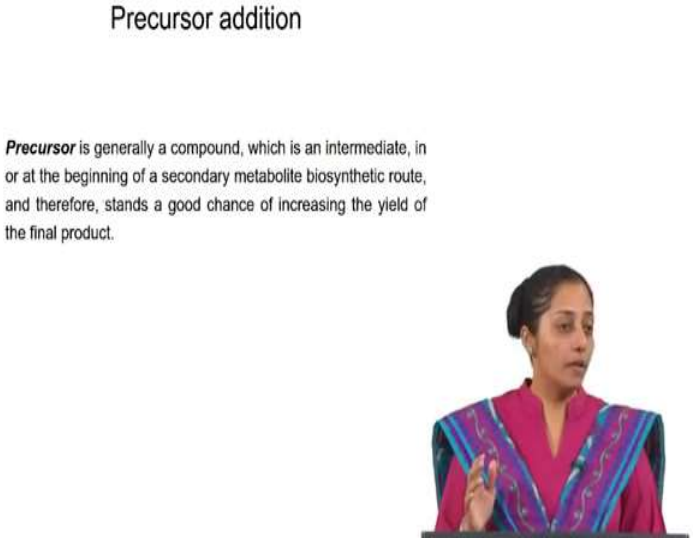

Now, talking about precursor addition; we know what is a precursor. Precursor is generally a compound which is an intermediate in or at the beginning of the secondary metabolite biosynthetic pathway and therefore, stands a good chance of improving the production of the downstream product.

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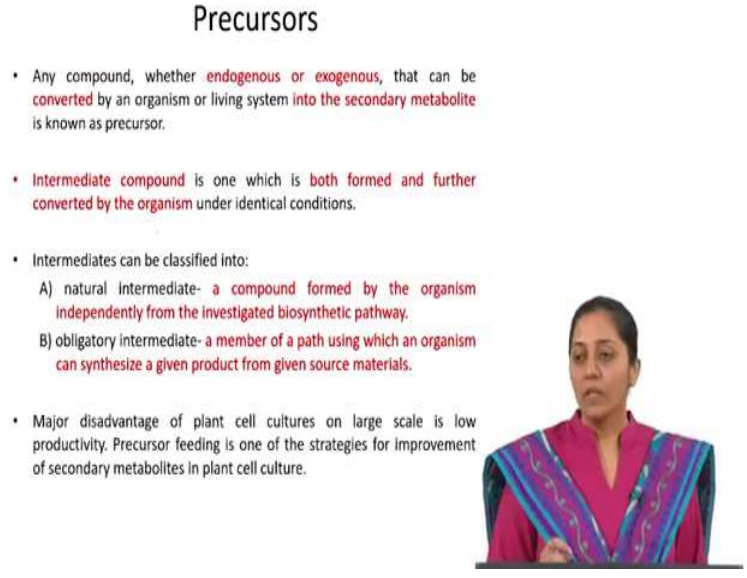

Now, there are different classes of precursors; they are classed as endogenous, exogenous; then they can be classed as indirect direct; they are classed as natural; then or obligatory intermediates; now what does that mean?

Any compound whether endogenous or exogenous that can be converted by an organism or a living system into the secondary metabolite of interest is known as precursor. Intermediate compound is one which is both formed and further converted by the organism under identical conditions which means that an intermediate compound will be formed and at the same time will be catabolized to form your product.

So, intermediates can be classified as natural intermediates; a compound formed by the organism, independently from the investigated biosynthetic pathway. Obligate intermediates - a member of the path although it is formed inside, but it is not directly participating in the biosynthetic pathway. Obligatory intermediate - a member of the path using which an organism can synthesize a given product from the given source material; so it is present inside as the intermediate. So, precursor feeding can be used to improve the yield of the secondary metabolite.

