Course Name: Basics of Crop Breeding and Plant Biotechnology

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Lecture-46: Agrobacterium Mediated Transformation in Tobacco and Rice

Welcome back. So, we will continue again. Now let us start our discussion on tobacco seed sterilization. First, why sterilization is needed? Once we have to do the plant transformation, then we need to do the transformation process under contained condition. We cannot take any seed from outside and we can initiate the transformation process. Because in outside, a lot of microorganisms are available.

Some fungal spores might be there, some bacterial contaminations might be there. So, by visual observation or by our naked eye, it could not be visible. But, once we will put those seeds on the media, on the enriched media in the tissue culture condition, then those bacteria, fungus could start its growth faster. Ok! And it can disturb the overall process of plant transformation.

So first, we need to do sterilization. We will discuss about the tobacco seed sterilization first. Tobacco seeds are very tiny in nature. So, the seeds are so much smaller. Ok! You can see over here, 1000 seeds are there.

So first, those seeds are initially washed with water to remove if any impurities are available over there, if any dust particles are available over there. After washing for a couple of times, we have to imbibe those seeds for 3 to 4 hours. So, the seeds will be swelled little bit and then, the rest of the process, sterilization process and germination would be better. So, then we have to initially treat those seeds with 70% ethanol for 30

seconds to 45 seconds for very few times, generally 30 seconds to maximum 1 minute. We can use for 70% ethanol sterilization.

By this sterilization, if any contaminants are there, if any fungal spores are there, or bacterial colonies are there, then it should be dyed. Then we have to use sodium hypochlorite. The sodium hypochlorite is a soft sterilizing agent. For tiny seeds or for soft tissues, sodium hypochlorite is mostly used for sterilization purpose. Ok! So, we have to use at least 3 to 5 minutes sterilization with sodium hypochlorite. So, but before that, we have to remove the 70% ethanol. Then, we have to wash it with sodium hypochlorite and once 3 minutes are over, then we have to wash it again and again, with double distilled autoclaved water for at least 4 to 5 times. We have to wash those seeds which are available in Eppendorf tube for 4 to 5 times with double distilled autoclaved water because the sodium hypochlorite or 70% ethanol, it should not be available at the final stage. Then basically, we have to distribute those seeds on the plates for germination. So, in this way, if we have to do the tobacco transformation, first we have to sterilize the tobacco seeds, then we have to grow the tobacco seedlings in the laboratory condition because once a seedling is being grown under laboratory condition under control tissue culture condition, then they are they are from the contamination will not come.

So our transformation chances or our transformation process will be more successful. So, then we will see the tobacco transformation process. Ok! So, for infection of tobacco plants for *Agrobacterium* mediated transformation of tobacco plants ok, the tobacco leaf disc method is used. So here, you can see in a magenta box, the tobacco plants have been grown. So initially, you have done seed sterilization, thereafter the seeds were placed on petri- plates.
Once they have been germinated, then eventually it has been transferred into magenta box, where again the solid media is there, in that media under contained environment this particular plant has been grown. So thereafter, for infection we have to make a particular construct and then construct has to be transferred into the *Agrobacterium* cell. Different strains, I have mentioned earlier for tobacco transformation, here we are going to use

LBA4404 strain. So, in that LBA4404 strain, our desirable plasmid has been inserted has been transformed. Ok! So first, the transformed or recombinant *Agrobacterium* cell has to be prepared, then that *Agrobacterium* cell harboring our desirable plasmid will be used for this plant transformation.

So before that, we need to grow the *Agrobacterium* for overnight, thereafter, we need to wash it in $MgSO_4$ salts and after that, we have to re-suspend it in different buffers. Later on, in the reference part you will see some literature wherefrom you will get the information regarding the different media compositions used over here. Ok! So, I am just going to show you the process. So, our *Agrobacterium* suspension is ready and then let us see how we should make the small pieces of this tobacco leaves for plant transformation. So, this is the *Agrobacterium* suspension.

The whole thing will be done in laminar air flow. So, the *Agrobacterium* suspension is poured in the petri-plate and thereafter using sterilized forceps, scissors, we have to cut a couple of leaves. We have to remove the, we have to remove the surface part of the leaf as well as the mid-rib part means our objective will be getting more wound because we know that if wounding is there and acetosyringone is available, *Agrobacterium* infection will be faster. So, we will try to create more wound in the leaf and we have to remove the mid-rib part also, because in the mid-rib, generally the *Agrobacterium* cannot infect easily. So, in this way, we have to chop the leaf into small pieces and thereafter, we have to put it in the *Agrobacterium* suspension.

