LESSON 14:
ANTHER/ POLLEN CULTURE

Objective

Why are Anthers/ Pollen Cultured in Vitro?

These are cultured primarily for the production of Haploid plants which find important application in the field of plant breeding (discussed later in the Lesson). Pollen culture is also termed as Microspore Culture. Haploids are sporophytes of higher plants with gametophytic chromosome constitution. It is possible to induce haploidy either using anther culture, henceforth reffered to as 'androgenesis' or from cultures of individual pollen grains.

Microspore Culture – A General Account

Haploid tissue can be cultured in vitro by using pollen or anthers as an explant. Pollen contains the male gametophyte, which is termed the 'microspore'. Both callus and embryos can be produced from pollen. Two main approaches can be taken to produce in vitro cultures from haploid tissue.

The first method depends on using the anther as the explant. Anthers (somatic

tissue that surrounds and contains the pollen) can be cultured on solid medium (agar should not be used to solidify the medium as it contains inhibitory substances). Pollen-derived embryos are subsequently produced via dehiscence of the mature anthers. The dehiscence of the anther depends both on its isolation at the correct stage and on the correct culture conditions. In some species, the reliance on natural dehiscence can be circumvented by cutting the wall of the anther, although this does, of course, take a considerable amount of time.

Anthers can also be cultured in liquid medium, and pollen released from the anthers can be induced to form embryos, although the efficiency of plant regeneration is often very low. Immature pollen can also be extracted from developing anthers and cultured directly, although this is a very time-consuming process.

Both methods have advantages and disadvantages.

Some beneficial effects to the culture are observed when anthers are used as the explant material. There is, however, the danger that some of the embryos produced from anther culture will originate from the somatic anther tissue rather than the haploid microspore cells. If isolated pollen is used there is no danger of mixed embryo formation, but the efficiency is low and the process is time-consuming. In microspore culture, the condition of the donor plant is of critical importance, as is the timing of isolation. Pretreatments, such as a cold treatment, are often found to increase the efficiency. These pretreatments can be applied before culture, or, in some species, after placing the anthers in culture.

Plant species can be divided into two groups, depending on whether they require the addition of plant growth regulators to the medium for pollen/anther culture; those that do also often require organic supplements, e.g. amino acids. Many of the cereals (rice, wheat, barley and maize) require medium supplemented with plant growth regulators for pollen/anther culture.

Regeneration from microspore explants can be obtained by direct embryogenesis, or via a callus stage and subsequent embryogenesis. Haploid tissue cultures can also be initiated from the female gametophyte (the ovule). In some cases, this is a more efficient method than using pollen or anthers. The ploidy of the plants obtained from haploid cultures may not be haploid. This can be a consequence of chromosome doubling during the culture period. Chromosome doubling (which often has to be induced by treatment with chemicals such as colchicine) may be an advantage, as in many cases haploid plants are not the desired outcome of regeneration from haploid tissues. Such plants are often referred to as 'di-haploids', because they contain two copies of the same haploid genome.

The above text gives you a general idea regarding what anther/ pollen culture is all about. Let us now look at each aspect in detail.

The methodology followed for Anther/ Pollen Culture in vitro is as below:

Haploid plants may be obtained from pollen grains by placing anthers or isolated pollen grains on a suitable culture medium; this constitutes anther and pollen culture respectively. The anthers may be taken from plants grown in the field or in pots, but ideally these plants should be grown under controlled temperature, light and humidity; the optimum condition may differ from species to species. Often, the capacity for haploid production declines with the age of donor plants.

Let us now follow the method employed for transferring anthers/ pollen to the culture medium.

Flower buds of the appropriate developmental stage are collected, surface sterilized, and their anthers are excised and placed horizontally on culture medium. Some workers prefer to partially embed the anthers in the culture medium. Flower buds with small anthers may themselves be cultured and, in some cases, the entire inflorescence has been cultured. Care should be taken to avoid injury to anthers since it may induce callus formation from anther walls. Alternatively, pollen grains may be separated from anthers and cultured on a suitable medium.

