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REVIEW ARTICLE

ANTHER CULTURE AS A SUPPLEMENTARY TOOL FOR POTATO BREEDING

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ABSTRACT

Anther culture is a technique for creating haploid lines from farmed potato (Solanum tuberosum L.), which possesses a tetraploid genome (2n = 4x = 48). Shoot regeneration via direct embryogenesis in anther culture is favored for producing dihaploid (2n = 2x = 24) potato lines that can be used in variety breeding. Not only in cultivated potato (S. tuberosum), but also in other genetically similar potato species, the anther culture methodology described in this chapter can be used.

KEYWORDS: *Haploid Line, Pollen, Donor Plant, Embryogenesis*

INTRODUCTION

The major cultivated species of the potato, Solanum tuberosum L. ssp. tuberosum is an autotetraploid (2n=4x=48). Other cultivated species sometimes used in genetics and breeding are: andigena (S. tuberosum L. spp. andigena Hawkes) tetraploid species, stenotomum (S. stemotomumJuz. Et Buk.) diploid species (2n=2x=24) and phureja species (2n=2x=24) (S. phurejaJuz. Et Buk.). In genetic studies and the anther culture, Chacoense (S. chacoense Bitt.), a wild diploid species, is sometimes used. There is a significant number of wild species ranging from diploid to hexaploid (2n=6x=72) of ploidy levels. Of the 176 chromosome counting species studied, 73 percent are diploid, 15 percent are tetraploid, and 6 percent are hexaploid. Hybrid animals that are triploid or pentaploid are the remainder. The world's collections of potato species and varieties are held by many broad gene banks.Up-to-date inventories have been published by German-Netherlands Potato GenBank in Braunschweig, Germany, and the USA Inter-regional Potato Introduction Project. The International Potato Centre, the German Netherlands Potato GenBank and the USA Inter-regional Potato Introduction Project carry out systematic assessments of germplasm collections. Dihaploid progenies (2n=2x=24) may be generated using the tetraploid varieties. For genetic research and breeding, dihaploids have many benefits. They are used to mating with populations of cultivated or wild diploids. The diploid hybrid progenies are tested for superior agronomic trait efficiency and more specifically, for their ability to generate unreduced 2n gametes. The 2n gametes are formed during meiosis due to the failure of either the first or second division. In genetic segregation, the first division restitution (FDR) and second division restitution (SDR) gametes have radically different effects.The FDR gametes are capable of retaining about 80% of a parent's heterozygosity and are thus useful when used to communicate with other tetraploid parents to maintain heterosis in the progenies. In many breeding programs, hybrid progenies are now produced from 4x x 2x mattings. Mono haploids (2n=x=l2) of potato can also be obtained from the tetraploid varieties. Two consecutive periods of decreases in chromosome numbers are involved. It reduces a tetraploid to a dihaploid. The I-dihaploid. Mono haploids and interspecific hybrids are also obtained from diploid specimens. For essential genetic analysis of the diploid S, mono haploids are valuable tools. A genus of tuberosum. Cytological proof of the fundamental chromosome number (.x=12) of the potato

genome is given by monoploids. To screen out harmful and lethal genes and identify beneficial mutants, mono haploid progenies are used. The development comes from the doubling of mono haploid chromosomesdiploids and tetraploids that are homozygous. Due to the easy (1:1) segregation ratio, the hemizygous state of mono haploids also makes mapping of molecular markers simpler. It is possible to generate both di- and mono haploids by two methods: parthenogenesis and androgenesis. The generation of dihaploids by parthenogenesis is a well-established method used to generate parents for 4x x 2x crosses in potato breeding. The preferred procedure for obtaining a large number of mono haploids tends to be another or microspore culture since the number of microspores much exceeds the number of ovules in an ovary [1]. Study projects have begun to concentrate on the introduction of isolated cell cultures worldwide. In this research, we aimed to improve the development of another culture of potato species.