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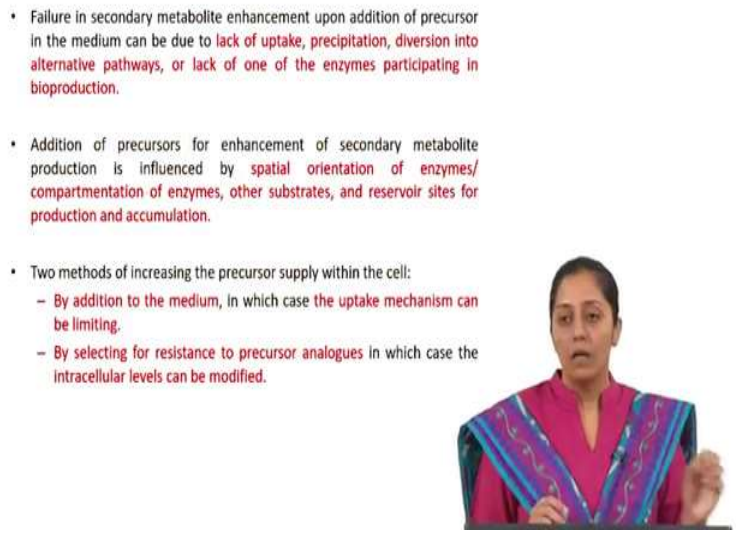

Now, suppose we add exogenously; now indirect precursors or I was talking about exogenous precursors which means that anything which you add, the cell can take up and then further metabolize it to produce the product. Now, why do you think adding a precursor does this? Sometimes it is seen that you add a precursor exogenously, although you know that it is a part of the biosynthetic pathway and ideally if you add it; it should drive the reaction forward. But sometimes it does not happen.

So, the reasons which are said to be responsible, despite adding precursors exogenously; there is no response by the cell in terms of yield enhancement. It is the lack of uptake; the cell is not able to exogenously take up the precursor, precipitation of the precursors - which means availability of the precursor to the cell can be a limitation; diversion into alternate pathways.

When added and exogenously added and taken up, it may get utilized in different other pathways because depending on which position it lies; because these are all branched pathways. So, and there is compartmentation. There is sometimes the entire biosynthetic pathway has been divided into different organelles. There is a transport of precursors which is taking place.

So, then if you provide the precursor from outside; this may get diverted towards any other pathway; what else can be a limitation? Maybe there is not enough enzyme which is available for the precursor to be utilized in that reaction. So, the expression level of the enzyme maybe a limitation or the activity of the enzyme which internally is expression based.

Now, addition of the precursors - when I say uptake; how is it facilitated? It has to cross the cell membrane barrier; it might be a toxic metabolite to the cell when it is present outside. The cell is able to overcome the toxicity - you remember I was talking about antimetabolites, where I gave an example of biotin. Pimelic acid when being a precursor of biotin in the biosynthetic pathway; pimelic acid is toxic to the cell.

So; what it does? What mechanism has happened? Maybe it is a detoxification process, that as soon as the pimelic acid is formed; it is transformed to biotin, biotin is a product of interest. So, if you add pimelic acid outside then it is seen that it is toxic to the cell. So, there it will not work or sometimes cyclodextrins are used in which the precursor is embedded such that it can facilitate the uptake of the precursor.

The toxic precursor is inside the cell because it will have the same hydrophilic hydrophobic ends; it will facilitate the uptake through the phospholipid plasma membrane or the cell wall. So, it may take up depending on the transport proteins; depending on the effect on the cell membrane, plasma membrane in order to facilitate the uptake.

So, some of the precursors, in some cases - they are provided as a complex such that they can be taken in by the cell, once in - then it can be utilized. Now addition of precursors for enhancement of secondary metabolite production; it is influenced by which factors? Spatial orientation of the enzymes, compartmentation of the enzymes, presence of other substrates,

reservoir sites for production and accumulation; all this will impact the effect of a precursor exogenously added in order to improve the product yield.

Now two methods of increasing the precursor supply within the cells by adding it into the medium - in which case what becomes limiting? The uptake mechanism becomes the limiting factor.

By selecting for resistance to precursor analogous; what does this mean? We have been talking about this; by selecting for resistance to precursor analogues in which case the intracellular levels can be modified. How? Let us take the example of pimelic acid which I gave. It's a precursor of biotin.