The following Figure 1. shows the developmental pathways of a pollen grain which after second Mitosis gives rise to a proembryonic mass. This proembryonic mass either directly (through embryo formation) or indirectly (through callus formation) may give rise to a haploid plant. In the next part of the lesson, these pathways are described in detail

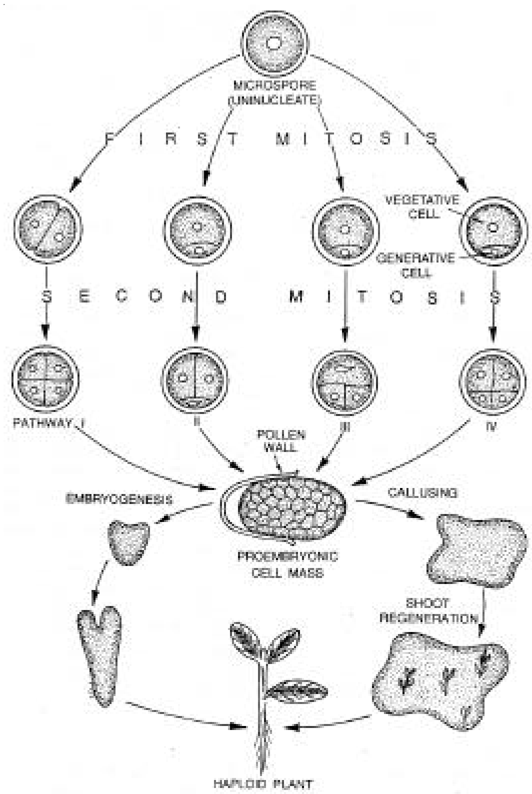

Fig. 1. The different patterns of early divisions in pollen grains during androgenesis and the subsequent development of proembryogenic cell masses so obtained.

In many plant species, SEs from the pollen grains of cultured anthers are directly produced, e.g., in Datura, Atropa, Brassica campestris, B. napus, several Nicotiana sp. (including N. tabacum and N. rustica), Petunia axillaris etc. In such cases, the plants obtained from germination of embryos are generally haploid, but some polyploids are also produced. But in many other species like rice (0. vulgare), barley (H. vulgare), wheat, tomato triticale etc. pollen grains produce callus from which plantlets may be regenerated under suitable culture conditions (Fig.1). In these cases, the ploidy level of plants varies considerably more than in those where embryos are produced.

Haploid plantlets have been regenerated from pollen grains of about 200 species of over 50 genera and 25 families. Of these, the following are examples of important crop species: potato (S. tuberosum), barley, wheat (Triticum sp.), rice, Brassica campestris, Triticale, many members of Solanaceae and some vegetables.

Pathways of Development

The early divisions in responding pollen grains may occur in one of the following four ways.

1. The uninucleate pollen grain may divide symmetrically to yield two equal daughter cells both of which undergo further divisions, e.g., Datura innoxia (Pathway I).
2. In some other cases, e.g., N. tabacum, Datura mete/, barley, wheat, triticale, chillies etc., the uninucleate pollen divides unequally (as it does in nature). The generative cell degenerates immediately or after undergoing one or two divisions. The callus/ embryo originates due to successive divisions of the vegetative cell (Pathway II).
3. But in few species, e.g., Hyoscyamus niger, the pollen embryos originate from the generative cell alone; the vegetative cell either does not divide or divides only to a limited extent forming a suspensor like structure (Pathway Ill).
4. Finally, in some species, e.g. Datura innoxia, the uninucleate pollen grains divide unequally, producing generative and vegetative cells, but both these cells divide repeatedly to contribute to the developing embryo/ callus (Pathway IV) (Fig.1).

Pollen grains of many crop species, e.g., tobacco, barley, wheat, etc., exhibit pollen dimorphism. Most of the pollen grains are bigger, stain deeply with acetocarmine and contain plenty of starch. But a small proportion (0.7%) of the pollen grains are smaller and stain faintly with acetocarmine; these are called S-grains. It is these S-grains which respond during anther culture; the frequency of responding pollen grains can be enhanced over that of S-grains by certain pretreatments, e.g., chilling.

Pollen grains of the cultured anthers show remarkable cytological changes during the first 6-12 days, called the inductive period. In tobacco, the gametophytic cytoplasm of binucleate pollen grains is degraded, ribosomes are eliminated and only few mitochondria and plastids remain. New ribosomes are synthesized following the first sporophytic division of the vegetative cell.

The responsive pollen grains become multicellular and ultimately burst open to release the cell mass. This cell mass may either assume the shape of a globular embryo and undergo the developmental stages of embryogeny, or it may develop into a callus depending on the plant species. In some species, e.g., rice, wheat, rye, maize etc., the pollen grains can be induced to produce embryos or calli by simply altering the medium composition.