Historical Trajectory of Anther Culture

In the cultivation of immature anthers of the solanaceous species Datura innoxia, the possibility of changing the usual gametophytic pathway of microspores to a sporophyte pathway facilitating haploid plant production through in vitro culture was first reported [7]. [8] Haploid plants have been successfully obtained from the production of isolated Nicotiana anthers. Since then, with many other crop species such as rice, haploid development using in vitro culture of anthers and isolated pollen has been successful, While microspore embryogenesis has been successful in model species such as barley, rapeseed, tobacco and wheat, the technique continues to be less receptive to certain other species which are scientifically or economically significant, such as Arabidopsis, woody plants and legume crops. In order to make this important method of forming haploids and diploids more robust, extensive research has been carried out. Anther or microspore culture should be able to allow the development of haploids in very large quantities from almost any species or genotype for the technique to be practically implemented in breeding programs [9]. Haploid plant development by anther culture in rice was first documented by [10]. Many researches have since been performed to develop different aspects of the culture of rice anther. Recent applications have been extended to promote other biotechnological methods, such as gene transformation, in addition to using another culture technique directly for dihaploid development [3]. In Japan and China, where the Japonica rice varieties are mainly used the technique of anther cultivation has been widely used to boost rice cultivation due to the suitability of the Japonica rice varieties to in vitro anther cultivation [5].However, due to the inherent recalcitrance associated with Indica strains, the use of this technique as a method for Indica rice breeding has been extremely limited. The ability of the Indica rice breeding technique is therefore yet to be completely unraveled [3].

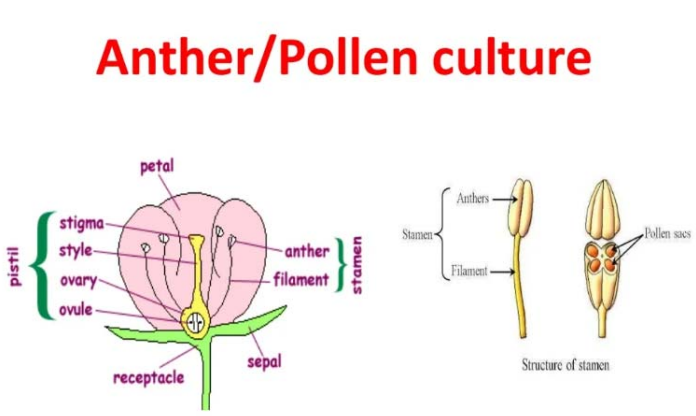

Figure 1.

Technique of Anther Culture

Normal gametophyte formation from microspores should be halted in order for androgenesis to be successful, and microspores are directed towards sporophyte growth. Pretreatments are usually needed to modify the normal pathway of pollen production and to activate the androgenic response. The unique androgenesis pretreatments that different species and even varieties within species need are very variable. Therefore, a single standard method cannot be generalized for androgenesis for a given species or even a variety. However, some common protocols to be followed during anther culture are well known and documented [4]. The short procedures are:

1. Collect the buds of potato at onset of flowering. Select the flower bud i.e. the length of sepals that of petals. Reject all buds which are beginning to flower.
2. Transfer the selected flower buds under the laminar air flow. Each flower bud contains five anther and this are normally surface sterilized in closed buds. The flower buds are surface sterilized by immersion in 70%ethanole for 10sec followed by 10min in 20% sodium hypochlorite. They are washed three times with sterile distilled water. Finally transfer the buds to sterile Petri dish.
3. To remove the anther slit the side of the buds with a sharp scapple and remove them with a pair of forceps, place five anthers with the filaments to another Petri dish. The filaments are cut gently. Damaged anthers should be discarded.
4. Anthers are placed on agar solidified MS basal medium.
5. The cultures are kept initially in dark. After 3-4 weeks, the anthers normally undergo pollen embryogenesis and haploid plantlets arise from the cultured anther. In some cases, anther may undergo, proliferation to form culls tissue which can be induced to differentiate to haploid plants.
6. At this stage the cultures are incubated at24-28^{o}C in a 14 hrs. day light regime at about 2000lux.
7. Approximately 50mm tall plantlets are freed from agar by gently washing with running tap water and then transferred to small pots containing autoclave potting compost. Cover each plant with glass beaker to prevent desiccation and maintain in a well-lit humid green house. After some weeks remove the glass beaker and the transfer the plant to large pots when the plant will mature and finally flower.