So in this way, small pieces are made, to make it more perfect, we can puncture each and every leaf disc using forceps also. So that, in between the leaf disc, the in between the leaf disc, our *Agrobacterium* can do infection also. Once the tobacco transformation is done, means once the tobacco leaf discs have been incubated with *Agrobacterium* strains, thereafter, we have to blot those leaf discs properly. In this way, we have to put a blotting paper at the lower part of the petri-plates, then we have to put the leaf disc which were being imbibed in the *Agrobacterium* suspension solution, it should be placed over there and thereafter, we have to put another blotting paper on the top of that and then we

have to press it little bit with forceps gently. So that, we have to remove if any excess *Agrobacterium* solution is available there, we have to remove it through blotting.

If we do not do this, once we will go to the subsequent steps, excess bacterial growth will be there and finally, our plants or the leaf disc will be killed completely by the over-growth of bacteria. So, this blotting process is very very needed. So, once the blotting is done, thereafter, we have to transfer those blotted leaf disc into the media and it will be grown over there and after 2 months, approximately 2 months, we can see such type of plant growth from some of the leaves. Ok! And definitely in the regeneration media, the regenerated plants are observed in regeneration media. So, in regeneration media, we must have to add proper antibiotics or the good term will be here, the proper selectable marker.

Earlier, we have discussed, sorry, different selectable marker genes are used in plant transformation like kanamycin, hygromycin. Ok! So, in this plate, particularly here hygromycin has been used. So, by using hygromycin, a selectable marker, from here nothing is being grown, from here very few cells are surviving, here it is showing almost green. So, some plantlets may come from here, while, from this particular part a lot of seedlings are coming. It means that, maybe in some tissues, in some cells, our transgene has been delivered and within that transgene, the hygromycin selectable marker is also available.

So, it can sustain the hygromycin containing media. Now, let us discuss the rice seed sterilization process. First of all, in rice seed sterilization, we have to unhusk the rice seeds. We know that in rice, a husk is available. In husk, a lot of dust particles could stay easily.

So, first we need to unhusk the rice seeds without disturbing the embryo part. If you just carefully see the husk, let us assume, this is the husk part of a rice and inside that, a rice seed is available. So, in rice seed, a small embryo part is available at a particular corner and most of the part is endosperm. So, through rice seed sterilization, basically,

we will see the callus formation from rice, basically callus will be initiated and from that particular callus eventually the callus will be embryonic in nature, and from that embryonic callus, from the transformed one, finally the plantlets could be grown under selection media. Ok! So, this process will be discussed over here and the callus is formed in this embryo part of the rice seed. Ok!

So, let us see how the rice seed sterilization is done. First, we have to unhusk the rice seeds and we have to wash it with water for a couple of times. The rice seed sterilization is mostly done in flask, because, a lot of water is needed and the seed sides are larger. So, it has to be washed properly. After a couple of washing, we have to use liquid soap and using this liquid soap we have to use, sorry, we have to wash the rice seeds for at least 4 to 5 minutes.

We have to continuously shake the flask, the frothing will be there, and after 4 to 5 minutes, we have to remove the frothing and we have to wash it with double distilled autoclaved water for a couple of times. Then, we have to use the main sterilizing media, over here, we are going to use mercuric chloride, that is $HgCl_2$. In rice seed sterilization, mercury chloride is used and generally 0.01% $HgCl_2$ is fine and it could be utilized for at least 3 minutes in case of rice. Thereafter, we have to wash it with water thoroughly for at least 4 to 5 times.

So that, no traces of mercury chloride or liquid soap will be available at the final stage and once this washing is done, we have to initially put those seeds on blotting paper, we have to soak the seeds using blotting paper. So that, not too much water is available over there and finally, we have to put the seeds on the media, on the callus inducing media that is abbreviated as CIM; callus inducing media. Basically, the seeds will be placed in such a way, so that in the rice seed, I have told, the embryo part is available, the seeds should be placed in such a way, so that the embryo part would be in touch of the media. Ok! So, the seeds will not be placed vertically, it should be placed in this way on the media. So now, we will discuss the rice callus transformation.

After 14 to 21 days of putting those rice seeds on CIM media, that is, callus inducing media, under dark, we can see such type of callus formation. What is callus? Callus are unorganized mass of undifferentiated cells. Ok! So here, you can see those are not organized in nature, some unorganized mass of cells are there and those are in undifferentiated condition, those are available in undifferentiated conditions, means no root no shoot is available over there. So, once the callus are ready, so first, we need to remove the callus from the seeds, we need to incubate this callus for a couple of days in CIM media once again, then we have to start the *Agrobacterium* mediated transformation of rice callus. Ok! So, over there, the overnight *Agrobacterium* culture is grown, once the *Agrobacterium* culture is grown for overnight and then we have to pellet down it and after precipitation, we have to re-suspend it in $MgSO_4$ buffer and thereafter, basically after washing with $MgSO_4$, we have to pellet down it once again and finally, we have to dissolve it in AAM media. Ok!