Careful studies in tobacco have shown that

i. over 80% of well developed embryos are associated with their radicular ends to the anther wall and that
ii. the exine of pollen grains must rupture in such a way as to expose the putative plumular ends of the developing cell mass for their further differentiation into SEs. The latter ensures that the exine lies between the would be radicular end and the supporting tissue to which the cell mass is adhered; this is believed to be important in establishing polarity which appears to be essential for SE differentiation.

During somatic as well as pollen embryogenesis an electrical polarity is established before the formation of bipolar SEs. Ionic currents flow inward from the site of future shoot, while they flow out from that of root. The electrical polarity is associated, at least in carrot SE regeneration, with an asymmetrical calmodulin (a protein involved in response of cells to Ca2+) distribution in the differentiating cell mass (the concentration is higher in the radicular zone). Many agents, including low voltage electrical fields and attachment to a substrate, that enhance polarity are known to promote SE differentiation.

Let us now discuss the culture requirements for anther/ pollen culture.

1. Culture Medium

Medium requirements may vary with:

1. Species,
2. Genotype,
3. Age of donor plants and anthers, and
4. Conditions under which the donor plants are grown.
   For example, pollen grains of Datura and tobacco produce embryos on an agar medium containing only 2-4% sucrose, while elaborate media e.g., N6 and Potato-2 media, had to be formulated for cereals. Sucrose is essential for anther cultures; the concentration may range from 3% for barely to 6% for wheat and potato, but 2-3% sucrose is most commonly used. For most plant species, a complete tissue culture medium is required. MS, LS (Linsmaer and Skoog) and some other tissue culture media are generally used. Media with dilute salt solutions, e.g., White's and Heller's media, are ordinarily supplemented with coconut milk. Sucrose plays a key role in the induction of embryogenesis, while other medium constituents appear to be needed for post-induction development of embryos. High sucrose level may play an osmoregulatory role during induction, but it is not necessary, or even detrimental, during embryo development.

2. Growth Regulators

In Solanaceous plants, pollen embryogenesis does not require any growth regulators, but low levels of auxins, cytokinins and even GA3 appear beneficial. 0.1 mgl/l IAA gave the best results. In Hyoscyamus niger an auxin, e.g., 2 mgl/l 2, 4-D, enhanced the frequency of responding calli but had no effect on the number of embryogenic pollen grains. In contrast, cytokinins (0.01-10 mg/l) reduced the number of pollen grains producing embryos most likely by interfering with cell division in induced pollen grains. In species where callus is formed, e.g., cereals, auxins and cytokinins are almost invariably used either in combination. or in sequence, but the role played by them is not known. It seems that different GRs may be required for best results with different plant species.

The presence of an auxin may determine the mode of subsequent development of androgenic cell masses. Wheat anthers cultured on a medium having 2, 4-D produce callus, while those kept on a coconut milk supplemented medium give rise to embryos. Similarly, when anthers of indica rice are cultured in the presence of an auxin, pollen grains begin to develop embryos, which continue in this mode if the anthers are transferred to an auxin-free medium. But if they are left on the auxinsupplemented medium, callus is produced.

3. Stage of Pollen Development

The optimum stage of pollen varies with the species. For many species, including Datura, tobacco etc., the optimum stage is just before or just after the first pollen mitosis, while the early binucleate stage is the most suitable for species like Atropa belladona and Nicotiana sylvestris, and is absolutely essential for Nicotiana knightiana. In cereals, the best stage appears to be the early or mid uninucleate stage, i.e., before the first pollen mitosis. In contrast, in species like tomato and Arabidopsis thaliana, the optimum stage is when the PMC's are in meiosis I, while in Brassica trinucleate pollen grains (at the time of pollen shedding) are the best. Pollen grains of Brassica remain responsive throughout the maturation phase, but their auxin requirement increases with the pollen age.

In tobacco, the beginning of starch accumulation in pollen grains marks the end of their embryogenic potential. Further, the presence of starch in early binucleate pollen is indicative of a lack of androgenic potential of the species, while in species having the potential starch is absent.

4. Culture Environment

Anther cultures are generally maintained in alternating periods of light (12-18 hr; 5,000-10,000 lux m^2) at 28°C and darkness (12-16 hr) at 22°C, but the optimum conditions vary with the species. The walls of responsive anthers turn brown and after 3-8 weeks they burst open due to the developing callus or embryos. After the seedlings (from embryos) or shoots (from callus) become 3-5 cm long, they are transferred to a medium conducive to good root development. Finally, they are transferred to soil in the same way as other in vitro regenerated plantlets.