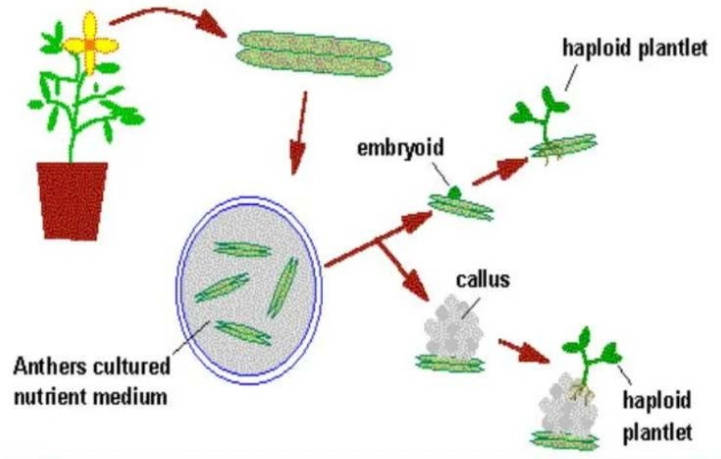

Figure 2.

4. Limitations Associated With Anther Culture

A number of limitations are correlated with the induction of haploids in potatoes. In order to use this technique equally well for potato breeding, fine tuning of another culture process addressing the constraints is needed. Although the anther culture technique was used to produce haploids from a variety of organisms, it is not possible to prove the success of the technique with regard to all genotypes of a crop species [11].The answer remains extremely varietal or genotype-specific, especially when it comes to the anther rice culture [1]. The issue is further exacerbated because the reaction of anther culture is influenced by the rising season even [4]. Many of the anthers struggle to develop in culture under in vitro conditions and thus repress the pollen from forming calluses. The early abortion of pollen and also in circumstances where pollen begins to divide and generate callus and necrosis or cell death occurs very early during the proliferation of callus are several reasons for failure. The ploidy of the resulting callus tissue is often associated with a degree of ambiguity since it can contain a chimera of diploid, tetraploid, and haploid cells. The formation of albino plants during regeneration is another problem that seriously affects anther cereal culture, and this can be described as the most restrictive stage in the process of anther cultivation [12]. Detailed research into protoplastides and the plastid genome of regenerated albino plantlets has shown that albinism is primarily due to incomplete membrane structure formation and multiple plastid developmental blockages [13]. Molecular studies carried out on anther culture of cereals such as wheat, barley, and rice have attributed the associated albinism to large-scale deletions and rearrangements in the plastid genome [14].

5.Factors Affecting Potato Anther Culture

Investigations on haploid induction through anther culture have been steadily increasing due to its importance as a supplementary breeding strategy. These studies are mainly driven with close monitoring of a number of factors that influence androgenesis in potato as described in detail below.

5.1 Genotype of the Donor Plants

Response to anther culture by Indian rice varieties is generally poor, and even among those that respond by producing callus, the in vitro morphogenic responses are highly genotype dependent. Similarly, potatoresponse is highly donor genotype dependent. The recalcitrance associated with the India types can be characterized mainly by poor callus induction response, poor renderability of green plants, and the occurrence of a large proportion of albinos [1].The use of optimal

media, specifically formulated for each of different genotypes, may help to improve the low response associated with some high valued varieties [13].

5.2 Anther Wall Factor

Act as conditioning factors and promote culture growth, report glutamine alone or in combination with serin and myoinositol could replace the anther wall factor.