Later on, in our reference, some papers have been mentioned some manuscripts have been mentioned, so you guys can go through it, if you would like to know about the detail process of or the compositions of different media. Ok! So, in this way in AAM media, basically, we have to finally dissolve the *Agrobacterium* cells, *Agrobacterium* pellets and over there we have to use acetosyringone, because acetosyringone is one of the important compound that will initiate in the *Agrobacterium* mediated gene delivery ok, so that is needed. So, thereafter, we have to put the callus on that particular media and we have to shake this plate gently without breaking those callus into small fragments, we have to shake it gently for approximately 25 minutes to 30 minutes and once this shaking is done, thereafter again, we have to do blotting. In rice, blotting is more crucial, because if you see here, the callus structure, no uniform structure is there, the callus structure is like this a lot of pores are there. So, in between these pores the *Agrobacterium* suspension might be available.

So, if we do not blot properly, within a couple of days, excessive bacterial growth could be occurred and it will kill all the callus easily. Ok! So, we have to use blotting properly using the blotting paper. So, once the blotting is done, then eventually, we have to put

those blotted callus into the petri-plates where the media is there. So, after infection, we have to put it in co-cultivation media. Ok! So, this is known as co-cultivation media.

In this co-cultivation media, basically, no antibiotic is given because in this media we are allowing the *Agrobacterium* to stay with the callus. So that, during this co-cultivation incubation period, the *Agrobacteria* can deliver the T-DNA into the callus. So, over here, no antibiotic is given ok, and acetosyringone should be given over there also and thereafter, we have to put it in other different media. After 48 hours, we have to remove the callus from the co-cultivation media and thereafter, gradually, we have to put it in selection media. In selection media, if in our construct hygromycin selectable marker gene is there, we have to add hygromycin antibiotics in the media.

If kanamycin is there, we have to add kanamycin in the media. Ok! In this way, the selectable antibiotic should be used in the selection media, and in addition to that, we have to use cefotaxime also, because this antibiotic can kill excess growth of *Agrobacterium* because thereafter, means after the co-cultivation media, we do not need *Agrobacterium* cell anymore. After, we need to get those callus or those plant samples where our transgene has been delivered during the co-cultivation process the transfer mechanism is mostly done. Ok! So, from here, we will put cefotaxime in the media also to kill the excess *Agrobacterium*. So, sorry, this is the co-cultivation media, for the initial days and this is the process of selection media.

Here you can see, the selection is going on, eventually, some callus are being killed completely, ok, while in some callus, a lot of survived cells are available, in some callus a lot of survived cells are available. So, the callus which are being killed, it means, in those cells, the transgene has not been delivered. Ok! So, thereafter, we have to put those things, those callus which are showing some promising criteria, we have to transfer it in regeneration media. In regeneration media, basically, we can see such type of shoot growth from those callus, because earlier, I have mentioned that callus are unorganized masses of undifferentiated cells.

So, it was undifferentiated in nature. In regeneration media, the differentiation is gradually being started. So, first, we will try to induce the shoot formation, ok, then eventually the root formation will be taken place. So, here, you can see from this callus over here, a seedling is coming over here, another seedling is coming. Ok! So, few transformed plants are being developed and once, in this way, we can initiate the root formation, then ultimately it is transferred in liquid media. Upto that, it is being grown in semi-solid conditions. Ok!

These are the different culture tubes containing putative transformed rice plants. This one, you can see, it has been fully killed in the liquid media where half strength Murashige and Skoog media is available, half MS it is commonly known as half MS, half strength of Murashige and Skoog media is available, along with that, hygromycin antibiotic is given. Ok! It is given 50 mg/liter, this concentration of hygromycin is also given over there and in this concentration, this plant is being killed. So, although at the callus stage, somehow it escapes the selection, over here it cannot escape means here our transgene has not been inserted properly.

This is surviving, this one is also surviving. So, after a couple of weeks, we can see whether they are still surviving or not. Over here, in this experiment, in first culture tube you can see among 3 to 4 plants, only one is almost surviving, rest of them are being killed, while these plants are surviving properly. It means, these plants might be a putative transformants. So, once we can identify the putative transformants through such type of screening, then eventually we have to go to PCR analysis by using gene specific primer, by using marker specific primer, that is hygromycin or kanamycin specific primer. We can screen these plants and thereafter, we can confirm it by different molecular techniques like southern, northern, real time PCR by these different molecular techniques, eventually we can screen our transgenic plants.

We can confirm our transgenic plants, these are the screening methods. Ok! These are the references of today's class. Thank you!