In tobacco, optimum temperature is around 25°C. Pollen embryos are not formed in D. innoxia anthers cultured at 20°C or below. Clearly temperature optima varies with the species. In some species, e.g., grape, potato, Datura etc. exposure of anthers to light during their first 24 hr of culture enhances the frequency of haploid callus or responding anthers. Light appears to be beneficial even in those species where anthers cultured in dark respond adequately. Isolated pollen grains require a relatively low intensity of light.

5. Pretreatments

Exposure of excised flower buds to a low temperature for some time e.g., at 3-5°C for 2 days or at 7-8°C for 12 days for tobacco, prior to removal of anthers for culture may markedly enhance the recovery of haploid plants. In some species, however, a brief exposure of anthers to a high temperature is reported to have a promotory effect, e.g., at 35°C for 24 h in Brassica campestris. It contrast, the best pretreatment for cereals like wheat, rice, barley etc. seems to be 3-28 days at 4-lO°C; this increases the frequency of green plants. The pretreatment temperature and duration may be considerably affected by plant species, genotype and stage of anther development. The mechanism of cold pretreatment response is not known but interference with starch accumulation in pollen and degradation of tapetum cellular matrix etc. may be involved.

In addition, pretreatments like centrifugation, irradiation with X-rays and gammarays, and reduced atmospheric pressure are reported to promote androgenesis. Production of haploid embryos or plantlet formation from pollen grains is referred to as androgenesis. '

6. Other Factors

Androgenesis in barley is promoted by the use of wheat or barley starch as gelling agent (in place of agar), and by the addition of ficoll in liquid medium. In many species, activated charcoal (in agar-gelled media) is promotive. In addition, amino acids like glutamine, proline, serine etc. enhance the frequency of responsive anthers. Anther extracts and media conditioned by culturing anthers for few days improve androgenesis; thus anther wall seems to provide some nutritional factors.

Pollen Culture

Isolated pollen grains, when cultured in vitro, give rise to haploid embryos or callus; this approach is called pollen culture.

The procedure followed for isolation of pollen and their culture is as follows:

Pollen may be isolated either by squeezing or float culturing the anthers. About 50 anthers may be placed in 20 ml of medium and squeezed with a glass rod. The solution is filtered through a nylon mesh of suitable pore size (25um for tomato to 100 um for maize), and centrifuged at 500-800 rpm for 5 min. The pollen pellet is collected, washed twice and suspended at a final density of 103-104 pollen/ml.

In float culture, excised anthers are floated on a shallow liquid medium in Petri dishes. The anthers dehisce in a few days releasing their pollen grains into the medium. These anthers continue to shed pollen so that their serial subculture yields pollen samples in different stages of androgenesis.

Initially, isolated pollen grains were cultured either in hanging drops or on a filter paper raft placed on cultured anthers. Subsequently, Nitsch and coworkers first replaced the nurse tissue by an extract of cultured anthers, and finally devised a completely synthetic medium for pollen culture, the crucial ingredients of which were glutamine, L-serine and inositol (for Solanum sp. asparagine and glutamine are needed).

Androgenesis can be markedly improved by a preculture of anthers for 4-7 days before pollen isolation. Successful pollen culture is reported for several species, e.g., tobacco, Datura, petunia, potato, barley, wheat, rice, maize, rapeseed etc.

Pollen culture offers certain advantages over anther culture due to the elimination of anther wall, e.g.,

i. studies on differentiation and development are easier and more precise,

ii. no callus formation can occur from wall tissue and

iii. products from different pollen grains ordinarily do not get mixed up (this eliminates the risk of chimera).

Conclusion

Anther/ pollen culture is a pathway for production of haploids in vitro. Haploids are useful in plant breeding programmes where induction of maximum genetic variability of germplasm is required to secure a wider scope for selection and introduction of better trait qualities in existing crop species. Androgenesis fulfills these requirements and haploids have been produced from about 250 species of plants, especially members of Solanaceae, Cruciferae, Gramineae, Ranunculaceae, etc.

Questions

1. What are the benefits from pollen or anther culture?
2. Define anther and pollen culture. Briefly describe the early patterns of cleavage in cultured pollen grains and the different modes of subsequent development of the proembryogenic cell mass so obtained.
3. Describe anther culture under the following heads:
   a. medium constituents
   b. stage of pollen development
   c. pretreatments
   d. pollen culture.

Note