5.3 Physiological Status of Donor Plants

The success of the anther culture is significantly influenced by the physiological state of the plants of the anther donor. This is mainly because physiology affects the amount of viable and healthy pollen grains developed within the anthers, the endogenous levels of metabolic pathway-regulating hormones, and the anther tissue nutritional status [4]. During maturation of the anther donor plants, environmental factors such as light intensity, photoperiod, temperature, nutrition, and CO2 concentration critically affect the growth and development. Also, pest infestations and control measures may have a detrimental effect on microspore development [13].

5.4 Pollen Development Stage

The process of pollen production is a crucial factor that greatly affects the performance of the culture of anther. In any stage of development, the induction of embryogenic calluses cannot be accomplished by cultured pollen, and the ability is limited only to particular stages of pollen maturity [4]. Therefore, during in vitro culture, a separate morphological indicator feature that correlates well with the stage of microspore maturity is widely used. In potato anther culture, the morphological trait that has been used is the measured distance between the nodes of the last two leaves: the flag leaf, and the penultimate leaf [18, 19]. In some cases, the panicle length at the time of harvest has also been used as a visually identifiable guide [20, 21].

5.5 Pretreatment of anther

Appropriate treatment required for good success of haploidproduction (depends on donor plant species).

5.6 Culture Media

In potato, the two main phases of anther culture, callus induction and shoot regeneration, require different nutrient regimes and regulators of growth. The medium of culture that best promotes the induction of callus is also not sufficient for regeneration. The transition of callus to a suitable regeneration medium must therefore be carried out at an appropriate time. Since the callus induction potential of a given rice variety is largely determined by the genetic makeup, significant levels of improvement in anther response cannot be expected by manipulation of nongenetic factors such as the culture medium. Nevertheless, the best responsive nutrient requirements must be chosen as an initial step in order to optimize anther culture, particularly if they are low responding Indica varieties [3].

5.6.1 Carbohydrate Source

In tissue culture media, a carbohydrate source is important since it serves as the key source of energy for the cultivated explant tissue. Like osmotic agents, carbohydrates are also essential. Osmotic pressure in the medium is normally controlled in potato anther culture by adding the carbohydrate source to the medium at a specific concentration. Very high concentrations when used during the later stages of culture seem to be deleterious for cereals [4]. The type of carbon source

directly influences the anther response.

5.6.2 Nitrogen Source

Inorganic nitrogen is commonly given in the form of nitrate and/or ammonium ions in cultural media. For the success of anther culture in rice, the ratio of the two nitrogen sources NO3-: NH4+ was found to be critical [3]. The N 6 basal medium most commonly used for rice anther culture has been formulated at particular concentrations for each of these nitrogen sources. However, Indica rice varieties perform much better when lower concentration of NH4+ ions than normal is used in the medium [1].

5.6.3 Plant Growth Regulators

Plant growth regulators have been widely investigated in anther culture. Appropriate in vitro culture media supplementation with effective growth regulators (auxins, cytokinin, or a combination thereof) is crucial for the success of androgenic reactions, especially from recalcitrant genotypes[4].The growth regulator 2,4-dichlorophenoxy acetic acid (2,4-D) is commonly used in the first phase of rice anther culture, and 2,4-D provided at fairly high concentrations (2 mgL−1) has produced improved rates of callus induction of up to 15% in some genotypes. In order to enhance androgenic response, the applicability of certain other auxins, such as naphthalene acetic acid (NAA), phenyl acetic acid, picloram and dicamba alone or in combination with 2,4-D, was also tested.

5.6.4 Culture Incubation Conditions

In the culture of plant tissue, crop temperature plays a significant role. Other cultures are typically incubated in the 24-27 °C temperature range. Light controls the morphogenesis of cultured pollen, and for embryogenic induction, darkness (low light intensity) or alternating light and dark conditions may be preferred. The effectiveness of culture conditions such as alternating periods of light with different temperatures (12–18 h; 5000–10,000 lux/m2 at 28°C and 12–6 h; in darkness at 22°C) [14]. Regeneration phase requires even more specific incubation conditions to achieve success, particularly for green shoot formation. Shoot regeneration from scutellum-derived callus of Indica rice was stimulated by applying osmotic stress conditions.