So, being toxic to the cell in order to select a high biotin yielding cell line, people add pimelic acid and try to acclimatize the cell or screen or select the cell. It is an invasive process now. So, they will select the cells which are able to survive under high concentration of pimelic acid exogenously added in the medium. How will that help in selection? Because only the cells which are able to take up and detoxify it to biotin will be able to survive and because they are able to convert them into biotin; they will be?

Higher yielding cell lines of biotin; so, that is the effect of adding precursor analogues and selection process.

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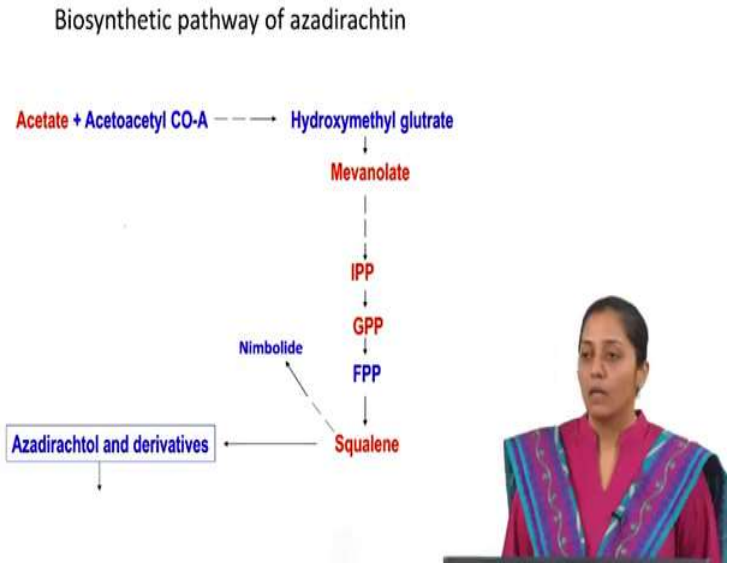

Now, as an example, this is the mevalonate pathway through which azadirachtin is produced. So, the red ones are in literature - some of the intermediates which have been added exogenously to enhance the production of azadirachtin.

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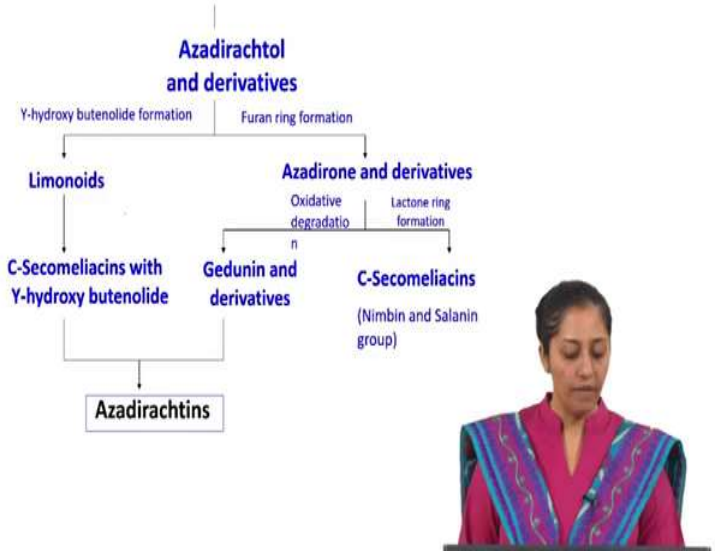

So, this is a part of the biosynthetic pathway which finally, leads to azadirachtins at the bottom. Now, the red ones I said have been used in literature for production of enhanced yield of azadirachtin. Now, acetate, sodium acetate salt if you provide it in large amounts or concentrations, these are also known as elicitors in literature. You will find that they are also stress enhancing compounds to the cell.

Now, how would you determine that anything which you are adding is a precursor? How will you find out, what kind of an experiment would you design to find out if what you added can be a precursor and not an elicitor. Precursor will be taken up; if it is an elicitor will it be taken up by the cell? No, if it is a precursor only it will be taken up by the cell.

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So, now coming on to cell permeability enhancers; cell permeability enhancers - they reversibly change the permeability of the cell. So, when I say reversibly what does that mean? How can anything change reversibly the cell membrane permeability? Either it should be able to disrupt or it should not be able to disrupt. Reversibly means what? Cell permeability enhancer means I am able to open such that things can go in; things can come out, but the minute I am able to open how is it reversible?

Student: After sometime it will again come back to the same place.

How?

Yes, that is true. Reversible means that we have exposed it to a limit for a time such that the damage is reversible; it does not become permanent. Now, what causes this damage to become reversible is depending on the exposure time, the concentration, chemical or technique you are using to create these transient pores.

Now, there are well defined defense mechanisms in place in the cell to overcome these transient pores. These transient pores can be caused by day to day damage to the cell membrane isn't it? Maybe because of the change in the cell fluidity because of the environmental factors; there can be a change in the cell permeability, membrane permeability or because of electrical shock, heat shock that's what you do; there can be pores formation.

So, there are more than one technique in the cell, sometimes which involve these membrane proteins - small portions; now these are called as caveolae. They are used to seal these damages. This is another kind of defense mechanism which is in place, which is used to seal up back the membrane. But because of the tension created in the phospholipid membranes, they remained; it is not that they are sewed together. Now because of the hydrophobic and the hydrophilic ends it is intact; any disturbance can cause transient pores.

So, there is a tension, the minute there is a transient pore there is tension among these phospholipid bilayers. This tension forces them to come back; only if even despite the fact that there is tension, they want to come back and they are not able to, it becomes permanent otherwise these are like elastic membranes.

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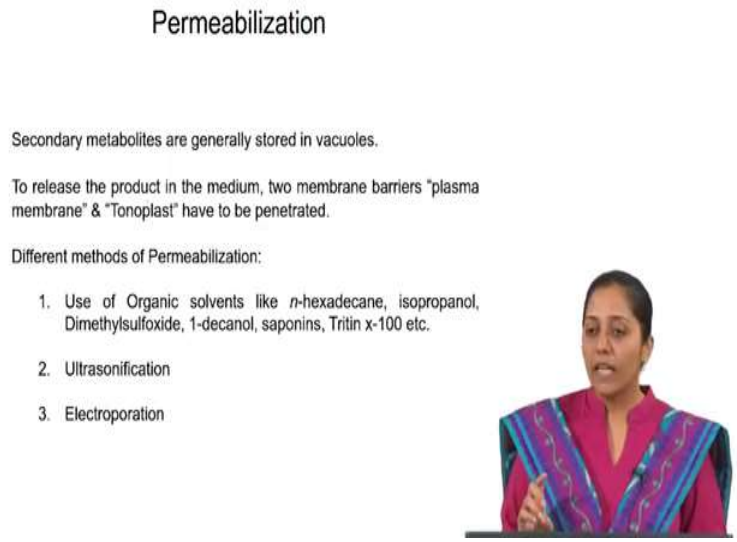

So, what kind of permeability enhancer? This is what we utilize under *in vitro* conditions in order to improve mass transfer; generally for the uptake of nutrients sometimes you would like to drive the product formation reaction forward by use of resins and the cell permeability enhancers so as to bring the product out from the cell and drive the intracellular concentrations of the product inside. Then they are also used for making the process continuous so that continuously you keep growing and the product is coming out. The intracellular product is coming out in the medium and it is getting collected. So that there is improvement in productivity of the plant cell bioprocess.

So, it will make the process continuous. So, what are the different kinds of permeability enhancers which are used? It includes ultrasonication, electroporation, use of surfactants or organic solvents like n-hexadecane or isopropanol or Triton X; these are some of them you will come across in literature where these kind of chemicals have been used as permeability enhancers.