6.Pretreatments To Trigger Androgenesis In Potato

A treatment typically applied to excised anthers, inflorescences, or anther donor plants prior to cultivation is necessary in many crop varieties, including cereals, to cause sporophyte production deviating from the normal pathway of pollen development. The form of the effective pretreatment, length, and the time of application vary with the species or even for different varieties [1, 4]. The different pretreatments which are in current applications for triggering the anther culture response, and they have been classified into three categories based on their utility as widely used, neglected, and novel [22]. High temperature and cooling, high humidity, water tension, anaerobic treatment, centrifugation, appetite for sucrose and nitrogen, ethanol, γ-irradiation, microtubule disrupting agents, electro stimulation, high medium pH and heavy metal treatment are all included in these pretreatments.

6.1 Temperature Pretreatment

Low-temperature application is the most widely used effective form of pretreatment for rice anther culture. Before culturing, harvested rice panicles are subjected to cold shock. The temperature and length, however, differ according to the range. By delaying the degeneration of microspores and anther wall tissue in rice, cold pretreatment given to rice anthers is known to increase the androgenesis potential [1, 3]. Generally, temperatures from 8 to 10°C for 8 days have been recommended to be optimal for many varieties of potato [23]. Panicle pretreatments longer than 11 days tend to increase albino production [24].exposure to high temperature (35°C for 10 min) before the cold treatment to enhance callus induction although it adversely affected green plant production.

6.2 Osmotic Stress

Osmotic shock has been described as another pretreatment that can replace or be used for the induction of androgenesis in conjunction with cold treatment. the treatment of anthers in 0.4 M mannitol solution to be effective for inducing androgenesis in microspore cultures of Indica and Japonica varieties [25]. Androgenesis in anther cultures of variety IR43 from 3 to 33.4 percent was encouraged by sole mannitol treatment without the cold pretreatment provided to anthers. It is described that when the anthers or isolated microspores are subjected to high osmolarity by incubating in metabolizable carbohydrates for short time, they start divisions during stress treatment and tolerate the following stress conditions [24].

6.3 Sugar Starvation

Sugar starvation was found to be successful in inducing embryogenesis not only in rice but also in many other crop species, such as tobacco, wheat, and barley [24]. Cold pretreatment could be partially substituted during androgenesis of Indica rice by sending microspores for sugar starvation for 3 days. In achieving high-frequency embryogenesis and plantlet regeneration [25], sugar deprivation could be extended to Indica and Japonica rice.

6.4 Irradiation

Irradiation penetration varies depending on the species and the morphology of the pollen and the thickness of the pollen wall [26]. Stimulation of the regeneration of green plants with the application of gamma rays at a dosage of 20 Gy[27]. By using irradiation of the 137Cs gamma rays, it may be possible to increase the green plant regeneration from two to three times, and the maximum response was elicited with a dose of 15 Gy.

7. Reagents and Equipment's Required for Potato Anther Culture

7.1 Reagents

- MS basal salts [15]
- Sucrose
- Charcoal
- Agar
- Wheat starch
- myo-lnosito1
- Thiamine HCL

- Ascorbic acid
- Glutamine
- Asparagine
- Lysine HCL
- IAA
- BA
- GA3
- And others

7.2 Equipment's

- Hotplate stirrer- Mixing & heating media and stock
- Erlenmeyer flasks- for culturing
- Cotton- for caping
- Aluminum foil- for wrapping
- Incubator – for incubation purpose
- Lax meter – light controlling system
- Forceps- for picking and harvesting embryo and anther
- Bottles-Storage of stock solutions, sterile distilled water, media
- Balance- Measuring out biochemicals and media
- Cylinder- Preparing stock solutions
- Microweb oven- To melt agar solidified media
- Magenta box- Culture vessel for Maintaining plant cultures
- Baby jar- Culture vessel for Maintaining plant cultures
- PH meter- Measurement and adjustment of media pH
- Autoclave- Sterilizing media and instruments
- And others

7.3 Media Type

Uhr85 [16] and MSU93 [17] liquid media (most recommended)

8. INDUCTION OF MORPHOGENESIS

In 60 mm (diameter) single well Petri dishes, the harvested embryos and/or calli from liquid medium are plated on a morphogenic medium. 8 g/L agar is used to solidify the medium. In a culture room, the cultures are preserved in a 16 h day and held at 24 ° C temperature.