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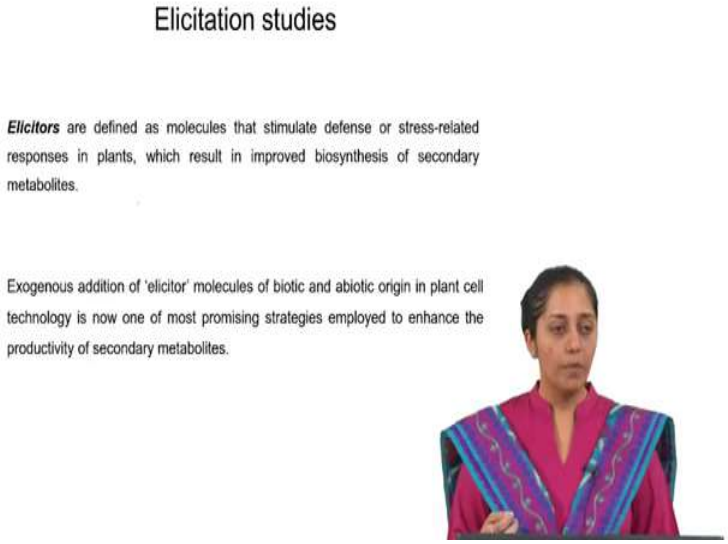

But the key there is, that the concentration in which you are using because even if you use surfactants. Now surfactants how do they increase the cell permeability? If you see the use of organic solvents some of them are surfactants.

Student: Solubilize the lipid layer

Right! solubilize because they also have hydrophobic and hydrophilic ends; so therefore, they will make transient pores. Now what is crucial here is that the concentration in which you use it and the time of exposure. So, therefore, when you are optimizing, one has to optimize the time of addition, because that will determine from the time of harvest - the exposure time and the concentration in which you are using it.

Talking about elicitors we now know what are elicitors; elicitors are defined as molecules that stimulate defense or stress related responses in plants which result in improved biosynthesis of secondary metabolites.

So, now this entire thing will include all that we have studied earlier in secondary metabolism. This can include PR proteins, this can include signaling molecules, this can include pathogenic components, this can include endogenously produced cell wall of plant cells or any other protein or defense related component which is produced by the plant cells. So, exogenous addition of elicitor molecules; now they can be biotic, abiotic depending on the origin; they can be endogenous, exogenous.

So, when I say biotic and abiotic; what does it mean? Biotic elicitors would be - can you make out from the word?

Student: microbes; biological origin.

So, which are - which have a biological origin; abiotic which are chemical. For example, can you give me an example; abiotic elicitor like?

Student: Calcium.

Abiotic elicitor like?

Student: Light.

Calcium is one of the medium nutrients. So, elicitor would be which can induce the participation of calcium signaling.

So, abiotic elicitors can be? These are all stress enhancers.

Student: Temperature; temperature.

Ok.

Student: Salinity

Salts, heavy metals, then?

Biotic elicitors can be fungal components, their cell wall components, chitin.

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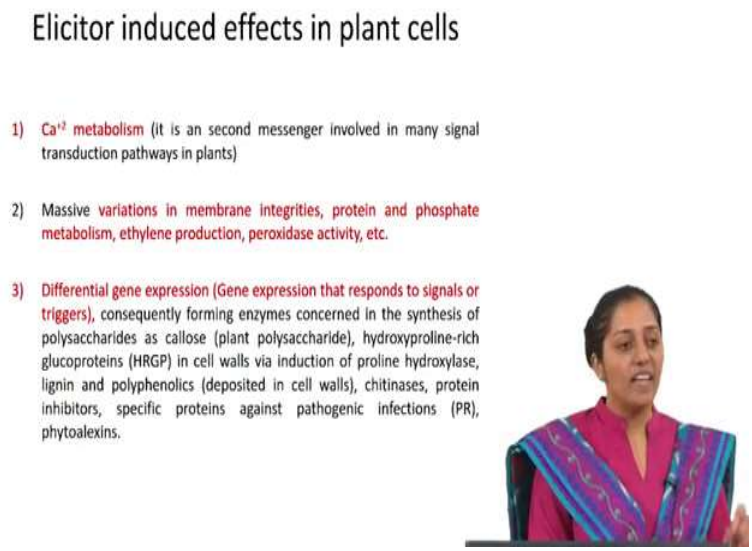

So, elicitor induced effects in plant cells include what? They can induce calcium metabolism. Now, calcium metabolism has a crucial calcium CMP pathway; it has a crucial role to play in inducing certain proteins and in transcription of certain enzymes which may be involved in the desired secondary metabolic pathway.

So, calcium metabolism, it is used as a second messenger in the cells; now second messenger is involved in many signal transduction pathways in plants. So, generally what happens- there can be an elicitor molecule which will come and bind to the receptor on the cell membrane. Ultimately, giving rise to certain kind of proteins which will then travel to endoplasmic reticulum and there through endoplasmic reticulum, it will open up the calcium channels. The intracellular concentration of the calcium ions will increase and these calcium ions will then bind to certain proteins which can act as inducers or repressors in your secondary metabolic pathways.

So, I am just giving you it in short; so this is how it can get induced, so calcium metabolism cyclic AMP is involved in that. So massive variation in membrane integrities, protein and phosphate metabolism, ethylene production, peroxidase activities; now these are the different ways in which an elicitor impacts the secondary metabolism inside the cell; it can give rise to more reactive oxygen species which means peroxidase metabolism.

It can give rise to signaling molecules like ethylene which we have already learnt now in the signaling - your secondary metabolism defense; induced defense or acquired defense. Then

differential gene expression is also impacted by elicitors; now gene expression that responds to signals or triggers consequently forming enzymes concerned in the synthesis of pathway.

Because now this is one of the ways which I said - calcium metabolism in turn can be related to transcription or translation; transcription of certain genes which are responsible for reducing those enzymes or expression of those enzymes which may be directly involved in the biosynthetic pathway or even in the production of PR proteins. Now PR proteins in general are a part of your defense.

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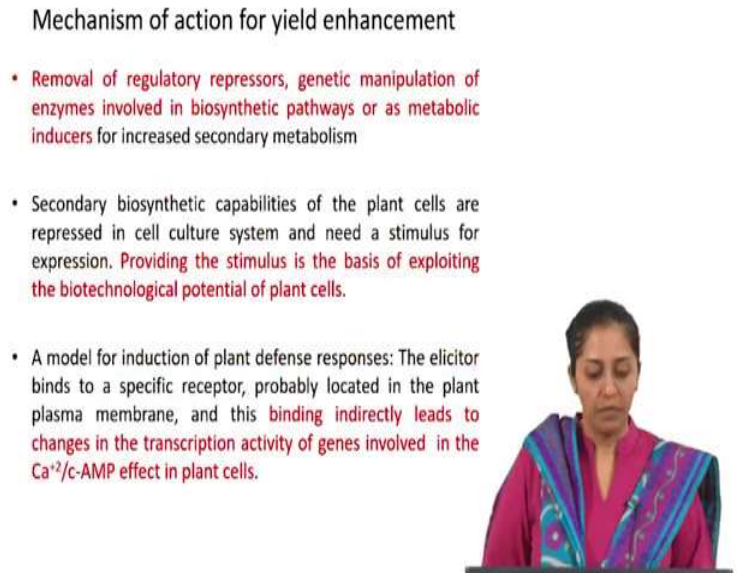

Now, mechanism of action - removal of regulatory repressors. So in turn this may also impact removal of repressors; I told you about cyclotides it is observed that under *in vitro* conditions if you elicit; add a stress signal, then you may end up getting compounds which are not known to be produced in the natural plant.

Why is it happening? That is the very indication that these elicitors or your environmental epigenetic factors may be responsible to remove the repressors or to induce the promoters, they might be acting as repressors and inducers in the secondary metabolic pathways; thereby leading to expression of certain genes which are responsible for production of novel metabolites.

So, these genes might be remaining cryptic; so, it may elicit those cryptic genes. So, removal of regulatory repressors, genetic manipulation of enzymes involved in biosynthetic pathway or

as metabolic inducers for increased secondary metabolism. These are the different ways in which the elicitors can play a role. Therefore, till date elicitor addition is one of the most promising strategies to enhance the yield of these *in vitro* cultures.

So, under *in vitro* conditions, they provide the stimulus which forms the basis of exploiting the biotechnological potential of these plant cells. Now, as I have already mentioned these elicitor molecules may bind to the receptors indirectly inducing the transcription activity of the genes involved in the calcium cyclic AMP signaling pathway.