8.1 Culture of Plantlets

Plantlets developed from the morphogenic medium are excised and maintained on a plantlet medium in Magenta GA-7 vessel. The plantlet medium promotes root and shoots formation.

8.2 Checking Ploidy Level of Plantlets

Three methods can be used to verify the ploidy levels of plantlets: chromosome counting, chloroplast counting in guard cells, and flow cytometry. Counting the chromosomes is the most reliable process. The root tips of the plantlets are immersed for 18-24 h in 0.002 M aqueous 8-hydroxyquinoline solution or ice-water. The root tips are then kept in a pre-chilled fixative at 4 ° C (I part 85% lactic acid: 99 parts 30 percent ethanol) for at least 4 hours. The tips are then hydrolyzed at 60 ° C for 7 min at 1 N HCI. The substance is blotted and flooded with Schiffs' reagent for 30 minutes after hydrolysis. The root tips are stained with lactoproprionic-orceinic on a glass slide (0.9 percent orcein in 45 percent propionic-lactic acid in equal parts). The tips are macerated, positioned under a cover slip and pressurized uniformly. Under a microscope, chromosomes are counted. For chloroplast counting a piece of the lower epidermis is removed and examined under light microscope. Plantlets with reduced ploidy have fewer numbers of chloroplasts in the guard cells than that of the donors.

8.3 Chromosome Doubling

For the treatment of colchicine, young plants (25-30 cm tall) are used. The axillary buds inside the leafaxils, along the stem, are carefully cut by a razor blade or ground-down forceps twenty-four hours before colchicine is applied. There is also the removal of leaf axils without axillary buds or pseudostipples. Six leaf axils per plant are housed, on average. It is possible to remove some of the terminal and outer lateral leaflets so that the stem can be encased in a polyethylene container. In the leaf axil, the oblong pellet of absorbent cotton is firmly placed. A strip of adhesive tape 3-4 mm wide is centered over the pellet and wrapped around the stem. The colchicine solution concentration is between 0.25-0.5 percent. When the cotton is soaked, it is added to the top of the cotton pellet with an eye dropper. The plant is immediately wrapped in a polyethylene bag and positioned for a fixed period of treatment in a shady area in the greenhouse. The bag and the pellets are then removed at the end of treatment. The plant is labelled and rinsed with water spray and grown on a greenhouse bench. When it reaches 6-8 cm, the shoot cultivated from a treated sub-axillary meristem is removed. It is treated with a rooting hormone (0.8 percent indol-3 butyric acid, Stim-Root No.1, Plant Products Co. Ltd., Bramalea, Ontario, Canada), grown to root in moist vermiculite, and then transplanted into a small pot of soil for tuberization (7-10 cm). The chromosomes are counted using root tips to determine if doubling has taken place.

9. Application of Anther Culture

Rapid production of homozygous lines may be equal to several generations of in breeding. Reduction of ploidy level leads to the elimination of undesired and lethal genes from the progenies in a genetic population. Generation of monohaploids provide the most effective tool toachieve this. Anther-derived plants provide materials with reduced ploidy levels for

mapping of molecular markers. Mapping of monohaploids, for example, avoids the problem of dominance in thesegregation of gene markers. Dihaploids are used to cross with diploid species. Diploid hybrid species are then used in *4x*x *2x* mating in the potato breeding program after screening the diploid hybrids (2n=2x=24)for ability to produce *2n* gametes. These plants are used to cross with the tetraploid S.*tuberosum* cultivars (2n=4x=48) to produce *4x* progenies.

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